Adhesion and Removal of Conidia of *Botrytis cinerea* and *Penicillium expansum* from Grape and Plum Fruit Surfaces

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ABSTRACT

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Adhesion and removal of dry and wet conidia of *Botrytis cinerea* and *Penicillium expansum* on dry and wet surfaces of grape and plum fruit were investigated. Conidia of *B. cinerea* adhered more strongly when applied in a water suspension or to the wet surface of grape fruit than when dry conidia were applied to a dry surface. Inoculation method did not consistently affect recovery of conidia of *P. expansum* from grape or plum fruit surfaces. None of the four removal methods (shake, sonicate, swab, and spatula) were consistently more effective over the range of inoculation methods and fruit surfaces used in this study.

Additional keywords: *Prunus domestica, Vitis vinifera*

Adhesion of fungal spores to plant surfaces is an important factor in the infection process and the epidemiology of plant diseases. Adhesion of fungal spores may involve secretion of fluids that prepare the infection court for the development of stages that are necessary for penetration (18). Extracellular mucilages are common on fungal germlings (17), including *Botrytis cinerea* Pers.:Fr. (9,15), and may even arise from the nongerminated conidium (19). A recent study of the adhesion of *B. cinerea* to several natural and artificial surfaces concluded that adhesion is a passive process and dependent, in part, on hydrophobic interactions (4). Adhesion significantly increased when spores were hydrated. Because fungal spores are often deposited on plant surfaces in water (rain, dew, or irrigation) as well as onto wet or dry surfaces from airborne inoculum (9,22), it is important to study the effect of these epidemiological factors on adhesion.

Many methods have been used to remove fungi and bacteria from plant surfaces and include sonication, agitation on a rotary shaker, and physically rubbing the plant surface. Swabbing has been reported to be less efficient than pulping for removal of yeasts from apple fruit surfaces (13). Swabbing introduces the additional problem of removing organisms from the swabs (24). Other methods for removal of microorganisms include washing samples in water (5,10,12,20), water plus Tween (6), or phosphate buffer (8), followed by vortexing or shaking the suspension for various amounts of time, then plating the suspension on agar media. A few reports document the use of ultrasound to facilitate harvesting and quantification of plant epiphytic microorganisms (6,14). Ultrasonication of fruit in sterile water dislodges 100 to 200% more yeast species from the fruit surface than shaking of fruit in sterile water for 1 to 2 h (14). None of these reports document the optimum time of sonication that is necessary for the detachment of most microorganisms from plant surfaces, and a comprehensive comparison of these removal methods has not been done.

The first objective of this study was to compare the effects of three methods of inoculation of wet or dry conidia of *B. cinerea* and *Penicillium expansum* Link on their adhesion to the surfaces of grape and plum fruit. The second objective was to compare four methods of removing conidia from fruit surfaces inoculated using the three methods above.

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MATERIALS AND METHODS

Fruit. Mature fruit of grape (*Vitis vinifera* L. cv. Dauphine) and plum (*Prunus domestica* L. cv. Santa Rosa) were surface-sterilized by immersion in 70% ethanol for 10 s, 0.35% sodium hypochlorite for 1 min, and then 70% ethanol for 10 s. In a series of preliminary experiments, this triple sterilization process did not affect adhesion and was used in all experiments to minimize contamination and facilitate colony counting. In each experiment, 15 replicate fruit were placed at 5°C for 4 h prior to application of dry spores (see below). Thirty fruit were held at 22°C prior to inoculation of 15 with a spore suspension and 15 with dry spores (see below).

Spore collection. Conidia of *B. cinerea* were harvested dry with a suction-type collector from 14-day-old cultures growing on synthetic grape agar (1.85 g of glucose, 1.95 g of fructose, 0.25 g of sucrose, 0.15 g of malic acid, 5.0 g of peptone, 2.0 g of yeast extract, 5.0 g of sodium chloride, and 15.0 g of agar per liter of distilled water). Conidia of *P. expansum* were harvested dry from 7-day-old cultures growing on potato-dextrose agar. Conidia were stored at 5°C until use (1 to 16 weeks). Storage time did not affect germination.

