Sources of Inoculum, Biology, Epidemiology, and Transmission of Sour Rot of Stone Fruit and Management of the Disease in the Orchard

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INTRODUCTION

In early July 2001, various samples of nectarine and peach fruit from orchards in northern Tulare and in Fresno counties and from packinghouses in this area were brought to our laboratory for diagnosis of an unusual decay. When the decay lesions originated close to the stylar end, leaking juice streamed from the lesions. When the lesion was on the stem end of the fruit and touched the packing box, it developed a ring-shape decay of 0.5 to 2.0 cm inner and 1.0 to 3.0 cm outer diameter. The leaking juice dissolved the cuticle, the epidermis, and the outer layers of the flesh, creating distinct furrows in the fruit tissue. Samples with similar decay lesions were observed and isolations were made with *Geotrichum candidum* consistently isolated from decayed fruit along with two other yeasts, which were identified as *Issatchenkia scutulata* and *Kloeckera apiculata.* Pathogenicity tests confirmed that each organism is pathogenic by itself or in combination, and in combination with one or both of the other organisms (Michailides et al., 2004). This research is focused mainly on *G. candidum* since it was the most frequently isolated and to be more aggressive.

During previous seasons, looking at the sources of inoculum in California stone fruit orchards and in the packing houses, significantly increased our understanding of the movement of the pathogen's spores from the field to the packing house and from the packing house to the field and of the impact this may have for the stone fruit industry. We also investigated other factors that affect disease, such as the possible role of insects in vectoring *G. candidum*, varietal susceptibility, the need for fruit wounding, fungicide insensitivity of isolates of *G. candidum* collected from various sources, and the role of cull fruit as inoculum source for further contamination of the orchard soil. In

addition, lately we used modern biotechnology tools to characterize strains of *G. candidum*. Specifically, the objectives in 2008 were:

OBJECTIVES

1. Determine differences in pathogenicity and polygalacturonase production by *G. candidum* as compared to *G. citri-aurantii,* corresponding to various levels of virulence.
2. Evaluate the effects of relative humidity on spore germination of *G. candidum* and disease development.
3. Identify and survey for resistant *G. candidum* isolates to Mentor® in the field and packinghouse.
4. Identify sanitation practices in harvest equipment and the field which can reduce sour rot decay.

METHODS AND RESULTS

1. Determine differences in pathogenicity and polygalacturonase production by G. candidum corresponding to various levels of virulence.

In 2007, a test was done to compare one isolate of *Geotrichum citri-aurantii* for its pathogenicity with that of isolates of *Geotrichum candidum* isolated from soil, decayed fruit, packing lines, and surface of peach leaves and fruits. The *G. citri-aurantii* isolate was pathogenic on stone fruits and caused a lesion size comparable to the ones produced by *G. candidum*. In 2008, we expanded this experiment with eight *G. citri-auranti* isolates which were isolated from lemon and navel orange fruits exhibiting sour rot symptoms. The isolates were collected from two different packinghouses in Fresno and Tulare counties. *G. citri-aurantii* was grown on acidified potato-dextrose agar for 72 hours, a 10^6 spores/ml suspension was prepared, and used to inoculate nectarine fruits. The isolates were tested in a randomized complete block design with four blocks. Summer fire nectarine fruits were surface sterilized by dipping them in a solution of 160 ml of chlorine, 160 ml ethanol, and 0.5 ml Tween 20 surfactant in 10 liters water for 4 minutes. Fruits were placed on a raised plastic mesh in plastic containers and water was added to the bottom of the containers to create high humidity (close to 100% relative humidity). Fruits were wounded and inoculated with 20 µl of 10^6 spores/ml. The plastic containers were incubated on a laboratory bench (74°±2°F). Pathogenicity of the isolates by measuring decay (lesion) diameter were recorded 5 days after inoculation.

Geotrichum citri-aurantii was found to be pathogenic on nectarine fruits and caused lesions that ranged from 7.7 to 18.7mm. Thus the citrus strain is considered as pathogenic as other pathogenic isolates of *G. candidum* causing sour rot on nectarine fruits (**Fig. 1).** The results suggest that the citrus strain may be as virulent as the isolates of *G. candidum* collected from decayed stone fruits, soil, and packinghouse which were tested for pathogenicity in 2006. This result also suggests that extra caution needs to be taken when processing stone fruits and citrus fruits with the same packing line at the end of the citrus packing and the beginning of the stone fruit season, as well as to dispose cull citrus fruits away from stone fruit orchards.

