FILAMENTOUS FUNGI FROM BLOSSOMS, OVARIES, AND FRUIT OF PICKLING CUCUMBERS [1, 2]

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(WITH 7 FIGURES)

For the last two decades, cooperative investigations into fermentations of various foods in brine and silage have been carried on by the U. S. Department of Agriculture and North Carolina State College (**19**). Those studies have been especially concerned with fermentation problems of commercial packers of cucumber pickles.

The main problem, which remained unresolved until the advent of the present studies on the filamentous fungi, was that of producing firm pickles. In spite of carefully executed sanitation and brining procedures, many packers were consistently unable to produce pickles of highest-quality firmness. Furthermore, in certain years, a number of them experienced the loss, through softening of stock, of the contents of some or all of the vats filled during the early part of the brining season.

The literature bearing on the bacteriology of the brine-fermentation of cucumbers in relation to enzymatic softening has recently been thoroughly reviewed by Demain and Phaff (**9**). They concluded that the much-studied aerobic bacilli, with their high-pH-optima pectinolytic enzyme systems, appear to play only a minor role in softening. In this

[1] Cooperative investigations of the Southern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, with the Department of Horticulture of the North Carolina Agricultural Experiment Station, and with the senior author and the late W. Lawrence White of Harvard University, who died shortly after these studies were begun.

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connection, the acid and salt content of curing brines—as well as their low oxygen tension—would provide highly unfavorable conditions for the development of aerobic bacilli; essentially the same would be true for the soft-rot bacteria.

The low pH optima (4.0) for pectinolytic enzymes associated with softening of commercially brined cucumbers (**4, 6**) would point to fungi as the enzyme source rather than to bacteria. Although yeasts are highly acid- and salt-tolerant and are active in cucumber fermentations (**2, 10, 11, 12, 16, 18**), enzymological studies on a large collection of isolates from brines gave no evidence that they would be incriminated as a potential source of the brine-stock softening enzyme, pectinase (**3**). Therefore, it was logical to assume that the softening enzymes known to be active in brines (**4, 6**) were introduced with the cucumbers rather than produced in the brine during fermentation.

Enzymatic studies of various parts of the cucumber plant (*Cucumis sativus* L.) disclosed the presence of pectinolytic enzymes in staminate and pistillate flowers (**1, 5**) although the specific causative agent(s) responsible for the enzyme activity was not determined. It was further observed (**13, 14**) that about five times as many early-harvested cucumbers retained their blossoms as did the fruit harvested later in the season. Since blossoms already had been indicated as possible carriers of the active softening enzymes, the specific source appeared to lie with microorganisms borne by them. A series of studies were initiated at the beginning of the 1952 season to investigate populations, identity and enzyme production of filamentous fungi associated with pickling cucumbers and to further substantiate the hypothesis that the requisite enzymes were chiefly of fungal origin.

Demonstrations (a) that blossoms do carry the softening agents into commercial vats and (b) that filamentous fungi can produce those agents have been presented earlier (**13, 14**). Brinings of cucumbers from which the blossoms had been detached produced firm pickles; when those blossoms were included, the resulting pickles were soft. Adding to the normal complement of retained blossoms further increased softening. A simple, inexpensive, but effective softening-enzyme control measure was evolved which consists of draining off the original cover brine 36–48 hours after the vats are filled and replacing it with a new brine (**13, 14**).

The present paper deals primarily with the isolation, classification, and frequency of filamentous fungi on blossoms, ovaries, and fruit of cucumbers from commercial fields, a greenhouse, and a commercial brining station. The remaining aspects will be subjects of other papers.

MATERIALS AND METHODS

Samples of cucumber blossoms, ovaries, and fruit (FIG. 2) were collected at weekly intervals from a commercial brining station at Ayden in eastern North Carolina, from five field locations within 20 miles of the station, and from a greenhouse at North Carolina State College, Raleigh (FIG. 1). Samples from the brining station consisted of cucumber fruit typical of stock being sent in by receiving and grading