Spore suspensions. Spores were added to sterile distilled water to prepare suspensions that ranged from 1×10^5 to 1×10^6 spores per ml. Each fruit was inoculated using 1 (grape) or 3 (plum) 20-µl drops. Evaporation time for the drops was about 2 h. Following inoculation, fruit were held at 22°C for 18 h before removal of spores.

Spore tower. For inoculation with dry spores, 3 to 5 mg of spores was dispersed into the top of an inoculation tower and allowed 20 min to settle onto the fruit, which were positioned on the floor of the tower according to the method of Salinas (21). The dimensions of the Plexiglas tower were 3 × 1 × 1 m (height × depth × width). Inoculation was done at 22°C and 40% relative humidity, and water rapidly condensed on the surface of 5°C fruit during inoculation. The surface of these fruit remained wet for about 1 h.

Spore removal. Actual spore density (conidia per mm^2) on the fruit surface was determined 24 h after inoculation from cyanoacrylate glue impressions, as described by Wilson and Pusey (25). Four methods were used to remove spores from fruit surfaces. In the first method, each fruit was placed in 20 (grape) or 50 (plum) ml of a 0.1% Tween 80 solution and shaken on a rotary shaker at 150 rpm for 3 min. In the second method, fruit were sonicated (Branson model B3, Branson Ultrasonics, Soest, Netherlands) for 3 min

in sterile distilled water using the same volumes as for shaking. In the third method, a cotton swab moistened in sterile distilled water was used to rub a 1 × 1 cm (grape) or 1 × 5 cm (plum) area of the fruit surface. The swab was rotated while moving it across the surface. The swab then was placed in 2 ml (grape) or 10 ml (plum) of sterile distilled water in a capped McCartney bottle and mixed 30 s on a vortex mixer. In the fourth method, a spatula (rubber policeman) was dipped in 2 ml (grape) or 10 ml (plum) of sterile distilled water and used to rub a 1 × 1 cm (grape) or 2 × 2 cm (plum) area of the fruit surface. Three cycles of alternately dipping to remove the conidia from the spatula, then rubbing the surface, were done on each fruit.

An aliquot of 0.2 ml of water containing spores removed by each of the four methods was plated on duplicate petri dishes containing potato-dextrose agar amended with streptomycin sulfate at 100 µg/ml. The dishes were incubated at 22°C for 2 (*B. cinerea*) or 4 (*P. expansum*) days before counting colonies. The experiment was done twice (grape) and three (plum) times. Three replicate fruit were used for each removal method and for the cyanoacrylate spore density determination for each inoculation method.

The spore density (conidia per mm^2 applied to the fruit surface) determined microscopically at 200× with the cyanoacrylate method was used as the basis for calculating percent recovery by the four methods. To calculate percent recovery, the total number (TN) of conidia removed from each fruit was determined based on the petri dish counts and the dilution factors. The surface area (SA) of each fruit from which conidia were removed was determined for application of dry spores as follows: shake and sonicate methods removed conidia from the inoculated top half of each fruit with area (A) = $4\pi r^2$; swab area was 100 mm^2 for grape and 500 mm^2 for plum; and spatula area was 100 mm^2 for grape and 400 mm^2 for plum. For each removal method, the number of conidia removed per mm^2 was calculated as TN/SA. For fruit inoculated with drops of conidial suspension, the surface area covered by conidia was 14.2 mm^2 measured with an ocular micrometer at 200×. Finally, percent recovery was defined as: number of conidia removed per mm^2/number conidia applied per mm^2 based on the cyanoacrylate method × 100. Percent values were transformed to the arcsine $\sqrt{\%}$ for statistical analysis (23). Data from each host:pathogen combination were analyzed as factorial experiments with trial, inoculation method, and removal method as main factors. Trial was considered a random factor in the analysis, and the error mean squares used to calculate the F values were calculated from the appropriate trial × factor sums of squares. Means were separated with the protected least significant difference (LSD) test. All significant differences are at P = 0.01 unless stated otherwise.