Studies on genetic diversity among isolates of *G. candidum* from soil, canopy, and stone fruit are being in progress with molecular procedures using sequence analysis. The ITS region and partial region including intron of the beta tubulin gene 1 of *G. candidum* from stone fruit were sequenced and analyzed. One isolate of the citrus strain was also used in this analysis. The ITS region sequence analysis showed high genetic diversity among isolates collected from different substrates, but sequences obtained from the beta tubulin gene 1 were more conserved and at least a sequence of a third gene is needed to resolve genetic diversity among *G. candidum* isolates. This analysis also showed that *G. candidum* is genetically distinct from *G. citri-aurantii*. The data obtained from this study can also be used as a data base for future designing of specific primers to be used as molecular tools to detect and quantify *G. candidum* in the soil, fruits, and packing lines.

In 2008, we tested 20 isolates from the population used to characterize *G. candidum* from California and found that only one isolate mated with the A1 mating type. This experiment is still in progress and it will be completed in 2009 by mating all the isolates with the two mating types in all possible combinations.

2. Evaluate the effects of relative humidity on spore germination of G. candidum and disease development.

In 2007, we compared the effect of four different relative humidities (75, 85, 99, and 100%) after wound inoculating the fruit. No significant differences in disease incidence and severity were detected among the various treatments. It seems that wounding creates conditions that made the decay development independent of the relative humidity, since the pathogen might have acquired moisture from the wound. Therefore, in 2008 we approached this first by looking at the effect of relative humidity on spore survival by testing the germination of arthroconidia of *G. candidum* under different levels of humidity. We used the protocol described by Smilanick and Mansour, 2007, with some modifications. A suspension of 10^6 of arthroconidia were placed on a 0.4µm Millipore paper and then allowed to dry in a sterile transfer hood. The filter paper was placed in chambers containing different relative humidity levels developed by various salt concentrations as described by Winston & Bates, 1960. The salts that were used were sodium chloride, ammonium sulfate, potassium chloride, and potassium sulfate. Then the chambers were placed in a 25ºC incubator. Spore germination was assayed at 0, 24, 48, and 72 hours by re-suspending the conidia from the filter paper into sterile water using a vortex at a high speed for 1 minute. A-200 µl aliquot of the spore suspension was then plated on plates with a semi-selective medium. These plates were incubated at 25˚C for 24-48 hours and spore germination was recorded as colony forming units. The data were expressed as percentages of the spores germinated at the initial time 0.

Survival of arthroconidia decreased with decreased relative humidity except at 100%. Surprisingly, the population increased at 100% humidity. Colony forming units decreased significantly with less than 88% RH (**Fig. 2**). The increase in colony forming units may suggest the spores of *G. candidum* germinated and produced mycelium and

spores under 100% RH. In future experiments, microscopic observations of the spores on the filter paper will be done to determine whether spores germinate, grow, and reproduce. This may suggest that if fruits were kept under very high RH (close to 100%) spores would germinate and reproduce on the fruit surface. We plan to test this theory in 2009 using fruits kept under different RHs.

3. Identify and survey for isolates of G. candidum resistant to Mentor® in the field and packinghouse.

In 2007, during an evaluation of the packing line as a major source of contamination, sour rot developed in a major portion of cull fruit that had been previously treated with Mentor® as a postharvest treatment. Fourty-two isolates of *G. candidum* were collected from the cull fruit. In 2008, we collected cull fruit and incubated them at 68ºF and >90% relative humidity for 5-6 days. Sixty isolates of *G. candidum* were also collected from the 2008 decaying cull fruit. Sensitivity of these isolates to Mentor® and also sensitivity of isolates (putative sensitive isolates) collected earlier than 2006 is being assessed to compare the two groups and determine if there is any development of any insensitivity to propiconazole. The detection of any emergence of resistant isolates of *G. candidum* to Mentor® is very important for the stone fruit industry because the industry would be one step ahead in disease management. If a resistant population starts to develop in a packinghouse, then there is a risk for development of a resistant population in the field through dumping of contaminated cull fruit back into stone fruit fields or the boarders of the fields.

