

# Colonization of Oak Wilt Fungal Mats by *Ophiostoma piceae* During Spring in Minnesota

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## ABSTRACT

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The colonization of *Ceratocystis fagacearum* fungal mats of different ages by *Ophiostoma piceae* on *Quercus* spp. was determined in three east-central Minnesota locations during the spring of 1995. The extent of the mat area colonized by *O. piceae* generally increased with mat age. Subsamples per mat yielding the fungus for the three locations averaged 11 to 27% for immature mats, 65 to 72% for mature mats, and 66 to 96% for aging and declining mats. On a mat-incidence basis, frequencies of *O. piceae* isolation from at least one subsample of an immature mat ranged from 30 to 53% compared with more than 90% for all other mat ages in the three locations. The average number of colony-forming units of *O. piceae* per square centimeter ranged from  $1 \times 10^3$  to  $1.2 \times 10^6$  and increased with mat age. The extent of *C. fagacearum* recovery from each mat was lower for aging plus declining mats ( $P < 0.03$ ) compared with other ages in two locations. On a mat-incidence basis, frequency of *C. fagacearum* recovery from at least one subsample of a mat averaged 99% for all ages of mats in all locations. The interaction between *O. piceae* and *C. fagacearum* on mats, nitidulids, and the oak wound surface is discussed.

Additional keywords: *Graphium pirinum*, insect transmission

Oak wilt is an important disease of oaks in the eastern United States and is the leading cause of oak mortality in Minnesota. The causal fungus, *Ceratocystis fagacearum* (T. W. Bretz) J. Hunt, is disseminated from diseased to healthy oaks by root grafts or overland by insect transmission (6). Sap-feeding beetles (Coleoptera: Nitidulidae) are considered the primary vectors in Minnesota (3,5). These beetles are attracted to *C. fagacearum* sporulation mats that form on recently killed oaks (*Quercus* spp.), primarily members of the red oak group (subgenus *Erythrobalanus*). After crawling over, burrowing into, and/or feeding on the mats, the insects leave with pathogen spores on and in their bodies (8,15) and may vector *C. fagacearum* to fresh wounds on oaks.

Oak wilt mats are often colonized by other microorganisms. The *Graphium* ana-

morph of *Ophiostoma piceae* (Münch.) Syd. & P. Syd. (12) has been observed as a common colonizer of spring mats in Minnesota and West Virginia (4,7). A *Graphium* sp. also has been observed on mats in Illinois (2). Limited observations on the frequency of *O. piceae* occurrence on mats in Minnesota have been reported (4,12). By visual assessment, Gibbs (4) found *O. piceae* on 90% of the mats collected from trees; the mat area covered by *O. piceae* ranged from synnemata present in only one area to total coverage. We have found that visual assessment underestimates actual colonization (9). Several other fungi, such as *O. pluriannulatum* (Hedgec.) Syd. & P. Syd., *Gliocladium roseum* Bainier, *Trichoderma viride* Pers.:Fr., and *Trichothecium roseum* (Pers.:Fr.) Link, have also been observed to colonize mats (4,13).

The observed low efficiency of overland spread of *C. fagacearum* in Minnesota may be partly the result of mat overgrowth by *O. piceae* (12). To investigate this hypothesis, Reutze and Parameswaran (12) suggested the relationship between *C. fagacearum* and *O. piceae* be further examined. Specifically, they suggested that factors influencing the establishment and growth rate of *O. piceae* on oak wilt mats be identified and the frequency of *O. piceae* occurrence in wounds and mats be determined. The frequency and extent of *O. piceae* colonization of mats of different biological ages during spring in Minnesota are reported here.

## MATERIALS AND METHODS

**Study sites and tree selection.** Infected northern red (*Q. rubra* L.) and northern pin (*Q. ellipsoidalis* E. J. Hill) oaks on scattered private properties in two northern suburbs of Minneapolis-St. Paul, MN (Ham Lake and Blaine) and in a park reserve near a southern suburb (Burnsville) were utilized for this study. Records of the diseased oaks that had wilted during July or August 1994 were obtained from city foresters or the park reserve staff. These trees were most likely to produce *C. fagacearum* mats the following spring. Weekly monitoring of the selected trees for mats commenced in mid-April 1995.