RESULTS

Cyanoacrylate glue impressions of the fruit surface provided a useful method to determine the actual spore density. However, spores sometimes remained on the fruit surface as well as becoming embedded in the cyanoacrylate glue, making counting tedious. Spore densities determined with this technique for application of conidial suspensions in drops were 86 and 662 *P. expansum* conidia per mm^2 and 327 and 1,400 *B. cinerea* conidia per mm^2 on plum and grape, respectively. Spore densities for application of dry spores in the tower were 32 and 45 *P. expansum* conidia per mm^2 and 4.5 and 5.5 *B. cinerea* conidia per mm^2 on plum and grape, respectively.

Inoculation method. The main effect of inoculation method was significant for recovery of *B. cinerea* from grape (Table 1), and recovery was least when conidia were applied in water suspension and greatest when warm fruit were inoculated with dry conidia for all removal methods (Table 2). When comparing recovery of *B. cinerea* conidia applied dry to cold versus warm grape fruit, recovery was significantly less from cold fruit when conidia were removed with the swab and spatula methods but not different with the shake and sonication methods (Table 2). Method of inoculation did not significantly affect recovery of *B. cinerea* conidia from plum fruit (Table 2).

Inoculation method did not affect recovery of conidia of *P. expansum* from grape fruit by shake, sonication, or spatula methods. Recovery by swabbing was significantly less from drop-inoculated grape than when dry conidia were applied to

Table 1. Analysis of variance of removal and inoculation methods for recovery of conidia of *Botrytis cinerea* and *Penicillium expansum* from the surface of grape and plum fruit

Source of variance	Grape			Plum		
		Mean square[x,y]			Mean square[x,y]	
	df	*B. cinerea*	*P. expansum*	df	*B. cinerea*	*P. expansum*
Trial (A)	1	2.7	590.8*	2	2,538.2*	82.6
Removal (B)	3	2,307.3*	236.9	3	922.1	375.4
Inoculate (C)	2	5,083.2*	202.2	2	867.0	2,102.7*
A × B	3	9.6	79.5	6	418.6	582.8
A × C	2	14.8	58.1	4	1,352.4*	753.1
B × C	6	526.7*	314.3*	6	445.1	506.9
A × B × C	6	55.8	81.2	12	175.5	256.5
Error I	48	42.8	13.5	72	121.1	69.0
Error II[z]	11	35.7	76.5	22	455.8	435.8

[x] Values are in arcsine $\sqrt{\%}$ recovery of conidia.
[y] * indicates significance at P = 0.05.
[z] Calculated from trial × factor sums of squares and used to calculate F values. Trial was considered a random factor.

Table 2. Comparison of three inoculation and four removal methods on recovery of conidia of *Botrytis cinerea* and *Penicillium expansum* from the surface of grape and plum fruit

Removal method	Percent recovery of conidia[x,y,z]											
	Grape						Plum					
	B. cinerea			*P. expansum*			*B. cinerea*			*P. expansum*		
	Drop	Dry-warm	Dry-cold	Drop	Dry-warm	Dry-cold	Drop	Dry-warm	Dry-cold	Drop	Dry-warm	Dry-cold
Shake	13.5Ba	32.1Ab	19.7Bab	31.9Ba	22.7Aa	14.7Aa	10.2Aa	4.1Aa	9.1Aa	39.4Ab	3.6Aa	3.4Aa
Sonicate	19.0Ba	64.1Bb	47.4Cb	36.7Ba	25.1Aa	22.5Aa	22.3Aa	10.3Aa	24.8Aa	17.0Aa	6.6Aa	6.8Aa
Swab	4.4ABa	83.1Cc	45.5Cb	8.3Aa	31.9Ab	15.9Aab	6.2Aa	27.9Aa	29.2Aa	24.7Aa	8.6Aa	9.0Aa
Spatula	2.1Aa	25.5Ab	6.7Aa	7.9Aa	24.4Aa	17.9Aa	2.4Aa	21.0Aa	23.9Aa	8.7Aa	4.5Aa	6.7Aa

[x] Spore density as determined with the cyanoacrylate glue method (25) was used as the basis for calculating percent recovery.
[y] Numbers followed by the same letter are not significantly different at P = 0.01 according to protected LSD. Capital letters are used to compare treatments within columns, small letters to compare treatments at different host:pathogen combinations within rows.
[z] Three inoculation methods were: drops of conidial suspensions and dry conidia applied in a tower to warm fruit or to cold fruit.

warm fruit (Table 2). For plum fruit, inoculation method did not affect recovery of *P. expansum* by sonication, swab, or spatula. Recovery with the shake method was significantly greater from drop-inoculated plum fruit than from fruit inoculated with dry conidia.