To determine disease incidence in cull fruits, sound cull fruits from four fields were arranged in two layers placed in industrial standard cardboard with 21 fruits per layer per field. Cull fruits were treated with Scholar® and Mentor® in the packinhouse and were evaluated for sour rot after incubating the fruit at 68ºF and >90% relative humidity for 5-7 days. In addition, we recorded the number of boxes showing at least one decayed fruit with sour rot. Percentages of cull fruits decayed by other major postharvest fungi were also recorded. The percentage of cull fruits decayed with sour rot ranged from 4.8 to 30.1% (**Table 1)**. Cull fruits also developed other major postharvest diseases such as brown rot, grey mold, and decay caused by *Rhizopus* and *Gilbertilla* spp. This clearly shows that cull fruit disposal in stone fruit fields may contribute to the introduction of insensitive isolates back in the fields as well as other fungi growing on fruits treated with Scholar® and Mentor®.

G. candidum isolates were grown on PDA for 4 to 5 days. Determination of *G. candidum* sensitivity to Mentor® was based on mycelial growth. Tests were carried out as described by Avenot and Michailides, 2007. The effective fungicide concentration to inhibit 50% of mycelial growth was calculated using regression analysis (**Fig. 3**). Propiconazole concentrations used to test sensitive isolates were 0, 0.015, 0.03, 0.06, and 0.125 ppm. To test insensitive isolates 0, 0.125, 0.25, 0.5, and 1 ppm were used. The EC_{50} for these two isolates were 0.03 and 0.47 ppm, respectively (**Fig. 3**).

In another experiment we inoculated Summer Fire nectarines with one each sensitive and “insensitive” isolates to test if there is any difference in the pathogenicity and

disease severity between the two isolates. Fruits were surface sterilized then wound inoculated with the two isolates as described earlier in the pathogenicity tests. The fruits were either treated with 135 ppm of propiconazole and then inoculated or inoculated first and treated then 18 to 20 hours after wound inoculation by dipping the fruits in fungicide for 30 seconds. Wound inoculated fruit without fungicide treatments were used as control. This experiment was done twice.

In 2008, we assessed the EC_{50} of 20 isolates of the baseline population and found the EC_{50} ranged from 0.022 to 0.121 ppm with an average of 0.05 ppm of a.i propiconazole, while the EC_{50} of 13 "insensitive" isolates collected from cull fruits treated with Mentor® ranged from 0.176 to 0.517 ppm with an average of 0.414 ppm. We are still trying to test more isolates of the two populations to establish the baseline sensitivity and compare the sensitive with the insensitive populations.

In untreated control fruits, the sensitive isolate caused bigger lesion in both experiments than the insensitive isolate; however, the insensitive isolate caused bigger lesion than the sensitive isolate after treating the fruit with propiconazole either before or after inoculation (**Fig. 4A**). The results of the second experiment showed similar trends as those of the first experiment (**Fig. 4B**). Obviously, isolates of *G. candidum* growing on cull fruits are less sensitive to propiconazole than isolates collected from stone fruit fields and fruit with sour rot in 2001 to 2006.

4. Identify sanitation practices in harvest equipment and the field which can reduce sour rot decay.

Soil population in relation to organic matter In 2008 we repeated the experiments from previous seasons to evaluate the distribution and the numbers of *G. candidum* propagules in the soil, on the leaves, and fruit surface in non-tilled orchards. Fruits were collected from commercial fields before harvest time, while leaves and soil samples were collected in September of 2008. Three composite soil samples were collected from the top first inch per field from 38 peach and nectarine orchards from Fresno, Kings, and Tulare counties. Twenty five leaves and 30 fruits collected per orchard were washed with 25 ml of water/25 leaves and 100 ml of water/30 fruits and 0.005% vol./vol. of Tween-20 was used as a surfactant. A 200µl aliquot of the washings were plated on each plate containing Novobiocin-amended PDA (Nov-PDA) supplemented with 1 ppm fludioxonil, the plates were incubated at 25˚C and the colony forming units of *G. candidum* were recorded after 2 days.

In 2007, a composite soil sample of representative fields with different propagules levels of *G. candidum* and soils of fields that did not yield any *G. candidum* were submitted to the UC Analytical Laboratory for organic matter analysis. The results showed that there was no correlation between the organic matter content and the number of propagules of *G. candidum* in soil. Similar soil samples collected in 2008 will be submitted for further analyses, after the completion of the soil population counts.