**Sample collection and processing.** Oak wilt mats were collected from 82 trees in the three locations between 15 and 31 May 1995. This collection period coincided with the peak sporulation period of that spring. Only mats that had sufficiently ruptured the tree bark to allow insect access were taken. Mats were exposed by removing the bark with a hatchet and mallet, and the biological age of each mat was assessed on-site. The categorization system described by Curl (2) was followed: immature, mature, aging, and declining. Differences in *O. piceae* colonization by biological age were suspected on the basis of an earlier report of the staggered arrival of nitidulid vector species according to different mat development and deterioration categories (11). The phloem side of each mat was removed for laboratory assessment of *O. piceae* colonization; tree number, mat number, and mat age were recorded for each collection. Mats were wrapped in aluminum foil, placed in polyethylene bags, and stored on ice in coolers during transport to the laboratory.

Either six (Blaine and Burnsville) or 10 (Ham Lake) subsamples were then taken from each mat to estimate the area that was colonized by *O. piceae* or that had viable *C. fagacearum* spores. A systematic, oval pattern was used to determine the location for subsample removal. Subsamples were taken with a heat-sterilized, 3-mm-diameter cork borer at a distance of 0.5 cm from the edge of the mat, placed in individual vials, and stored in the dark at  $-4^{\circ}\text{C}$ .

The presence of viable propagules of *O. piceae* and *C. fagacearum* on subsamples was determined by serial dilution techniques. Each subsample disk was shaken into a glass vial with 4.5 ml of sterile distilled water. The vial was capped and

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vigorously agitated on a Vortex Genie 2 (Fisher Scientific, Pittsburgh, PA) for 15 s to dislodge fungal propagules. Three serial 10-fold dilutions of the resulting suspension were made in sterile distilled water, and 0.5-ml aliquots of each dilution were spread on acidified (10% lactic acid) potato dextrose agar (Difco, Detroit, MI) in petri dishes and incubated in the dark at 22°C. Colonies were counted after 7 and 14 days. *O. piceae* colonies were generally well developed after 1 week, but many *C. fagacearum* colonies needed 12 to 14 days to develop. All subsamples were processed within 4 months after collection. Mycelial and spore characteristics were used to identify the pathogen. Synnemata and mycelial conidia were used to identify *O. piceae*, and the identification was confirmed by E. B. Smalley, University of Wisconsin at Madison, by pairing 10 selected isolates with known mating types of the fungus.

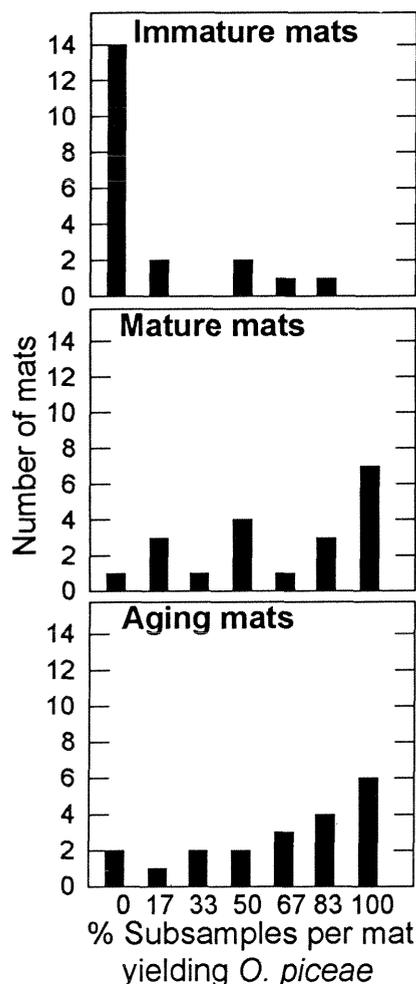
**Summary and analyses of data.** All data were summarized by mat age for each location. For frequency analyses, a sub-

