Etiology of Preharvest Colonization of Bing Cherry Fruit by Fungi

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ABSTRACT

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During 1991–1993, the colonization of cherry fruit (*Prunus avium* 'Bing') by *Alternaria* spp., *Aureobasidium pullulans, Cladosporium* spp., and other fungi was detected soon after petal fall and thereafter until harvest, when 87–100% of surface-disinfested fruit yielded fungi. Taxa most frequently recovered were pathogenic to ripe, wounded fruit. Receptacular and stylar scars were frequently colonized. Histological sections of receptacle and stylar scar tissues from mature cherries revealed hyphae in walls and lumina of necrotic cells and vascular tissues.

Additional keywords: *Arthrinium, Botrytis, Epicoccum, Penicillium,* postharvest pathology, *Stemphylium, Ulocladium.*

Postharvest losses of sweet cherry fruit due to fungal decay often occur despite postharvest application of fungicides and other recommended control measures (35). Since the Japanese market was opened to U.S. cherries in 1978, the volume of exported cherries has steadily grown, and there has been an increasing need to reduce postharvest losses at the terminal markets. Losses in exported lots generally do not become evident until arrival at the destination, after the costs of production, fumigation, packing, and shipping have already been incurred.

Alternatives to fungicide use for postharvest decay control in cherries are needed, because Japan has established no regulatory tolerance for postharvest fungicide residues. Chemical and biological control experiments in our laboratory yielded variable levels of postharvest disease control when treatments were applied either postharvest or just before harvest (R. G. Roberts, *unpublished*). Accordingly, we developed a working hypothesis that sweet cherry fruit in eastern Washington are colonized in the field prior to harvest by the fungi responsible for postharvest decay. Preliminary data collected in 1991 showed that colonization of cherry fruit by fungi began soon after petal fall and continued throughout the fruit development period. Therefore, more comprehensive experiments to determine the origin, identity, and prevalence of fungi in cherry fruit tissues were conducted in 1992 and 1993.

MATERIALS AND METHODS

Fruit sampling. Samples of 100 sweet cherry (*Prunus avium* L. 'Bing') fruit without visible injury or disease were gathered weekly from two to six trees at each of three locations (Columbia View, Orondo, and Stemilt Hill) within 40 km of Wenatchee, Washington. Cherries from all trees sampled at a location were pooled to provide a single sample for each location at each sampling date. Sampling began 1 wk after petal fall and continued through week 6 in 1991, and through harvest (weeks 10–11) in 1992. In 1993, samples of 100 fruit from eight trees were collected weekly for 8 wk prior to harvest at Columbia View. Fruit were placed on ice immediately after collection and transported to the lab.

Fungicide application at each site varied but typically included one or two cover sprays of myclobutanil (Rally 40W at 183–584 ml/ha), two or three cover sprays of fenarimol (Rubigan 1EC at 440–913 ml/ha), and up to one cover spray of wettable sulfur (Microthiol sulfur at 6.72 kg/ha) during the growing season.

Isolation and maintenance of fungi. Stems were removed and fruit were surface-disinfested by immersion for 3–4 min, including sonication for 1 min, in NaOCl at 100 μg/ml with low-chlorine demand surfactant (LT150 Rinse Aid, Savolite Corp., Seattle, WA 98108) at 100 μg/ml. In 1991 and 1992, disinfested fruits 1–5 wk old were bisected and placed cut-surface down on malt extract agar (MEA) (34) and/or half-strength V8 agar (2) plates and held in an incubator at 25 C. After the fifth week, whole fruits were incubated at 25 C on plastic trays that kept fruit well separated. Trays were stored in moist chambers for 10–14 days; then fruit on plates and trays were examined at 10–60× for fungal growth. In 1991 and 1992, most isolates were obtained from receptacular and stylar scars; so in 1993, stylar scars and receptacles were excised, plated directly on agar, and incubated as above.

Small bits of mycelium were transferred from colonized fruit to half-strength V8, MEA, or potato-dextrose agar (PDA)(2) and incubated at 20–25 C until growth nearly covered the plate. Some plates were placed approximately 45 cm from 15-watt fluorescent and 15-watt near-ultraviolet lights (General Electric Co.) to enhance sporulation.

In 1991 and 1992, isolates were preserved for later identification. Propagules were harvested from plates, transferred to silica gel (9), and stored at −16 C. The locations on the fruit from which the isolates grew were recorded. Fungal isolates were coded according to sample site, week of collection, and the cherry from which the isolate was obtained.