G. candidum was detected on the leaves in 13.9% of the fields and on the fruit surface in 23.5% of the fields sampled in 2008. The pathogen was detected in the soil of 71% of the fields sampled. These results confirmed the results from previous seasons that soil is a major source of inoculum of *G. candidum* that can reach the canopy (leaves and fruit) and move to the packing house on plant material or harvesting equipment. Results from three seasons are represented in Table 2.

Effect of soil depth and survival of spores in orchard soils In 2008, we started an experiment to study the effect of soil depth on the survival of *G. candidum* in orchard soils at different depths. To determine propagule survival of *G. candidum* in orchard soils, a spore suspension was prepared from 30 fruits decayed with sour rot by macerating the decayed fruit in a blender with 3 liters of water and then adding the mixture in 25 kg of soil collected from a stone fruit orchard. The mixture was distributed into wire baskets (opening 1 × 1 cm) and the baskets were buried at 4 and 8 inches below the soil surface as done with *Mucor piriformis* (Michailides and Ogawa, 1987). Changes in the levels of *G. candidum* propagules at the two depths in comparison with the initial level from the first sampling is being monitored twice a month. Monitoring will continue for 12 months. After each soil sampling from the wire baskets, the soil was air dried at room temperature on a laboratory bench and a spore suspension was prepared as described earlier. The soil suspension was plated on semi selective media, the plates were incubated at 25˚C, and the CFU were counted after 24-48 hours. CFUs per gram of soil were expressed as percentages of the levels determined in the initial soil sampling (time zero).

At 4 inches deep, spore population declined to 19.5% after 2 weeks, increased to 137% after 1 month, and then decreased to 1.8% after 2 months. Subsequently the levels of *G. candidum* increased to 43.4%, and decreased again in the following sampling periods (**Fig. 5**). On the other hand, for the 8 inches depth, *G. candidum* population decreased to 0.3% after 1 month and remained very (0.5 to 1.5%) during the following sampling periods (**Fig. 5**). If the population at 8” stays this low, then burying *G. candidum* spores at that depth would affect survival levels of the pathogen.

Effect of soil depth Soil samples were collected from the same fields used in 2007 except one field (field #2), where the trees were pulled out. So in total four fields were sampled at three soil depths 1, 2, and 4 inches deep and population levels of G*. candidum* were determined as described earlier. In 2007 and 2008, we recovered propagules also as deep as 4 inches. In 2008, we did not detect any propagules in field #1 while in 2007 the population decreased as the depth increased. The results for field’s #3 and #5 had the same trends in the 2 years as the population increased in field #3 and decreased in field #5 as the soil depth increased. Results from field #4 showed inconsistencies (**Fig. 6**). In general *G. candidum* population recovered from the fields in 2008 was not consistent with the results from 2007 except the population in fields #3 and #5. On the other hand, results from spore survival at 4 and 8 inches deep showed that the populations do decrease as depth increase and results were are more consistent for the 8 inches than 4 inches depth (**Fig. 5**).

Packinghouses and preconditioning rooms

In 2008 we also investigated the populations of sour rot pathogen in packing houses. A packing line in each of seven packing houses was sampled seven times during the 2008 season. Samples were taken by sampling randomly the surfaces of the line at different locations (**Fig. 7**) using Rodac plates containing potato dextrose agar amended with novobiocin (Nov-PDA) and supplemented with fludioxonil. The locations where sampling was done included the fruit dumping location, the brushes, belt after the brushes, and finally the fruit sorting tables.

Propagules of *G. candidum* were detected on all four areas sampled in all seven packinghouses (**Fig. 8**) at some point during the whole period of sampling. In three packinghouses where good sanitation measures were applied, the frequency and *G. candidum* population was less than in other packinghouses (**Fig. 8C**, **D**, & **E**). In those three packinghouses, the packing line was cleaned every day after operation. These data confirmed data from previous seasons and suggested that the brushes may create minute wounds that allow nutrients from the fruit tissues to leak and support the growth and reproduction of *G. candidum* and "inoculation" of the fruit with the pathogen could occur in this way. The results also show that whenever good sanitation practices are taken, then the frequency and numbers of *G. candidum* will be reduced, and thus the risk to have sour rot problems in the packing house will also be reduced.