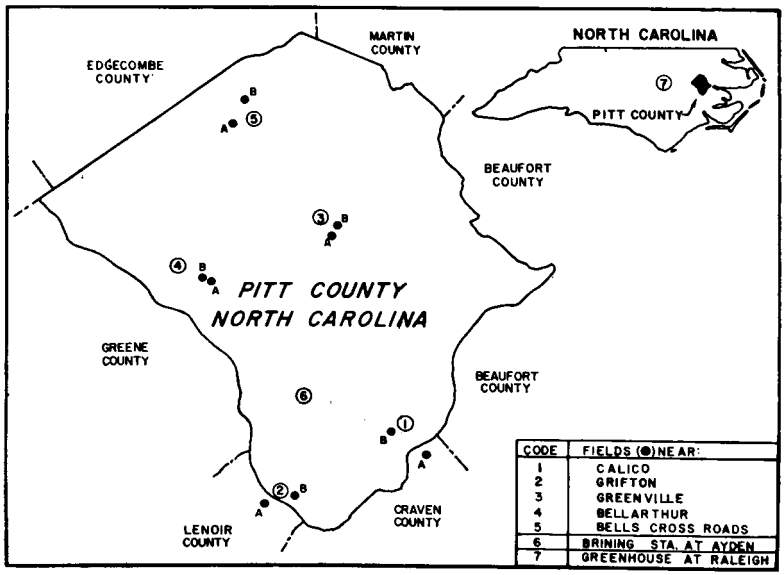

FIG. 1. Map showing origin of the samples; sampling locations are explained in the legend. Field samples were collected from the "A" fields except that the "B" fields were the source for samples collected in the last three periods at field location 1 and in the final period at field location 5.

stations which supply the cooperating company, and of blossoms which had been retained by the fruit. Greenhouse samples were of newly opened pistillate perianths and of the ovaries which supported them. Field samples, in addition to the fresh blossoms and ovaries, also included fruit typical of that being harvested for brining. Information about the collections is summarized in TABLE I.

Aseptic precautions were observed throughout. All samples were placed in sterilized paper bags by means of sterile forceps. At the end of each day of collecting, the samples were refrigerated until the fol-

lowing morning, at which time they were processed to produce inocula for culturing.

Each blossom sample, after being weighed, was blended in 0.85% saline for one minute at 14,000 rpm in a sterile, 50 ml Omnimixer [7, 8] blending-chamber. For the fresh blossoms, the dilution employed was 1:10 (w/v); however, a 1:50 dilution was found more convenient for the desiccated perianths collected at the station.

TABLE I

ORIGIN, NATURE, AND WEIGHT OF SAMPLES COLLECTED

Source	Type	Units per sample	Average weight (g)		Weekly samplings		Total
			Sample	Unit	Starting date	Ending date	
Five commercial fields[a]	Blossoms[e]	25	3.51	0.140	6/5	7/17	7
	Ovaries	25	12.00	0.480	6/5	7/17	7
	Fruit[d]	20	265.4	13.3	6/12	7/10	5
Brining station[a]	Blossoms[e]	45–100	0.5–2.0	0.017	6/12	7/10	5
	Fruit[d]	20	313.8	15.7	6/12	7/10	5
Greenhouse[b]	Blossoms[e]	25	4.15	0.166	6/26	7/17	4
	Ovaries	25	9.68	0.387	6/26	7/17	4

[a] Packer variety of cucumbers.
[b] Model variety of cucumbers.
[c] Perianths of freshly-opened, pistillate flowers.
[d] No. 1 size, 1 to 1½ inches in diameter.
[e] Detached from the fruit at time of sampling.

Weighed samples of ovaries were shaken in 125 ml Erlenmeyer flasks with equal weights of saline for 10 minutes on a rotary shaker [8, 9] at 210 oscillations per minute.

Fruit samples were similarly diluted but were placed in one-liter flasks which were shaken 100 times by hand.

Inoculation was accomplished by withdrawing and streaking on agar plates 0.01-gram aliquots of several different dilutions of each inoculum by means of a calibrated platinum loop. The media used for the isolation of filamentous fungi were Littman's oxgall agar with streptomycin (20) and Wickerham's nitrogen-base broth (30) with glucose, acidified

[7] Manufactured by Ivan Sorvall, Inc., Norwalk, Connecticut.

[8] It is not the policy of any of the organizations represented by the authors to recommend the products of any company over similar products of any other company. The name is supplied for informational purposes.

[9] Model V, capacity 40 flasks, manufactured by the New Brunswick Scientific Co., New Brunswick, New Jersey.

FIGS. 2–7.

to pH 3.5–3.7 with tartaric acid and solidified with agar, as previously described (17). Both media facilitate isolation of fungi by restricting the spread of most colonies and inhibiting the growth of many bacteria.

After incubation, usually for four days at 28° C, the sets of plates which had received the most extreme dilutions containing fungi were selected, the colonies were counted and recorded, and isolations were made by transferring bits of mycelia directly from the colonies to potato-dextrose-agar slants. This medium was used to maintain the cultures and to grow them for identification purposes. Other media employed in identification were Czapek's agar for the Aspergilli and Penicillia (22, 27) and de Vries's gallic-acid agar in the case of *Cladosporium cladosporioides* (Fres.) de Vries (28).