sample was deemed positive for *O. piceae* if at least one colony was detected on any of the three petri dishes used per subsample for the dilution series counted. To investigate whether the extent of *O. piceae* colonization of oak wilt mats differed by mat age within study locations and whether data within mat ages could be combined across locations, data were analyzed by using the ordered categorical Kruskal-Wallis method (1,10). Because of low cell frequencies, STATXACT 3 (10), a program that computes exact, small-sample *P* values, was used to approximate these values rather than chi-square distribution. When cell frequencies were low for aging and declining mats, the categories were combined. The same methods were used to determine whether extent of recovery of *C. fagacearum* from the same mats differed by mat age. Quantitative data involving colony-forming units of *O. piceae* or *C. fagacearum* isolated from mat subsamples were averaged for each mat and grouped according to mat age and location. Only values obtained from positive subsamples were used in the calculation. Analyses of variance were performed on transformed numbers ( $\ln [CFU + 1]$ ), and mat age comparisons were made (Fisher's least significant difference) when the *F* statistic was significant (14). When an outlier was detected, the mat was omitted from the summary and analyses.

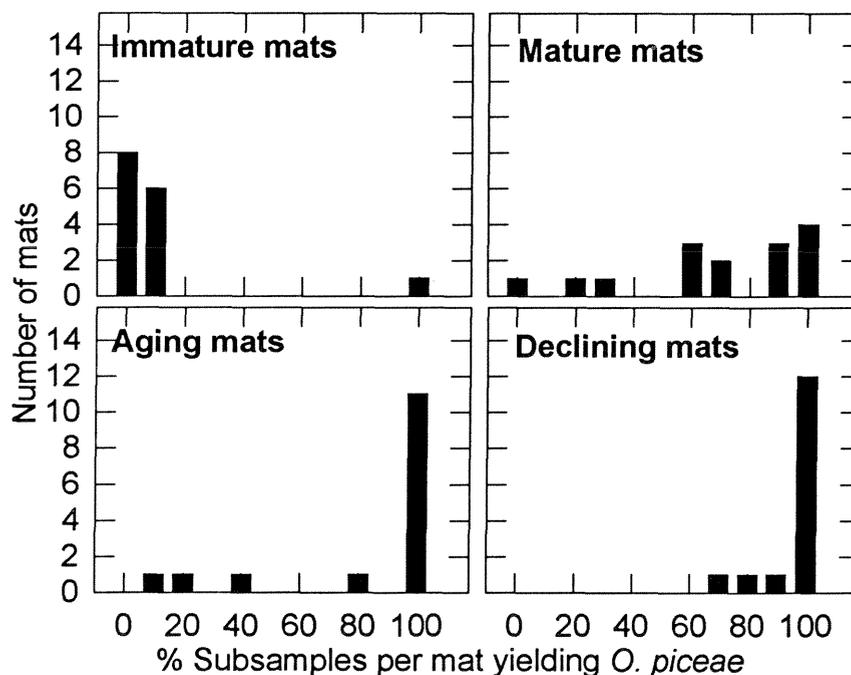
## RESULTS

Colonization of Burnsville, Ham Lake, and Blaine oak wilt mats by *O. piceae* increased with mat age; this determination

was based on the percentage of subsamples for each mat yielding the fungus (Figs. 1–3). Location effects on the extent of mat colonization were found between Burnsville and Blaine data; Ham Lake data were not compared because of the different number of subsamples tested. Thus, results are presented by location. In general terms, 16 of the 20 immature mats from Burnsville had  $\leq 17\%$  of associated subsamples with *O. piceae*; half of the aging mats had 83 or 100% of the six assayed subsamples per mat yielding the fungus (Fig. 1). A similar trend was observed with the Ham Lake mats: 14 of the 15 immature mats had  $\leq 10\%$  of the associated subsamples yielding *O. piceae*, while all the subsamples from 12 of the 15 declining mats yielded the fungus (Fig. 2). Slight differences were found for the Blaine collections: 12 of the 15 immature mats had 33% or fewer subsamples from each mat with detectable *O. piceae* compared with 13 of the 15 mature and aging plus declining mats with 67% or more positive subsamples (Fig. 3). The Burnsville subsamples yielding *O. piceae* for three mat ages averaged 14%, immature; 65%, mature; and 66%, aging. Only the percentage of immature mat colonization differed from the others ( $P < 0.001$ ). The Ham Lake mat subsamples yielding *O. piceae* for the four mat ages averaged 11%, immature; 69%, mature; 83%, aging; and 96%, declining. Extent of colonization of immature mats differed from the others ( $P < 0.001$ ), while the extent of colonization of mature mats was similar to that of aging ones ( $P > 0.051$ ) but different from that of declining mats ( $P < 0.002$ ). Similar