Histology of infected fruit. In 1992, unblemished cherry fruit were collected and placed on ice weekly 1–6 wk prior to harvest, surface-disinfested as described above, and incubated at 20–25 C until examination at 25–60× revealed hyphae in receptacles and stylar scars. In 1993, other fruits were collected in a similar fashion and stored 6 days at 1 C. Stylar scars and receptacles were excised,

fixed under vacuum in phosphate-buffered 2% glutaraldehyde, then either dehydrated in an ethanol/*n*-butanol series prior to infiltration with glycol methacrylate (14) or dehydrated with ethanol prior to infiltration with Spurr's medium (33). After polymerization, methacrylate-embedded tissue was sectioned at 7–20 μm on a Spencer rotary microtome, and Spurr's-embedded tissue was cut at 2 μm on a Reichert-Jung Ultracut E microtome. Methacrylate sections were stained with periodic acid and Schiff reagent. Spurr's resin sections were stained with Paragon stain (29). All sections were counterstained with aniline blue-black and photographed at 100–1,000× with a Zeiss Axioplan microscope.

Identification of isolates. Pure cultures of isolates growing on half-strength V8, MEA, PDA, or in the case of *Aureobasidium* isolates, liquid cultures of 3% glucose yeast extract peptone (LiqGYP) (20), were examined at 200–1,000×. Some genera were readily recognizable at 40–60× and were classified to genus on that basis. Reference slides made with glycerin jelly, or Shear's mounting medium (9) for other isolates, were examined with compound microscopes. Hermanides-Nijhof (17) was followed for identification of *Aureobasidium* and *Hormonema*; substrate utilization tests (8) were conducted for representative *Aureobasidium* isolates. Booth (3) was followed for identification of *Fusarium* isolates, Pitt (26,27) for *Penicillium* isolates, and Klich and Pitt (19) for *Aspergillus* isolates.

Pathogenicity tests. Groups of five to seven ripe Bing cherries were surface-disinfested as described above. Fruit within each group were either stab-inoculated with spores from a fresh culture of a representative isolate of a frequently isolated fungal taxon, or were stabbed with a sterile needle (negative controls). Fruit were incubated on trays in moist chambers as described above. Fungi were recovered from inoculated cherries by transferring a small mass of tissue from decayed cortex or epidermis to agar media. Resultant growth was examined at 400–1,000×. Isolates used were *Aureobasidium pullulans* (de Bary) G. Arnaud AV7-16a, AV7-24a, AV7-30b, AV7-44b, AV7-47a, AV7-73, AV7-85a, AV7-86a, and AV7-88c; *Cladosporium herbarum* (Pers.:Fr.) Link AV7-49, AV7-85b, ST6-66a, and ST6-91; *Cladosporium cladosporioides* (Fresen.) G.A. De Vries ST9-27; *Alternaria alternata* (Fr.:Fr.) Keissl. AV7-13, CV7-59, CV7-71, and CV7-73; *Cladosporium malorum* Ruehle AV9-70, CV7-82, ST6-27, and ST6-100; *Arthrinium arundinis* Dyko & Sutton CV6-62a and AV5-53; *Ulocladium atrum* G. Preuss AV8-30, CV10-42, ST10-73, and ST11-36; *Epicoccum nigrum* Link CV10-16, ST11-28a, and ST11-78; *Stemphylium botryosum* Wallr. ST9-95a and AV9-73; and *Aspergillus ustus* (Bainier) Thom & Church AV10-28, AV10-37, and ST7-17. Tests with *Aureobasidium, Alternaria, Cladosporium,* and *Arthrinium* were repeated a minimum of two times.

Statistics. Untransformed percentage colonization data were analyzed by linear regression in SigmaPlot (Jandel Scientific, San Rafael, CA 94912-7005).

RESULTS

Isolation of fungi from fruit. In all 3 yr and locations, colonization of fruit by fungi was detected soon after petal fall. In 1991, 61, 84, and 77% colonizations were observed at Columbia View, Orondo, and Stemilt Hill, respectively, in the fifth or sixth week (Fig. 1). At harvest in 1992, the percentage of fruit colonized by fungi was 87, 100, and 100% at Orondo, Columbia View, and Stemilt Hill, respectively; and in 1993 at Columbia View, the percentage was 100%.

Regression analysis revealed a positive relationship between percentage colonization of cherry fruit by fungi and weeks after petal fall (Figs. 1A–C and 2A–C) from all locations in all years. Comparison of the slopes of the 1991 and 1992 regression lines revealed a similar positive relationship of fungal colonization to weeks past petal fall at all three locations in the same year, but the slopes differed by year at all locations. At Columbia View (Fig. 2A) and Orondo (Fig. 2B) in 1991, colonization of cherries by *Cladosporium* and *Aureobasidium* increased more rapidly than colonization by *Alternaria*. At Stemilt Hill in 1991, colonization by *Cladosporium* increased more rapidly than by either *Aureobasidium* or *Alternaria* (Fig. 2C).