Each of the dilutions used for inoculation was tested for pectinolytic activity. Also, many of the subsequently isolated fungi were investigated for pectinolytic activity, and some for cellulolytic activity. The methods employed were those used by Bell et al. (3, 4, 6) and by White and Downing (29), respectively. The results are discussed in detail in a companion paper devoted to populations and softening enzyme activity of fungi from the cucumber plant (15). However, summarized information for the action of the various identified species on pectin and for certain species on cellulose, based on the above report, is included herein.

RESULTS

NATURE AND OCCURRENCE OF FILAMENTOUS FUNGI

A total of 1032 isolates of filamentous fungi was obtained. These represented at least the 83 different entities listed in TABLE II. The number will vary according to the systems of classification employed; e.g., if the isolates representing the genus *Fusarium* had been segregated in accordance with the species-concept of Wollenweber and Reinking

FIGS. 2–7. FIG. 2. Left: the blossom and ovary of a freshly-opened pistillate flower of the cucumber (*Cucumis sativus* L.); right: a No. 1 size (1 to 1⅛-inch diameter) fruit with retained perianth. The illustrated structures are typical of those collected in the samplings. Approx. 0.5 ×. FIGS. 3–7. Unidentified sporulating fungi; magnification 800 ×. FIG. 3. Conidia and seta of unidentified sporulating fungus A. FIG. 4. Unidentified sporulating fungus B. The distinctive but variable spores and the spindle-shaped "hyphal" cells can be seen. FIG. 5. Chains of conidia and two single phialides of unidentified sporulating fungus C. FIG. 6. Conidiophores of unidentified sporulating fungus D with spore-heads borne by phialides in the second (left) and third (right) types of configuration mentioned in the text. FIG. 7. Several conidiophores of unidentified sporulating fungus E. Note the peculiar, hyaline spore-bearing tips.

TABLE II

NAMES, NUMBERS, FREQUENCY, SEASONAL DISTRIBUTION, AND PECTINOLYTIC ACTIVITY OF THE FUNGI ISOLATED

Organisms	No. of isol.	Frequency of isolation from all samples					Seasonal distribution	Pectinolytic activity
		Sampling by weeks			by media[a]			
		Fields (35)[b]	Station (5)[b]	Greenhouse (4)[b]	LO (41)[b]	NB (41)[b]		
PHYCOMYCETES								
Choanephora cucurbitarum (B. & R.) Thaxt.	4	3	1	—	3	1	ML[c]	—
Cunninghamella echinulata (Thaxt.) Thaxt.	1	—	1	—	1	—	M	N[d]
Mucor sylvaticus Hagem	19	5	4	—	9	4	EM	P
Mucor sp. 2	1	1	—	—	—	1	E	P
Mucor sp. 3	1	1	—	—	1	—	E	P
Rhizopus arrhizus Fischer	1	1	—	—	—	1	E	P
R. stolonifer (Ehrenb. ex Fr.) Lind	4	—	2	—	1	1	EL	P
ASCOMYCETES								
Chaetomium globosum Kunze	2	—	—	2	2	—	M	N
Pseudonectria sp.	1	1	—	—	—	1	E	P
Rosellinia vel aff.	1	1	—	—	—	1	E	N
Thielavia basicola (B. & Br.) Zopf	3	3	—	—	2	1	E	P
FUNGI IMPERFECTI[e]								
Alternaria teuuis auct.	88	31	2	4	29	23	EML	P
Ascochyta cucumis Fautr. & Roum.	159	35	4	—	34	37	EML	P

[a] The media are Littman's oxgall agar (LO) and Wickerham's nitrogen-base agar (NB).

[b] The figures in parentheses indicate the total number of times which an entity could possibly occur in that category.

[c] The symbols E, M, and L refer to Early, Middle and Late portions of the harvest according to the following sampling weeks: E = the first two; M = the third through the fifth; and L = the last two.

[d] Representative cultures were screened for ability to bring about glycosidic hydrolysis of pectin; N means negative; P, positive; NP indicates that some of the tested strains gave negative results, others positive; — means not tested.

[e] Most of these organisms undoubtedly represent Ascomycetes but were placed here because no perfect-stage fruiting was found. None of the sterile fungi had clamp connections.