Removal method. The main effect of removal method was significant for recovery of *B. cinerea* from grape fruit (Table 1). The spatula method was the least effective, and sonication and swabbing were most effective. However, the removal × inoculation method interaction was significant, and swabbing was not significantly different from spatula removal of *B. cinerea* conidia applied in a water suspension (Tables 1 and 2). Also, with drop inoculation, no significant differences were observed between the shake, sonication, and swab methods. For recovery of *B. cinerea* conidia applied dry to warm grape fruit, swabbing was significantly more effective than other methods, and shake and spatula were least effective (Table 2). There were no significant differences between methods of removal of *B. cinerea* conidia from plum fruit (Table 2).

Recovery of conidia of *P. expansum* from grape was significantly better with shaking and sonication than the swab or spatula methods from fruit inoculated with conidia in water. No difference was observed between any of the removal methods when inoculation was with dry conidia (Table 2). For recovery of *P. expansum* from plum, none of the removal methods were significantly different.

When comparing adhesion of *B. cinerea* with that of *P. expansum*, adhesion of *B. cinerea* was significantly greater ($P = 0.05$) than *P. expansum* on both fruit types when applied as a suspension but significantly less than *P. expansum* for dry-inoculated spores.

DISCUSSION

Other researchers showed that hydration of conidia of *B. cinerea* by chilling the surface after inoculation improved adhesion (4). We found similar results on grape but not on plum. Fruit surfaces that were wet with moisture of condensation remained wet for only 1 h; whereas inoculum suspensions applied as drops remained wet for 2 h. Conidia of *B. cinerea* started germinating (swelling) in less than 2 h but did not form germ tubes under the conditions used in this study. This could account for the increased adhesion of spores applied in water drops. *P. expansum* conidia, however, germinated more slowly; and adhesion of these conidia was not improved by hydration. At 20°C, less than 47% of *P. expansum* conidia germinate after 16 h (1).

Release of extracellular mucilage from nongerminated conidia has been reported to occur within 5 to 20 min of hydration for *Nectria haematococca* (11) and *Cochliobolus heterostrophus* (2). Conidia of *Uncinuliella australiana* are coated with a thin layer of mucilage that instantly forms adhesion pads upon hydration (16). The basis for adhesion of conidia of *B. cinerea* is not fully understood but appears to involve a passive mechanism (4). We could find no previous studies concerning adhesion of *P. expansum* to plant surfaces.

The methods of spore removal used in these studies all were nonrigorous and provided only partial removal of spores. Thus, differences in adhesion between fungi, method of inoculation, and method of removal could be detected. The surface impressions made with cyanoacrylate glue were sharp and detailed but were laborious for determination of initial density of inoculum.

None of the four removal methods were consistently more effective over the range of inoculation methods and fruit surfaces in this study, although the spatula method often was the least effective. Perhaps the best approach would involve a combination of two or more removal methods. Recently, it was shown that a combination of shaking for 5 min followed by 5 min of sonication was highly effective for removing yeasts from the surfaces of pome fruit (3).

Conidia of *B. cinerea* and *P. expansum* are deposited on plant surfaces in both water suspensions, such as rain or packinghouse dump tank water, and as dry, airborne spores (9,22). If recovery from all three types of inoculation is considered, only twice in 48 removal method–fungus–host combinations was more than 50% of the overall spore deposit removed. Thus, spore removal methods similar to those reported here and used to study population dynamics or sanitation efficacy, or to obtain biocontrol agents, may greatly underestimate the populations of epiphytic microorganisms. Hirano and Upper (7) discussed the difficulties used to enumerate epiphytic bacteria and agreed that published procedures are less than 100% efficient. The efficacy of spore removal methods must be carefully determined and, based on our results, will be affected by surface moisture as well as by spore type. It is possible that some of the most effective biocontrol microorganisms or others important in microbial ecology adhere so tightly to the plant surface that they cannot be detected, even by the most stringent methods of removal. Additional research is necessary on removal techniques, including longer treatment times than those used in this study as well as combinations of the various removal techniques.

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