Colonization of cherry fruit in 1992 by individual taxa varied by location but, with the exception of *Cladosporium* at Stemilt Hill, there was a positive relationship between colonization by fungi and weeks after petal fall, as in 1991 (Fig. 3). The slopes of the regression lines for each fungus and location in 1992 were shallower than in 1991, however, which is also reflected in the shallower slope for total fungi in 1992 (Fig. 1). The colonization data for individual taxa in 1992 (Fig. 3) were less consistent than data for total fungi from all years (Fig. 1), which is reflected in the relatively lower r^2 values. *Penicillium* was isolated most frequently from Columbia View and was nearly absent in fruit from Orondo. *Ulocladium* was isolated most frequently from Stemilt Hill and was also nearly absent from Orondo.

The following fungi were isolated from cherries sampled at three locations in 1992: *Acremonium* spp., *Allewia eureka* (E. Simmons) E. Simmons, *Alternaria alternata, Arthrinium arundinis, Ascochyta* spp., *Aspergillus nidulans* (Eidam) G. Wint., *A. puniceus* Kwon & Fennel, *A. ustus, A. versicolor* (Vuill.) Tiraboschi, *Aureobasidium pullulans, Botryodiplodia* sp., *Botrytis cinerea* Pers.:Fr., *Camarosporium* spp., *Chaetomium globosum* Kunze:Fr., *Cladosporium cladosporioides, C. herbarum, C macrocarpum* G. Preuss, *C. malorum, Coniothyrium* spp., *Cryptosporiopsis* sp., *Curvularia inaequalis* (Shear) Boedijn,

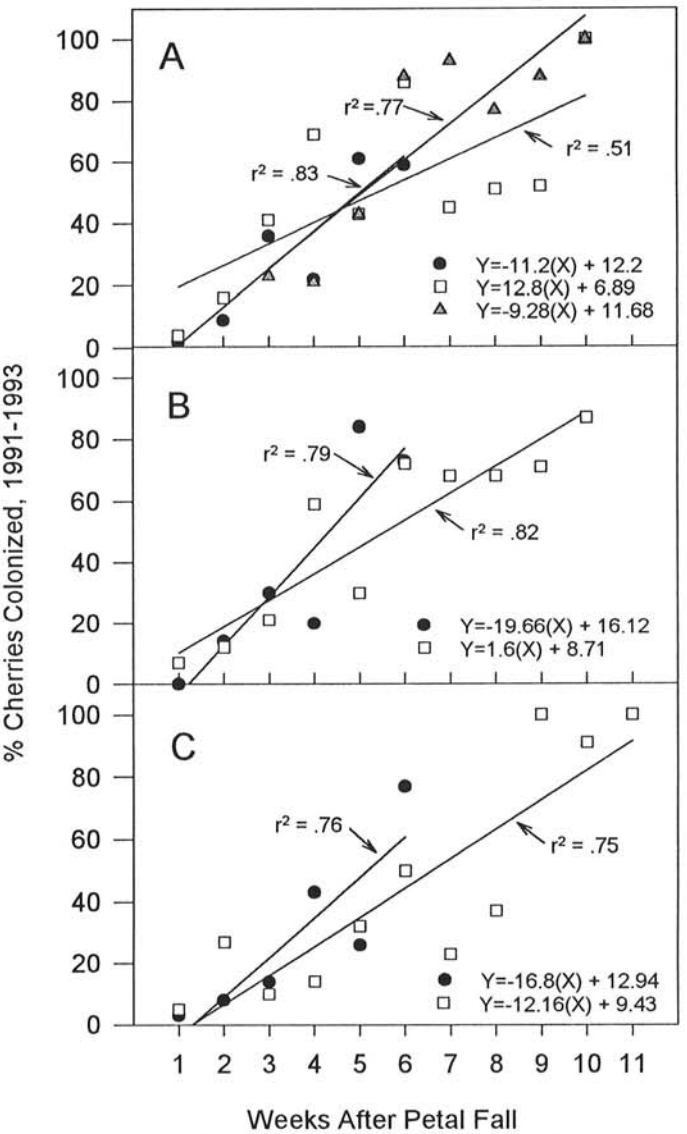

Fig. 1. Percentage of cherry fruit colonized by fungi at **A,** Columbia View; **B,** Orondo; and **C,** Stemilt Hill during 1991(●), 1992 (□), and 1993 (△).