TABLE II—*Continued*

Organisms	No. of isol.	Frequency of isolation from all samples					Seasonal distribution	Pectinolytic activity
		Sampling by weeks			by media[a]			
		Fields (35)[b]	Station (5)[b]	Greenhouse (4)[b]	LO (41)[b]	NB (41)[b]		
Ascochyta sp.	1	1	—	—	1	—	L	N
Aspergillus elegans Gasp.	1	—	—	1	1	—	L	P
A. fumigatus Fres.	7	7	—	—	1	6	EML	P
A. niger Tiegh.	1	1	—	—	1	—	M	P
A. sydowi (Bain. & Sart.) Thom & Ch.	1	—	—	1	1	—	L	P
A. terreus Thom	1	—	1	—	—	1	M	P
A. unguis (Em.-W. & G.) em. Thom & Raper	1	1	—	—	1	—	E	P
A. versicolor (Vuill.) Tirab.	2	1	—	—	1	1	E	P
Cephalosporium sp. 1	5	3	1	—	4	—	EML	NP
Cephalosporium sp. 2	1	1	—	—	1	—	E	P
Cercospora sp.	1	1	—	—	—	1	M	N
Cladosporium cladosporioides (Fres.) de Vr.	104	31	5	3	37	13	EML	P
Colletotrichum lagenarium (Pass.) Ell. & H.	8	7	1	—	8	—	EML	P
Curvularia trifolii (Kauff.) Boed.	4	3	—	1	3	1	ML	P
Fusarium episphaeria (Tode) em. S. & H.	1	—	—	1	—	1	L	P
F. moniliforme Sheld. em. S. & H.	10	6	2	—	5	4	EML	P
F. oxysporum Schl. em. S. & H.	60	19	5	0	19	18	EML	P
F. roseum Lk. emend. S. & H.	106	27	5	1	31	12	EML	NP
F. solani App. & Wr. em. S. & H.	58	25	4	—	21	18	EML	P
Fusarium sp.	14	10	2	2	5	9	EML	—
Geotrichum sp.	3	—	2	—	2	—	M	N
Gliocladium roseum Bain.	2	2	—	—	2	—	M	N
Helminthosporium sp.	2	2	—	—	—	2	ML	P
Heterosporium terrestre Atk.	1	1	—	—	1	—	E	N
Humicola fusco-atra Traaen	1	1	—	—	1	—	E	P
Metarrhizium anisopliae (Metsch.) Sor.	2	2	—	—	—	2	EM	N
Myrothecium roridum Tode ex Fr.	4	3	—	—	3	—	L	NP

TABLE II—*Continued*

Organisms	No. of isol.	Frequency of isolation from all samples					Seasonal distribution	Pectinolytic activity
		Sampling by weeks			by media[a]			
		Fields (35)[b]	Station (5)[b]	Greenhouse (4)[b]	LO (41)[b]	NB (41)[b]		
M. verrucaria (Alb. & Schw.) Ditm. ex Fr.	9	8	—	—	8	—	EML	N
Myrothecium sp.	2	2	—	—	2	—	ML	NP
Papularia arundinis (Cda.) Fr.	1	1	—	—	1	—	E	N
Penicillium atramentosum Thom	1	1	—	—	—	1	M	P
P. chermesinum Biourge	1	1	—	—	—	1	E	P
P. duclauxi Delacr.	2	1	1	—	—	2	L	P
P. expansum Lk.	8	0	3	—	1	3	EML	P
P. frequentans Westl.	1	—	—	1	—	1	L	P
P. funiculosum Thom	2	—	—	2	—	2	ML	P
P. gladioli Mach.	1	—	1	—	—	1	M	P
P. herquei Bain. & Sart.	1	1	—	—	1	—	E	P
P. implicatum Biourge	1	—	1	—	—	1	L	P
P. janthinellum Biourge	16	10	1	—	4	9	EM	P
P. nigricans (Bain.) Thom	5	3	—	—	—	3	E	—
P. oxalicum Curr. & Thom	162	29	5	1	30	31	EML	P
P. piscarium Westl.	2	2	—	—	2	—	EM	P
P. restrictum Gilm. & Abb.	2	2	—	—	—	2	EM	P
P. rugulosum Thom	1	—	1	—	—	1	M	P
P. steckii Zal.	11	1	4	1	2	5	EM	P
P. sublateritium Biourge	7	5	—	—	4	3	EML	P
P. thomi Zal.	2	1	—	—	1	—	L	P
P. urticae Bain.	1	—	—	1	1	—	M	P
P. variabile Wehm.	2	1	1	—	1	1	M	P
Penicillium sp.	1	1	—	—	1	—	E	P
Pestalotiopsis macrotricha (Kleb.) Stey.	3	2	—	—	—	2	EL	P
Phoma sp.	7	2	5	—	1	6	ML	P
Pullularia pullulans (de Bary) Berkh.	3	1	1	—	—	2	E	P