**Fig. 1.** Extent of colonization of oak wilt fungal mats by *Ophiostoma piceae*, Burnsville, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported for three mat ages (2). Twenty mats of each age were collected from 10 trees. Data were derived from serial dilution plating of six subsamples per mat.



**Fig. 2.** Extent of colonization of oak wilt fungal mats by *Ophiostoma piceae*, Ham Lake, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported for four mat ages (2). Fifteen mats of each age were collected from 8 to 11 trees. Data were derived from serial dilution plating of 10 subsamples per mat.

colonization was found for aging and declining mats. The Blaine subsamples yielding *O. piceae* for three mat ages averaged 27%, immature; 72%, mature; and 78%, aging plus declining. Only the extent of immature mat colonization differed from the others ( $P < 0.005$ ). On a mat-incidence basis, the frequencies of immature mats with *O. piceae* occurring in at least one subsample of a mat were 30, 47, and 53% for Burnsville, Ham Lake, and Blaine, respectively. For all other mat ages in all locations, frequency of mats with at least one subsample with *O. piceae* was 90% or greater.

Recovery of *C. fagacearum* propagules from subsamples of mats from Burnsville, Ham Lake, and Blaine generally appeared to decrease with mat age; this observation was based on the percentage of subsamples per mat that yielded *C. fagacearum* (Figs. 4–6). Differences across mat age, however, were significant only for the Ham Lake ( $P = 0.002$ ) and the Blaine ( $P < 0.03$ ) collections. In the Ham Lake collection, re-

covery (percentage of subsamples with viable *C. fagacearum* per mat) was less from declining mats (59%) than from immature and mature mats (both 97%) but similar to recovery from aging mats (64%). Extent of recovery of *C. fagacearum* from Blaine aging plus declining mats was significantly different only from that of the mature mats ( $P < 0.05$ ). Location effects on the extent of *C. fagacearum* recovery were found between Burnsville and Blaine data; thus, data from different locations were not combined. On a mat-incidence basis, the frequency of *C. fagacearum* recovery from one or more subsamples of the mat was very high (average, 99%) for all mat ages in all locations.

More than 99% of the mature, aging, and declining mats from all three locations had one or more subsamples that yielded both fungi upon isolation. When frequencies of isolation of *C. fagacearum* only, *O. piceae* only, or both were studied, it was

found that propagules of *O. piceae* were never isolated without *C. fagacearum* also being recovered. The frequency of *C. fagacearum*-only isolation was highest for immature mats and negligible for all others.

The average number of *O. piceae* colony-forming units determined per square centimeter of mat area ranged from  $1 \times 10^3$  to  $1.2 \times 10^6$  (Table 1). Propagule numbers varied by mat age ( $P < 0.01$ ) but not by location ( $P > 0.70$ ). Recovery of *O. piceae* colony-forming units increased with mat age with differences ( $P < 0.01$ ) found between ages:  $3 \times 10^3$ , immature;  $52 \times 10^3$ , mature; and  $640 \times 10^3$ , aging and declining mats. Large numbers of *C. fagacearum* colony-forming units ( $>8.6 \times 10^4/\text{cm}^2$ ) were commonly recovered from the same mat subsamples. Because propagule numbers varied by location ( $P < 0.01$ ), data were further analyzed within each location. Numbers of *C. fagacearum* propagules increased with mat age on Burnsville mats ( $P = 0.04$ ), while no differences were found for Ham Lake ( $P > 0.06$ ) and Blaine ( $P > 0.30$ ) mats.