C. lunata (Wakk.) Boedijn, *C. pallescens* Boedijn, *Cytospora* spp., *Dendrophoma* sp., *Drechslera* state of *Cochliobolus spicifer* R.R. Nelson, *Drechslera verticillata* (O'Gara) Shoemaker, *Embellisia eureka* (E. Simmons) E. Simmons, *Emericella nidulans* (Eidam) Vuill., *Epicoccum nigrum, Fusarium oxysporum* Schlechtend.:Fr., *F. sporotrichioides* Sherb., *Geomyces pannorus* (Link) Sigler & Carmichael, *Geotrichum* sp., *Hormonema* state of *Pringsheimia* or *Dothiora, Humicola fuscoatra* Traaen, *Leptodiscella africana* (Papendorf) Papendorf, *Leptosphaeria* sp., *Libertella* sp., *Mucor* sp., *Mycovellosiella* sp., *Paecilomyces lilacinus* (Thom) R.A. Samson, *P. ochraceus* Onions & Barron, *P. variabilis* Barron, *Penicillium aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. expansum* Link, *P. fellutanum* Biourge, *P. glabrum* (Wehmer) Westling, *P. implicatum* Biourge, *P. janthinellum* Biourge, *P. raistrickii* G. Sm., *P. roquefortii* Thom, *P. rugulosum* Thom, *P. simplicissimum* (Oudem.) Thom, *P. variabile* Sopp, *P. viridicatum* Westling, *Periconia igniaria* E. Mason & M.B. Ellis, *Perisporium funiculatum* Preuss, *Phaeoramularia hachijoensis* Matsushima, *Phaeoseptoria phalaridis* (Trail) R. Sprague, *Phaeosphaeria eustoma* (Fuckel) L. Holm, *P. fuckelii* (Niessl) L. Holm, *Phoma complanata* (Tode:Fr.) Desmaz., *P. nebulosa* (Pers.:Fr.) Berk., *P. pomorum* Thuem., *Phoma* spp., *Pithomyces chartarum* (Berk. & M.A. Curtis) M.B. Ellis, *Pleospora helvetica* Niessl, *P. herbarum* (Pers.:Fr.) Rabenh., *P. media* Niessl, *P. tragacanthae* Rabenh., *P. vagans* Niessl, *Pseudoseptoria donacis* (Pass.) Sutton, *Spiniger* sp., *Spicaria violacea* Abbott [non Petch], *Sporormia subticinensis* Mout., *Sporormiella intermedia* (Auersw.) Ahmed & Cain, *Stemphylium botryosum* Wallr., *Trichoderma atroviride* Karsten [non Bissett], *T. koningii* Oudem., *Truncatella angustata* (Pers.) S.J. Hughes, *Ulocladium atrum,* and *U. consortiale* (Thuem.) E. Simmons. Most fungi isolated from cherry samples from the three locations in 1992 were Hyphomycetes, but Coelomycetes and Ascomycetes were common. Only one Zygomycete was isolated. Approximately 90%