TABLE II—*Continued*

Organisms	No. of isol.	Frequency of isolation from all samples					Seasonal distribution	Pectinolytic activity
		Sampling by weeks			by media[a]			
		Fields (35)[b]	Station (5)[b]	Greenhouse (4)[b]	LO (41)[b]	NB (41)[b]		
Sporotrichum pruinosum Gilm. & Abb.	3	2	—	1	1	2	EM	P
Stagonospora sp.	1	—	—	1	—	1	M	P
Stysanus sp.	1	—	—	1	1	—	L	N
Trichoderma viride auct.	16	11	1	—	6	7	EML	NP
Truncatella sp.	1	1	—	—	—	1	E	P
Undet. sporulating fungus A	28	17	—	—	17	—	EML	—
Undet. sporulating fungus B	15	5	1	—	—	6	E	—
Undet. sporulating fungus C	2	2	—	—	—	2	M	N
Undet. sporulating fungus D	2	2	—	—	—	2	EL	P
Undet. sporulating fungus E	1	1	—	—	—	1	M	N
Undet. sterile fungus A	11	10	—	—	8	2	EML	NP
Undet. sterile fungus B[f]	5	4	—	—	—	4	ML	N
Undet. sterile fungus C	2	2	—	—	—	2	EM	N
Undet. sterile fungus D	1	1	—	—	1	—	E	P
Undet. sterile fungus E	1	1	—	—	1	—	M	P

[f] The information given for this entity is incomplete; it was believed not to be a filamentous fungus and cultures were isolated only from plates of the sampling date 6/26/52.

(31) instead of the Snyder and Hansen concept (24, 25, 26), the list would have been much longer. Identification of the *Fusaria* was facilitated by the fact that the collection assembled by the late Dr. W. Lawrence White of Harvard University contained many cultures which had been determined by Sherbakoff or Snyder. Also, the authors are indebted to Dr. C. W. Hesseltine of the Northern Utilization Research and Development Division (USDA), Peoria, Illinois, whose provision of numerous strains of *Penicillium* greatly assisted in the determinations for this genus.

The following is a brief discussion of isolates by genera in alphabetical order:

Alternaria. All of these isolates were taken to represent the single species *A. tenuis* auct., *sensu lato.* This was one of the two most ubiquitously and abundantly distributed fungi found. However, it was encountered in only two of the five sampling weeks at the brining station. Strong pectinolytic activity and high frequency both indicate that this entity is important as a source of softening enzymes in samples of cucumber material collected at the brining station (cf. **15**).

Ascochyta. All but one of the 160 isolates were assignable to *A. cucumis* Fautr. & Roum., which is the imperfect stage of *Mycosphaerella citrullina* (Smith) Gross., the widespread attacker of cucumber vines and fruit. Despite the fact that this entity was absent from greenhouse samples, it occurred with greater frequency than any other fungus in the study. Isolates tested were strongly or very strongly pectinolytic. This organism is considered to be another major source of softening enzymes observed for cucumber material (cf. **15**).

Aspergillus. Members of this genus, consisting of 14 isolates representing seven species, were considered of too limited occurrence to be very important in softening. The pectinolytic activity of most species was moderate.

Cephalosporium. These isolates proved weakly pectinolytic and were notable only for the fact that none was derived from the nitrogen-base medium.

Cercospora. The single isolate showed no pectinolytic activity.

Chaetomium. The two isolates of *C. globosum* Kunze were from greenhouse samples; they were strongly cellulolytic but negative for action on pectin.

Choanephora. The four isolates of the cucumber blossom-blight organism, *C. cucurbitarum* (Berk. & Rav.) Thaxt., died before they could be tested for action on pectin; however, this fungus occurred too rarely to be important here.

Cladosporium. All of these isolates were placed in *C. cladosporioides* (Fres.) de Vries, a species morphologically similar to *C. cucumerinum* Ell. & Arth. Twenty-five of them were examined by Prof. N. N. Winstead at North Carolina State College, and were excluded by him from *C. cucumerinum* on the basis of pathogenicity tests on cucumber and tomato plants. De Vries's gallic-acid test similarly excludes them.

This organism is the other of the two most ubiquitously and abundantly distributed fungi isolated. Like *Alternaria tenuis,* it occurred in all five of the field samples; unlike that organism, it was isolated from the brining-station samples for all of the five sampling weeks. It was obtained almost three times as frequently on Littman's agar as on the nitrogen-base medium.

Cultures tested for pectinolysis were strongly or very strongly active. This fungus was undoubtedly one of the more important sources of softening enzymes found for cucumber material (cf. **15**).

Colletotrichum. Only one of the eight isolates of *C. lagenarium* (Pass.) Ell. & Halst., the anthracnose organism, was obtained from brining-station samples. For this reason, and also because of its comparative infrequency, it is considered to be of minor importance as a source of pectinolytic enzymes, despite strong activity.

Cunninghamella. The single isolate of *C. echinulata* (Thaxt.) Thaxt. was negative for pectinolysis.

Curvularia. C. trifolii (Kauff.) Boed. occurred too rarely to be important here even though it was strongly pectinolytic.