We observed that among the packinghouses that used good sanitation practices, the frequency of *G. candidum* levels was less than in the other packinghouses where sanitation specific for sour rot was not a priority. Compared to the results in 2007, we found that the population incidence has reduced in three packing houses. Through personal communication with the owners of the packing houses, we know that cleaning of the packing lines was done more frequently now than last season. In three packing lines, we found an increased incidence of *G. candidum* and this increase mainly occurred in the last sampling period in August.

CONCLUSIONS

1. It is clear that *Geotrichum* isolated from decayed citrus fruit can be pathogenic on stone fruit by causing sour rot.

2. Relative humidity has an effect on the survival and reproduction of *G. candidum* spores. The importance of this finding is to monitor relative humidity during postharvest practices (i.e., preconditioning, fruit enclosed in boxes, etc.) and keep relative humidity to recommended levels.

3. *G. candidum* growing on cull fruits treated with Scholar® and Mentor®, resulted mainly from contamination of the packing line with *G. candidum* propagules. Disposing the cull fruits back in stone fruit orchards may introduce *G. candidum* inoculum back in the field as well as isolates insensitive to fungicides.

4. A comparison of the effective fungicide concentrations to inhibit 50% of mycelium growth (EC_{50}) among isolates of *G. candidum* shows development of insensitive isolates. Investigations of resistance development among populations of *G. candium* are still in progress and need to be continued.

5. In general, the insensitive to propiconazole isolate of *G. candidum* caused larger lesions on treated fruits than those caused by the sensitive isolate after treating the fruits with propiconazole.

6. In 2007, we showed *G. candidum* propagules contaminated the packing lines, particularly at the fruit dump area and the area at the brushes and after the brushes. This process resembles to an artificial “inoculation” of fruit that originated from orchards whose soil and fruit were free of any *G. candidum* propagules. Good sanitation practices and frequent cleaning of the packing lines results in reduced levels of the pathogen’s inoculum.

7. Population of *G. candidum* at different depths was not consistent. Generally, there was a trend that the population will decrease as soil depth increases. Plowing the soil in the orchard may help reduce the propagules of the pathogen.

8. It is very clear after three seasons of soil sampling that soil is the main source of inoculum of *G. candidum* and dirt brought in the packing house as dust on the fruit surface and/or leaves from the trees can contaminate the packing lines.

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Table 1. Incidence of major postharvest diseases on nectarine cull fruits in 2008.

Field	Sour rot[1] (*Geotrichum candidum*)	Brown rot[1] (*Monilinia fructicola*)	Grey mold[1] (*Botrytis cinerea*)	*Rhizopus* spp.& *Gilbertella* spp.[1]
1	7.0	12.9	3.8	7.9
2	30.1	10.0	0.2	10.0
3	4.8	4.5	0.3	0.5
4	6.3	5.4	0.0	0.0

[1]Data are expressed as percentages of cull fruits with postharvest decay.