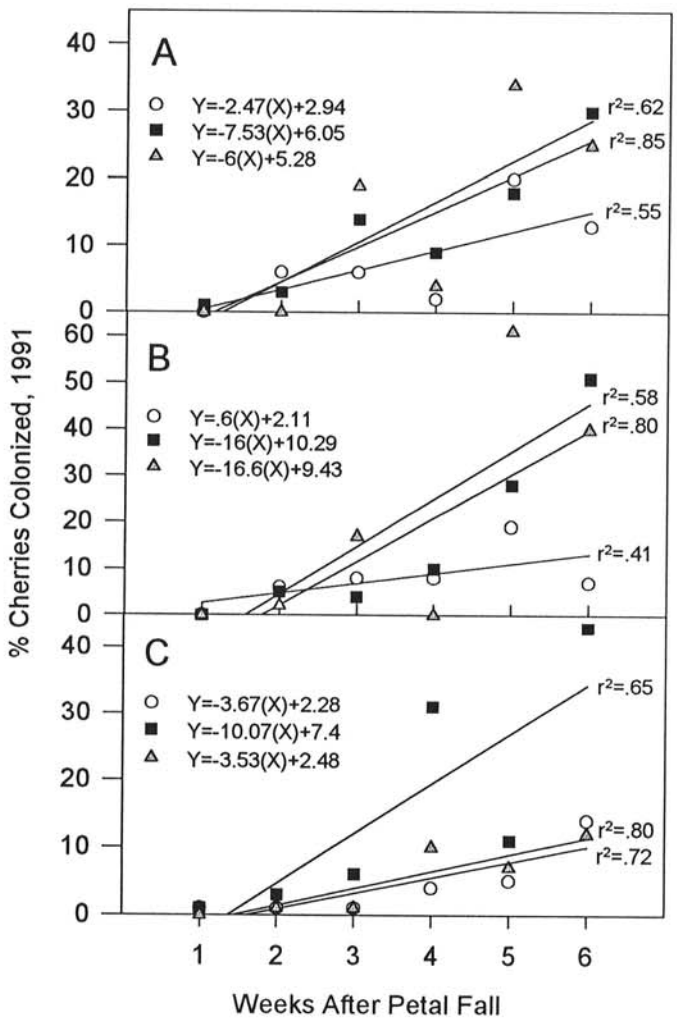

Fig. 2. Percentage of cherry fruit colonized by *Alternaria* (○), *Cladosporium* (■), and *Aureobasidium* (△) at **A,** Columbia View; **B,** Orondo; and **C,** Stemilt Hill in 1991.

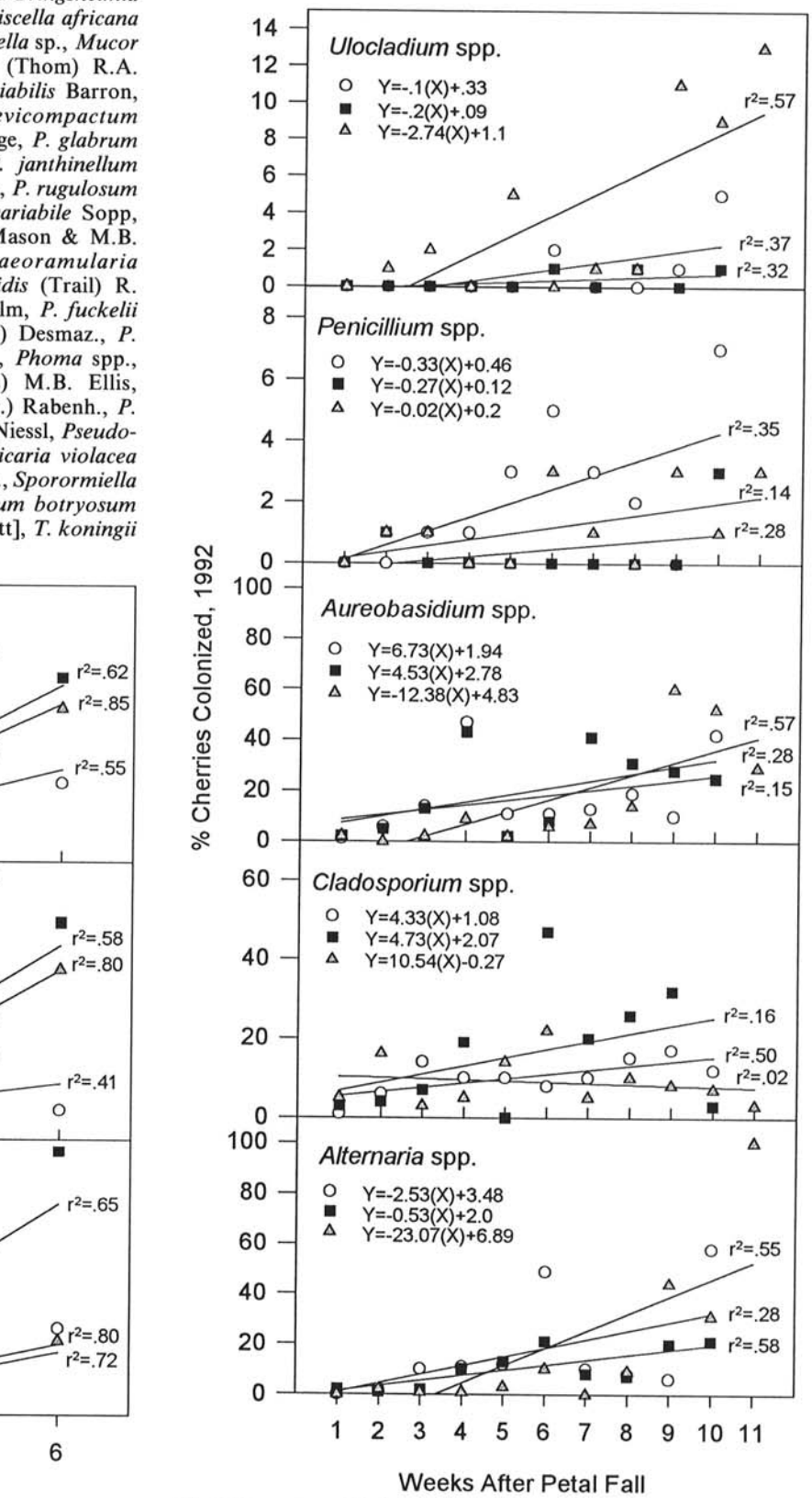

Fig. 3. Percentage of fruit colonized by *Alternaria, Cladosporium, Aureobasidium, Penicillium,* and *Ulocladium* at Columbia View (○), Orondo (■), and Stemilt Hill (△) in 1992. The total percentage can be >100% because some fruit were colonized by more than one fungus.