Fusarium. Most of the 249 isolates represented one or another of the three most common species, *F. oxysporum* Schl. emend. Snyder & Hans., *F. roseum* Lk. emend. Snyder & Hans., and *F. solani* App. & Wr. emend. Snyder & Hans. Those three species were of frequent occurrence except from greenhouse samples.

Thirty isolates, representing the five species listed in TABLE II, were tested for pectinolytic activity; all but one was positive. Members of this genus obviously are important as a potential source of softening enzyme activity observed for samples of material (cf. **15**).

Geotrichum. All three isolates were pectinolytically negative.

Gliocladium. G. roseum Bain.; the single isolate tested was negative for pectinolytic activity.

Helminthosporium. The two isolates tested were strongly positive for pectinolysis but this entity was too rare to be important here.

Humicola. The single isolate of *H. fusco-atra* Traaen showed moderate pectinolytic activity.

Metarrhizium. Both isolates of *M. anisopliae* (Metsch.) Sor., the "green muscardine" which attacks many insects, were pectinolytically negative. This fungus was present in the samples from one field for two consecutive weeks. Although its presence is readily explained by assumption that infected insects were associated with the plants sampled, and although it clearly is unimportant here, it should be pointed out that published information shows that this organism can be an active saprophyte.

Mucor. All but two of these isolates were typical of *M. sylvaticus* Hagem. Five isolates in this species were tested for pectinolysis and all were found moderately active. Because of this and the fact that its frequency at the station was relatively high, this species may be a moderately important contributor of softening enzymes.

Myrothecium. Two of the 11 isolates tested were weakly pectinolytic; the remainder were negative. Although members of this genus apparently contribute very little as sources of pectinolytic softening enzymes, they may be important in any cellulolytic softening.

Of the 15 isolates representing this genus, all but two were readily assignable to *M. verrucaria* (Alb. & Schw.) Ditm. or *M. roridum* Tode ex Fr. The other two had spores which were similar to those of *M. roridum* but much longer.

Papularia. The single isolate of *P. arundinis* (Cda.) Fr. was considered negative for pectinolytic activity.

Penicillium. Although all of the isolates which were tested proved strongly positive for pectinolytic activity, only those representing *P. oxalicum* Curr. & Thom were considered sufficiently abundant to be of substantial importance as a source of softening enzymes. Seven isolates of this species were tested for cellulolytic activity and all were able to reduce the tensile strength of cotton tape by 90 or 95%. A culture of *Myrothecium verrucaria* and one of *Chaetomium globosum,* which were selected for comparison, caused 100% and 90% loss of strength, respectively.

As can be seen from TABLE III, *P. oxalicum* was one of the more abundant fungi in the field samples; of even greater significance is the fact that it was the most abundant in the brining-station samples. There is little reason to doubt that this species was an important contributor to the concentration of pectinolytic enzymes found in samples of cucumber material, particularly flowers, during the 1952 season (cf. **15**).

The frequency of occurrence of *P. janthinellum* Biourge, *P. steckii* Zal. and *P. expansum* Lk. in field and station samples was high enough so that they are considered of moderate importance.

Pestalotiopsis. P. macrotricha (Kleb.) Stey. occurred too rarely to be of importance and was only weakly pectinolytic.

Phoma. The seven isolates assigned here probably represent a single species. They do not represent *Macrophomina phaseoli* (Maubl.) Ashby, the charcoal-stem-rot fungus. The three cultures tested were positive for pectin degradation.

TABLE III

FREQUENCY (%) OF OCCURRENCE OF THE SEVEN MOST IMPORTANT FUNGI IN SAMPLES FROM THREE SOURCES

Organism	Frequency of isolation from samples collected at:						
	Brining station		Commercial fields			Greenhouse	
	Blossoms %	Fruit %	Blossoms %	Ovaries %	Fruit %	Blossoms %	Ovaries %
Penicillium oxalicum	90	70	86	71	100	—	17
Cladosporium cladosporioides	50	20	71	64	90	38	17
Fusarium roseum	50	60	64	43	70	12	—
Fusarium oxysporum	60	70	57	36	80	—	—
Fusarium solani	30	70	50	71	80	—	—
Ascochyta cucumis	60	40	93	100	100	—	—
Alternaria tenuis	10	30	93	86	70	25	33

Pseudonectria. The single isolate is noteworthy for the fact that the imperfect stage belongs to *Fusarium;* no such relationship has been reported previously for these genera. This fungus has been described recently as *P. diparietospora* by Miller et al. (21).

Pullularia. P. pullulans (de Bary) Berkh. occurred infrequently; the three isolates tested were positive for action on pectin.

Rhizopus. Although all of the isolates of *R. stolonifer* (Ehrenb. ex Fr.) Lind and *R. arrhizus* Fischer were active pectinolytically, they were of such limited occurrence that they must be considered to have only minor roles as contributors to enzyme softening of cucumbers.