## DISCUSSION

Except on immature mats, *O. piceae* was found on at least one subsample of 90% of all mats collected during the spring in the three east-central Minnesota locations. This agrees with an earlier report by Gibbs (4), if it is assumed that mature, aging, and declining mats were examined in his sample. The extent of colonization of oak wilt mats by *O. piceae* generally increased with progression of biological age of the mats, but statistical differences ( $P < 0.05$ ) in the extent of colonization were consistently found only between immature mats and all others. The lower rate of isolation of *O. piceae* from immature mats is not surprising. The rupture in the bark that allowed insect access had presumably occurred more recently in the immature mats than in the mature, aging, and declining mats. *O. piceae* is apparently introduced into the mats via contaminated nitidulids carrying the fungus (8,12). The general patterns of coverage across mat ages were similar for Burnsville and Blaine. The number of *O. piceae* propagules recovered from mat subsamples increased with mat age, attesting to the ability of *O. piceae* to quickly overgrow and sporulate on oak wilt mats. These results agree with those reported earlier for mats collected daily for 1 week from one tree (12). However, our study results differ from those of this earlier report in that we never isolated *O. piceae* without parallel recovery of *C. fagacearum*, even on the oldest mats examined. The difference in results may be explained by the isolation techniques used in the two studies. Direct plating onto 2% potato dextrose agar of mat pieces or ascospores exuded from perithecia was used in the earlier study, while serial dilution plating

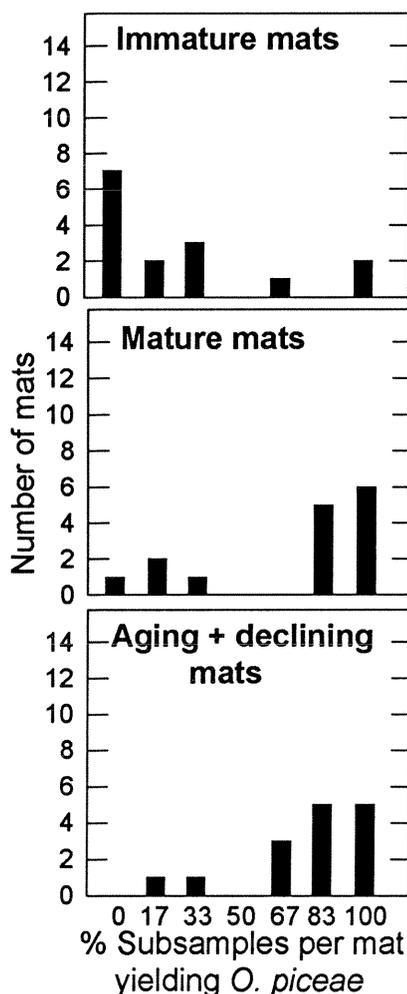


Fig. 3. Extent of colonization of oak wilt fungal mats by *Ophiostoma piceae*, Blaine, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported by mat ages (2). Fifteen mats of each age were collected from 5 to 10 trees. Data were derived from serial dilution plating of six subsamples per mat.

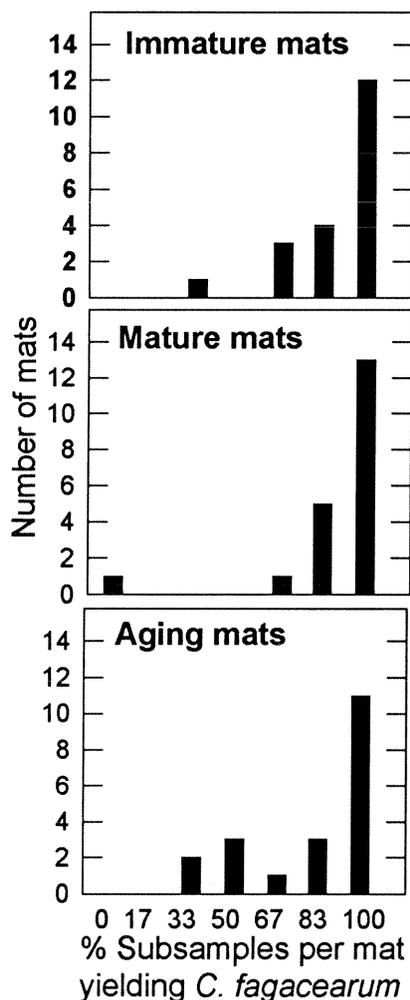


Fig. 4. Extent of *Ceratocystis fagacearum* recovery from oak wilt mats collected from Burnsville, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported for three mat ages (2). Twenty mats of each age were collected from 10 or 11 trees. Data were derived from serial dilution plating of six subsamples per mat.

of washings from mat subsamples was used in our study.