of all isolates were obtained from stylar and receptacular scars. The genera most frequently isolated from symptomless fruit in 1991–1992 were *Alternaria, Cladosporium,* and *Aureobasidium.* Other genera, most notably *Penicillium* and *Ulocladium,* were isolated less consistently (Figs. 2 and 3). In 1992, *Epicoccum* was isolated from 12 samples and colonized 13% of the fruit in one sample, *Botrytis cinerea* was isolated in six samples, and *Arthrinium* was isolated in only four samples but was found in 10% of the fruit in one sample. One or two *Phoma* isolates were present in 26% of the samples in 1992.

Pathogenicity tests. All isolates of *Cladosporium herbarum, C. cladosporioides, C. malorum, Alternaria alternata, Arthrinium arundinis, Ulocladium atrum, Epicoccum nigrum, Stemphylium botryosum,* and *Aureobasidium pullulans* tested were pathogenic to wounded cherry fruit. *Aspergillus ustus* was not pathogenic. Fungi reisolated from rotted fruit were morphologically identical to those with which the fruit were inoculated.

Histology. Hyphae were detected in scars and receptacles in locations affording probable protection from surface-disinfestation at 100 μg/ml NaOCl. Intraluminar and intramural hyphae were observed in necrotic stylar scar and receptacular tissues. Hyphae penetrated several cell layers deep into vascular tissues contiguous with stylar scars or receptacular margins (Fig. 4A–F). When disinfested fruit were incubated prior to fixation, penetration of stomata adjacent to the stylar scar by hyphae originating in scar tissue was observed (Fig. 4F). Hyphae originating in stylar scar tissue also grew under the cuticle in incubated fruit. No hyphae were seen in the interior receptacular tissues, although fungi, especially *Alternaria* and *Aureobasidium* spp., were isolated from distal portions of surface-disinfested stems in related experiments (R. G. Roberts, *unpublished*).

DISCUSSION

Results from this study demonstrate that moribund fruit tissues can serve as colonization sites for normally saprotrophic fungi that are capable of causing postharvest decay. We demonstrated that those fungi most frequently isolated from these moribund and asymptomatic tissues are pathogenic to wounded cherry fruit, and that penetration of cherry fruit epidermis can occur without wounding when mycelia growing from these colonized loci penetrate stomata or enter healthy tissue by subcuticular hyphal proliferation. Wounds incurred during harvesting and handling could be readily colonized by these resident fungi, which because they are protected by host tissue, would not be affected by hypochlorite or other nonsystemic treatments.

The repeated isolations of fungi from 87 to 100% of unblemished fruit at harvest, and the demonstration of hyphae in histological sections of stylar scars and receptacles and adjacent tissues, confirm the hypothesis that symptomless fruit is colonized by fungi prior to harvest in north central Washington. In related experiments, we obtained similar isolation frequencies when cherry fruit were immersed in 70% ethanol prior to immersion in NaOCl, and the concentration of NaOCl was raised to 5,250 μg/ml (F. M. Dugan and R. G. Roberts, *unpublished*). Similar but more stringent techniques are used for the isolation of plant endophytic fungi (25) and are useful here to demonstrate that surface-contaminating fungi were not problematic, although no technique short of axenically producing cherry fruit can guarantee a complete absence of contaminating fungi on fruit surfaces. The question of internal vs. external fungi and the interpretation of data from studies that employ surface sterilization techniques (5,7) is not new. The histological demonstration of fungi in cherry fruit tissues, however, provides unambiguous evidence of the presence of internal fungi in cherry fruit, although the identity of the fungi in sectioned material is not known. We have also used Petrini's technique (25) to demonstrate the presence of endophytic *Alternaria alternata* in asymptomatic cherry peduncles (R. G. Roberts and F. M. Dugan, *unpublished*). Results of our histological studies also suggest that pathogenic fungi may readily move from dead scar tissue into the fruit as they mature, either through the original site(s) of colonization or from secondary sites such as stomata. That the majority of isolations yielded fungi that were pathogenic to wounded fruit indicates that the presence of these fungi in cherry fruit at harvest is not trivial and is likely to predispose colonized fruit to postharvest decay and to limit their storage life.