Rosellinia, vel aff. The single isolate was pectinolytically negative.

Sporotrichum. Isolates of *S. pruinosum* Gilm. & Abb. were rarely obtained and only weakly pectinolytic.

Stagonospora. The single isolate was pectinolytically positive.

Stysanus. The one culture was negative for pectinolysis.

Thielavia. T. basicola (B. & Br.) Zopf, represented by three isolates, was pectinolytically weak.

Trichoderma. T. viride auct. was common enough from field samples to warrant consideration. However, only one isolate in every three was positive for action on pectin and none was present in station blossom

samples, and so it was concluded that this entity is of only minor importance here.

It was interesting to note that during the first four sampling weeks, eight of the ten cultures were isolated from Littman's agar, while all six obtained during the final three weeks were derived from the nitrogen-base medium. No corresponding shift in morphological characteristics was found.

None of the isolates manifested the loose, tuftless mycelial growth or the *Gliocladium*-like conidiophores which frequently occur in strains that produce gliotoxin or viridin, and which Miller et al. (21) recently described as *Gliocladium virens*.

Truncatella. The single isolate was pectinolytically positive.

Unidentified sporulating fungi. Of the next five entities discussed, several generic names are known for one of them but not accepted, whereas the genera to which the other four belong are "new" to these writers. Because an entity unknown to some often is known to others, the authors prefer not to diagnose five new genera. The five are here illustrated and briefly described with the hope that readers who may be familiar with them or are working with similar organisms will be benefitted by having this information. Cultures will be available upon request.

Unidentified sporulating fungus A. This interesting fungus probably is relatively unimportant as a source of softening enzymes. Although it was moderately abundant in the field samples, it was never obtained from station or greenhouse samples. All isolates came from Littman's agar.

The cultural appearance of this fungus is similar to that of *Verticillium albo-atrum;* i.e., there is a paucity of aerial hyphae, and the dominant feature is the black aspect presented by myriads of small, black sclerotial bodies on the surface of the agar. Microscopically, however, there is less similarity; short, black setae occur among the sclerotia, and the numerous curved, hyaline spores, suggestive of *Menispora,* frequently become several-septate at maturity (FIG. 3).

Unidentified sporulating fungus B. This organism has been the subject of discussion among the authors of this paper. Some regard it as a filamentous fungus, others would classify it among the yeasts. The spores are quite unyeastlike but the mycelium can be interpreted as being composed of spindle-shaped cells which form false hyphae with false branching. FIGURE 4 illustrates the variable spores and some hyphal cells which have been slightly disoriented by pressure on the coverslip.

The 15 isolates of this fungus came from cucumber material representing only one of the sampling periods. It was present, however, during most or all of them and was abundant during these investigations. All of the isolates were obtained from the nitrogen-base medium.

Unidentified sporulating fungus C. This fungus is congeneric with the imperfect state of *Cephalotheca sulfurea* Fuckel [10] (8) and with certain of the fungi which were described as species of the untenable genus *Spicaria* and which more recently have been transferred to *Paecilomyces* (7). This latter disposition is not accepted because the fungi included in that concept of *Paecilomyces* can be segregated into at least three groups which are distinguished by their phialides and conidia. A paper in which a more satisfactory disposition will be proposed is now in preparation.

Characteristic of the group which the fungus represents are the tapering narrow-tipped phialides and, particularly, the chains of spores connected by extremely narrow isthmi (FIG. 5).

Unidentified sporulating fungus D. This entity exhibits a pattern of sporulation which has superficial similarities to that of *Cladosporium* but which results in heads rather than chains of spores. The greatest similarity is in the elliptical phialide cells which may arise singly on undifferentiated aerial hyphae and sporulate immediately, which may proliferate into chains of such cells and terminate in spores, or which may be organized on verticillately branched conidiophores (FIG. 6). Both conidia and phialides remain unicellular with smooth, light-brown walls.

Unidentified sporulating fungus E. This fungus also has a distinctive method of sporulation. The conidiophores are stout, erect, simple or branched, and with dark-brown walls. They are terminated by abruptly narrowed, irregularly flexed hyaline structures which bear the conidia in the manner of a *Tritirachium* in which each stalk is so short that its spores are compacted into a ball. Similar structures usually develop subsequently from near the apices of one or more of the subterminal cells of each conidiophore (FIG. 7).

Undetermined sterile fungi. None of these had the clamp connections which would have indicated that they were Basidiomycetes. Of the five entities in this section, only sterile fungus A was of more than rare occurrence and it was isolated only from field samples. Three of four cultures of this entity proved pectinolytically positive. It is considered to be of minor importance here.