The interaction between nitidulids and *O. piceae* and *C. fagacearum* on oak wilt mats and in fresh wounds on healthy trees has been discussed by others (4,7). Reutze and Parameswaran (12) stated that the colonization of mats by *O. piceae* could reduce or prevent contamination of potential insect vectors with *C. fagacearum*; however, the authors made no mention of direct harm to *C. fagacearum* mats by *O. piceae*. Colonization of oak wilt mats by *O. piceae*

should conceivably hasten deterioration of the mats, as would colonization by secondary invaders and other contaminating microorganisms (13). We do not consider *O. piceae* to be a mycoparasite of *C. fagacearum*, and our parallel recovery of *O. piceae* and *C. fagacearum* at somewhat similar levels of colony-forming units from mat subsamples (Table 1) suggests that the two fungi coexist quite well. Concerning these fungi on insect vectors, Jewell (7) proposed that *O. piceae* (under the name *G. rigidum*), *O. pluriannulatum*, and *C.*

*fagacearum* share a symbiotic relationship with nitidulids; however, *C. fagacearum* is probably the newest member of this relationship.

The interaction between *O. piceae* and *C. fagacearum* on a wound surface has been described as competitive by Gibbs (4). No infection occurred when a fresh xylem-penetrating wound on a healthy oak was inoculated with *O. piceae* 24 h before challenge inoculation with *C. fagacearum*. On the basis of this finding, Gibbs speculated that *O. piceae* may be a naturally occurring biological control agent that prevents overland transmission of *C. fagacearum* at the fresh wound surface. Considering the more exposed position of *O. piceae* synnemata with conidial masses compared with the more embedded position of *C. fagacearum* perithecia with exuded ascospores on the mats as well as hyphal overgrowth of ascospore masses on perithecial tips, Reutze and Parameswaran (12) speculated that potential vectors

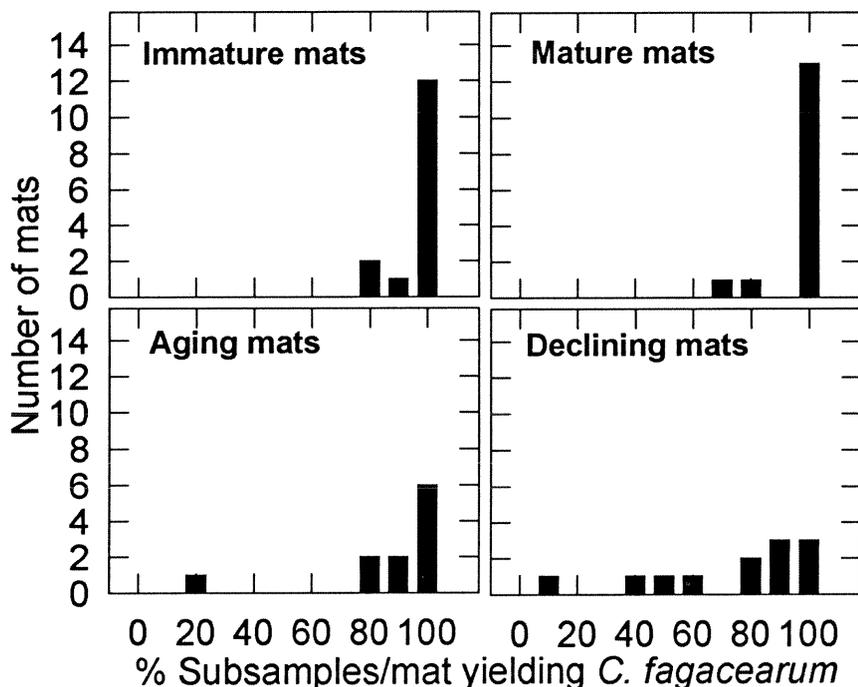


Fig. 5. Extent of *Ceratocystis fagacearum* recovery from oak wilt mats collected from Ham Lake, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported for four mat ages (2). Fifteen mats of each age were collected from 8 to 11 trees. Data were derived from serial dilution plating of 10 subsamples per mat.