Alternaria and *Cladosporium* have long been known to cause postharvest losses of cherry fruit (16). The large numbers of *Alternaria* and *Cladosporium* isolates obtained from fruit prior to harvest are consistent with the predominance of these genera in decayed cherries from commercial packing houses (R. G. Roberts, *unpublished*) and terminal markets (16). Patterson (24) reported that commercially handled (uninoculated) sweet cherries developed 52.3% decay when held at 5 C for 8 wk. De Vries-Paterson et al (6) reported the growth of *Alternaria* spp. from symptomless, uninoculated cherry fruit in Michigan. Johnson et al (18) reported the isolation of endophytic, pathogenic *A. alternata* from mango stem tissue prior to flowering and thereafter. *Alternaria* isolates from our study keyed to *A. alternata* in Neergaard (22) and matched the description for *A. alternata* in Ellis (11). However, *A. alternata* is considered a species complex of a dozen or more species (30), and some isolates disposed of as *A. alternata* in our collection are undoubtedly representatives of *A. arbusti* Simmons (31). Pathogenicity of *A. arbusti* to cherry fruit was not evaluated.

There are also records of *Aureobasidium* infections of stone fruits, including cherry (21). Diehl (10) reported that *Aureobasidium* caused lesions on leaves of stone fruits. These *Aureobasidium* infections are currently attributed to *A. prunicola* (Ellis & Everh.) Hermanides-Nijhof, not to *A. pullulans* (13). Other investigators have also obtained isolates, identified only as *Pullularia* (*Dematium*) sp., from naturally occurring cherry fruit lesions but did not identify them further or conduct pathogenicity tests (12). Our *Aureobasidium* isolates are best accommodated in *A. pullulans* fide Hermanides-Nijhof (17).

Noteworthy by their absence were *Rhizopus* spp. and *Monilia* spp. Brown rot is not an economically important disease of cherry in central Washington, although its importance elsewhere in the western United States is well documented (23). *Rhizopus* is a well-known postharvest pathogen of cherries and other stone fruits (32), and its absence from these studies is consistent with early work by Brooks (4), who showed the occurrence of Rhizopus rot of peach is a function of postharvest handling practices.

The diversity of genera and species recorded during this study is typical of the diversity found in other studies when agricultural commodities were extensively evaluated for the occurrence of fungi (15,28). Some genera isolated are normally associated with other substrates or are generally regarded as saprophytes. Such genera were not responsible for appreciable percentages of colonization in this study, although some taxa (*Ulocladium atrum, Arthrinium arundinis, Epicoccum nigrum*) were isolated from 10–13% of some samples and were pathogenic to ripe cherries. Probably because stylar and receptacular tissues become necrotic during the abscission of floral parts, these sites were readily colonized by many fungi usually considered saprophytes. Other known pathogenic fungi (*Botrytis cinerea, Penicillium expansum,* and others), although isolated with low frequency here, may nevertheless possess special pathogenic significance because of their ability to spread in storage (16,32). This is the first report of pathogenicity to cherry fruit by *Arthrinium arundinis* and *Cladosporium malorum.*

Preharvest colonization of fruit by fungi is an important factor that affects the efficacy of chemical control measures. In the absence of strongly systemic eradicative or prophylactic control technologies, prevention of colonization and subsequent postharvest decay will depend on control strategies that must begin early in the growing season. Current Washington State University crop protection recommendations do not address control of these fungi during the growing season (1), and current extension publications (35) that address integrated management of postharvest diseases of tree fruits likewise contain no recommendations. As integrated approaches to management of postharvest diseases of cherry fruit are developed and implemented, the colonization of