[10] Chesters's culture was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

COMPARISON OF ISOLATION MEDIA

Of the 83 entities listed in TABLE II, 28 were obtained only from Littman's agar, and 29 only from the acidified nitrogen-base agar. Of the 26 fungi which occurred on both media, seven were more common on the one, eight were more common from the other, and eleven occurred in approximately equal proportions on both agars. It must be pointed out, however, that most of the entities occurred too infrequently to justify the drawing of conclusions from only the figures given above. It is necessary to consider those organisms which were abundant.

Of the ten most common fungi which were obtained from both field and station samples, two occurred more frequently on one medium, two on the other, and six were about equally frequent on both.

It must be concluded, therefore, that the two isolation media were about equally effective in permitting enumeration of the common members of the fungal flora of pickling cucumbers. If either had been used alone, the same entities would have been found abundant—although possibly in a slightly different order of frequency—but the list of rare fungi would have been smaller. For the purpose of designating the important fungal sources of softening enzymes, either medium would have been satisfactory. However, if isolation were to be repeated for that purpose, the nitrogen-base agar would be selected; on it, the fungal colonies remained small and restricted much longer (several days) than was the case with Littman's agar.

DISCUSSION AND CONCLUSIONS

The 83 entities of fungi to which the isolates were assigned are easily divided into two categories based on their importance as potential contributors to enzymatic, particularly pectinolytic, softening of cucumber material. The occurrence of an organism in station samples is probably of greater significance than is its abundance in field samples, because of the fact that the softening enzymes, resulting chiefly from fungal growth, are carried into the vats predominantly on blossoms retained by the cucumbers. The fungi which are considered to be of major importance as sources of softening enzymes in cucumber material are those which were common in both station and field samples; those which occurred infrequently obviously belong in the category of minor or no importance.

TABLE III lists, in what is presumed to be their approximate order of importance, the seven species which are considered to have been major contributors to softening enzyme activity of cucumber brines in

eastern North Carolina during the 1952 season. The great frequency of *Penicillium oxalicum* in brining-station blossom samples necessitates placing this potent fungus high in the list. *Cladosporium cladosporioides* and the three common species of *Fusarium* are also placed high, for the reason that those four fungi were found to be about 1000 times as abundant on station blossoms as on field blossoms. The order of increase in propagative units on station perianths over field perianths was 200 times for *Ascochyta cucumis* and 500 times for *Alternaria tenuis*. It is possible that much or most of the increase in propagules resulted from sporulation as the blossoms dried on their way to the station. Although differences between sporulating and non- or lightly-sporulating fungi should have been minimized by the blending, they still may have been great enough to have caused these last two entities to be placed lower in the list than they belong. Also, it is realized that there may be differences in enzyme concentrations between spores and hyphal cells, or, for that matter, in the amount of enzyme secreted into the blossom by fungal growth. The list is arranged on the basis of frequency and *in vitro* enzyme potency and is subject to rearrangement when additional information becomes available.

The other category comprises the rest of the species isolated; these are of minor importance as individual sources of brine-stock softening enzymes but they still may have a role, if only a small one, as contributors to softening activity. More than half of the minor species were positive when tested for pectinolytic activity and most are known (**15**, **23**) to produce cellulolytic enzymes.

It is believed that the origin of softening enzyme activity of commercial cucumber brines under the conditions described is brought about by enzymes contributed by filamentous fungi which enter the vats chiefly on blossoms retained by the cucumbers. Many fungi contribute to softening but the seven species listed in TABLE III stand out as being the most important.

SUMMARY

Results are presented for the identification of fungi isolated from samples of blossoms, ovaries and immature fruit of the cucumber (*Cucumis sativus* L.) collected throughout one season from five commercial fields, a brining station and a greenhouse, all of which were located in North Carolina. Of the 1032 cultures isolated, 964 (93.4%) were reduced to 73 species in 34 genera; the remaining 68 isolates (6.6%), representing 10 distinct entities, were not fully identified. Thirty-one species in five major genera represented 80.50% of the total isolations;

42 species in 29 minor genera accounted for 12.9%. Seven species in the five major genera—*Penicillium, Ascochyta, Fusarium, Cladosporium* and *Alternaria*—represented 71.4% of all isolations. The species were: *Penicillium oxalicum,* 15.7%, of isolates; *Ascochyta cucumis,* 15.4%; *Fusarium roseum,* 10.3%; *Cladosporium cladosporioides,* 10.1%; *Alternaria tenuis,* 8.5%; *Fusarium oxysporum,* 5.8%; and *Fusarium solani,* 5.6%. These species comprised most of the total fungus populations of field blossoms and station blossoms collected throughout the season and were considered to be an important source of pectinolytic and cellulolytic enzymes.

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