Table 1. Colony-forming units (CFU) of *Ophiostoma piceae* and *Ceratocystis fagacearum* isolated from oak wilt fungal mats in three locations, spring 1995<sup>a</sup>

Location Mat age <sup>b</sup>	Mats yielding <i>O. piceae</i>		Mats yielding <i>C. fagacearum</i>	
	Total	Average CFU/cm <sup>2c</sup> (×10 <sup>3</sup> )	Total	Average CFU/cm <sup>2c</sup> (×10 <sup>3</sup> )
Burnsville				
Immature	6	2	19	86
Mature	18	31	19	200
Aging	18	240	19	280
Ham Lake				
Immature	7	6	14	190
Mature	13	61	14	260
Aging	15	910	11	500
Declining	15	1,200	12	400
Blaine				
Immature	8	1	13	340
Mature	14	69	15	290
Aging + declining	15	625	14	544

<sup>a</sup> Data were derived from isolation of each fungus from 3-mm-diameter fungal mat subsamples by serial dilution plating techniques. Either six (Burnsville and Blaine) or 10 (Ham Lake) subsamples were tested per mat.

<sup>b</sup> Mat ages are based on descriptions by Curl (2).

<sup>c</sup> Values are based on isolation from mats from which one or more subsamples yielded the fungus.

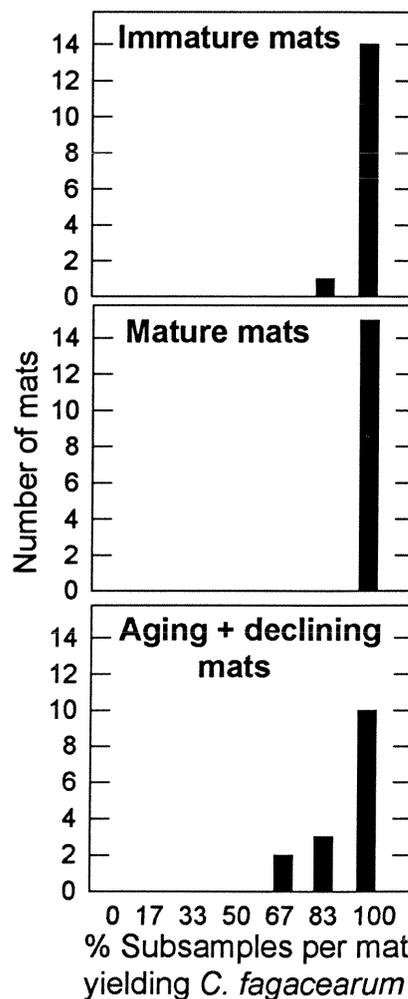


Fig. 6. Extent of *Ceratocystis fagacearum* recovery from oak wilt mats collected in Blaine, MN, spring 1995. The numbers of mats with different frequencies of isolation are reported by mat age (2). Fifteen mats of each age were collected from 5 to 10 trees. Data were derived from serial dilution plating of six subsamples per mat.

leaving *O. piceae*-colonized mats are likely to carry *O. piceae* spores more frequently than pathogen spores and in many instances would arrive at fresh wound surfaces with *O. piceae* before *C. fagacearum* arrived. Thus, *O. piceae* overgrowth of oak wilt mats would indirectly reduce the number of effective transmissions of the oak wilt fungus. In this sense, nitidulids would also appear to play a role in the biological control of overland transmission of *C. fagacearum* in nature (8).

In an attempt to test these hypotheses, we are currently investigating the frequency of *O. piceae* and/or *C. fagacearum* on nitidulids collected from mats differentially colonized by *O. piceae*. Free-flying nitidulids were previously found to be carrying *O. piceae* and *C. fagacearum* at similar frequencies (8).

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