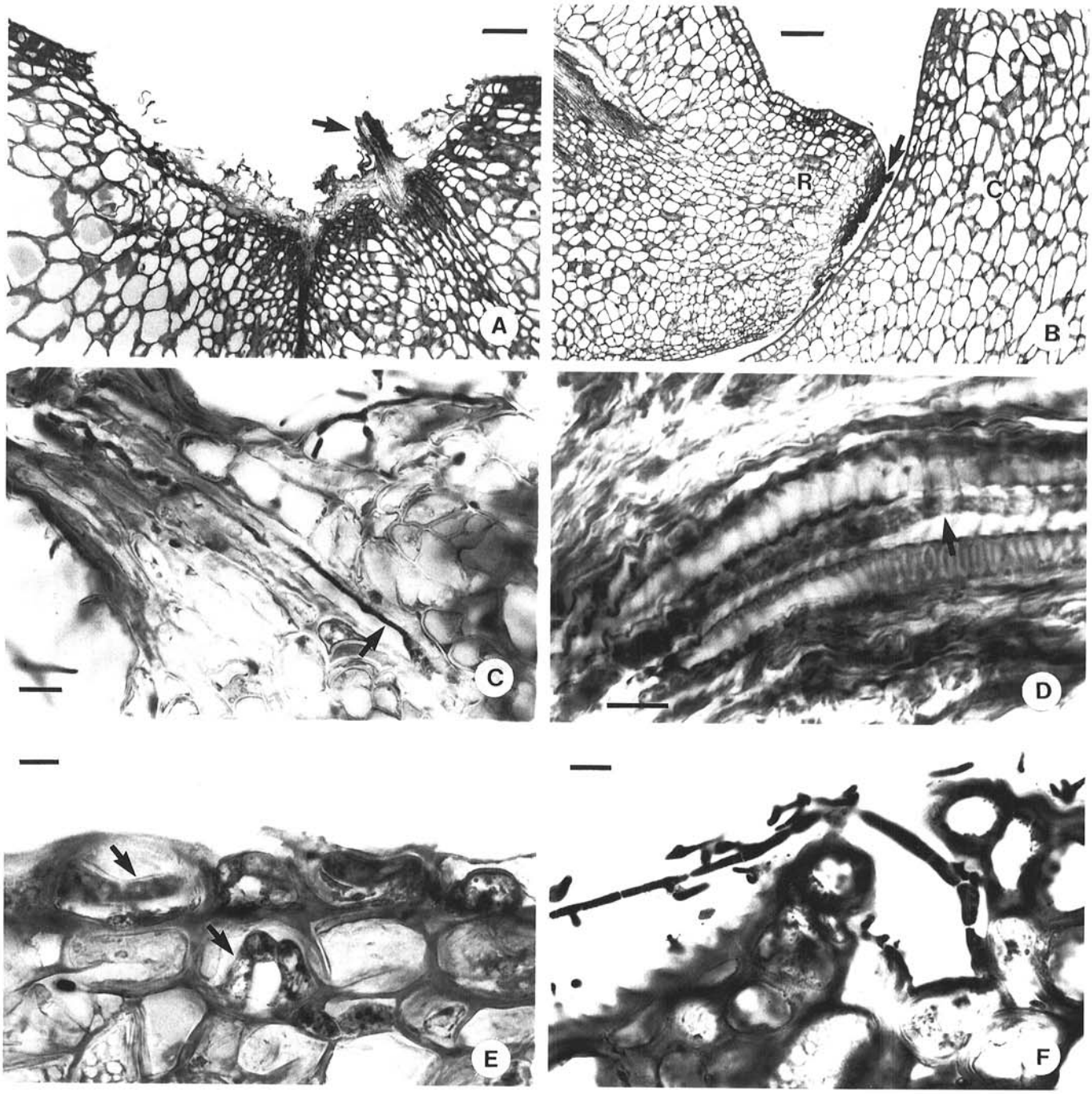

Fig. 4. Typical colonization sites and instances of colonization or infection. **A,** Necrotic cells in stylar scars, especially those where vascular tissue projected into scars (arrow) was colonized by time of harvest; **B,** necrotic cells at margin of receptacle (arrow) were typically colonized by fungal hyphae; **C,** hypha (arrow) inside vascular tissue associated with stylar scar; **D,** hypha (arrow) inside vascular tissue of receptacular margin; **E,** hyphae (arrows) in epidermal and subepidermal cells at receptacular margin; and **F,** stoma penetrated by hyphae. Cells adjacent to substomatal chamber show signs of necrosis. Abbreviations: R = receptacle; C = fruit cortex. A–D, longitudinal sections; E, tangential section. All sections are from glycol methacrylate-embedded tissues cut at 7–10 μm. Bars = 100 μm in A and B, 10 μm in C–F.

cherry fruit by fungi before harvest must be recognized as potentially affecting fruit quality, and new control strategies must be developed. Unfortunately, the fungicides routinely applied for mildew control in north central Washington have little effect on the isolation frequencies of fungi from cherry fruit (F. M. Dugan and R. G. Roberts, *unpublished*). We will present in a subsequent paper results of field experiments that evaluate how the application of fungicides effective against the predominant fungi isolated during this study affect preharvest colonization and postharvest shelf life. Field experiments to evaluate cultural and biological controls for preharvest fungal colonization should be conducted to establish effective and commercially viable control measures.

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