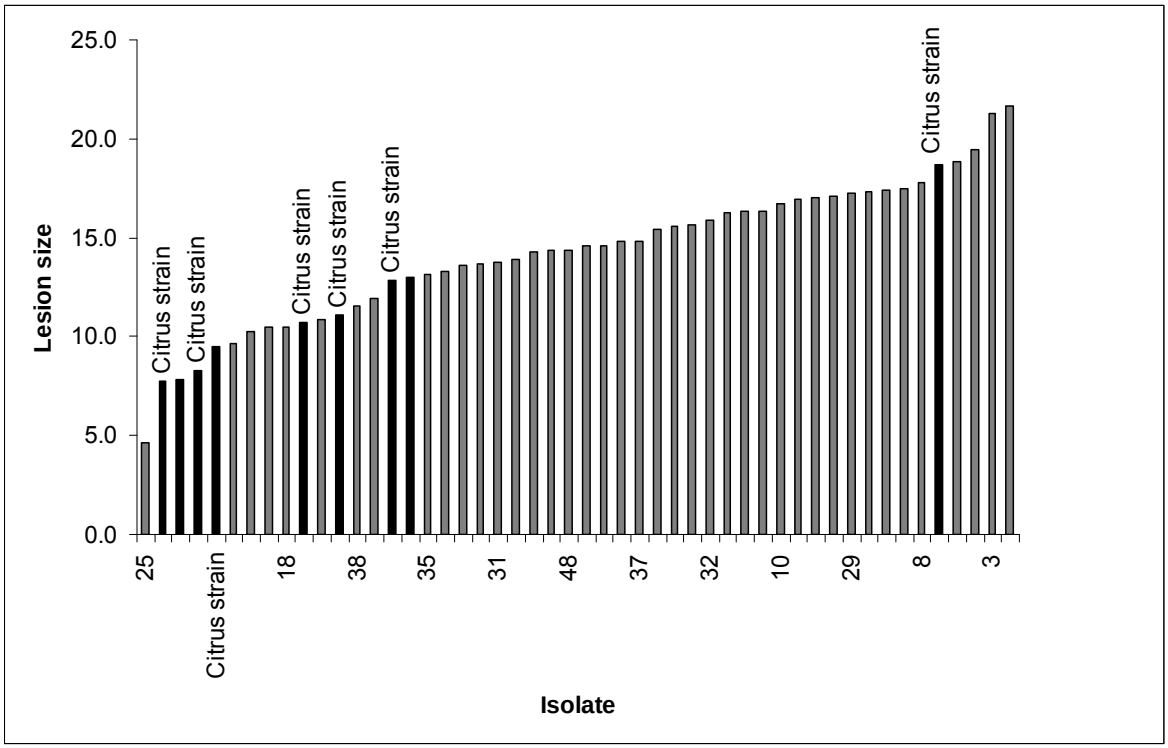

Figure 1. Pathogenicity tests and disease severity of *Geotrichum citri-aurantii* isolated from decaying citrus fruits compared to pathogenicity and disease severity of isolates of *G. candidum* isolated from leaves, fruit surface, soil, decayed nectarines and peaches, tomato, and from packing lines in commercial packinghouses. (Decay was evaluated after 5 days incubation at 74°±2°F.)

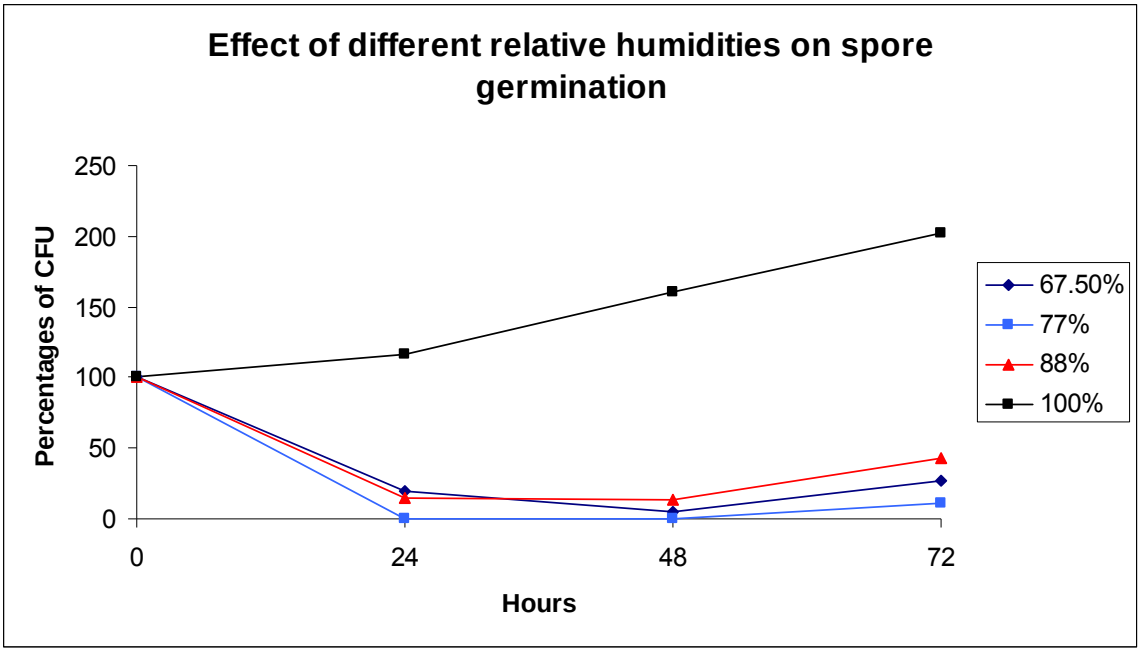

Figure 2. Effect of various levels of relative humidity on survival and germination of *Geotrichuum candidum* spores.

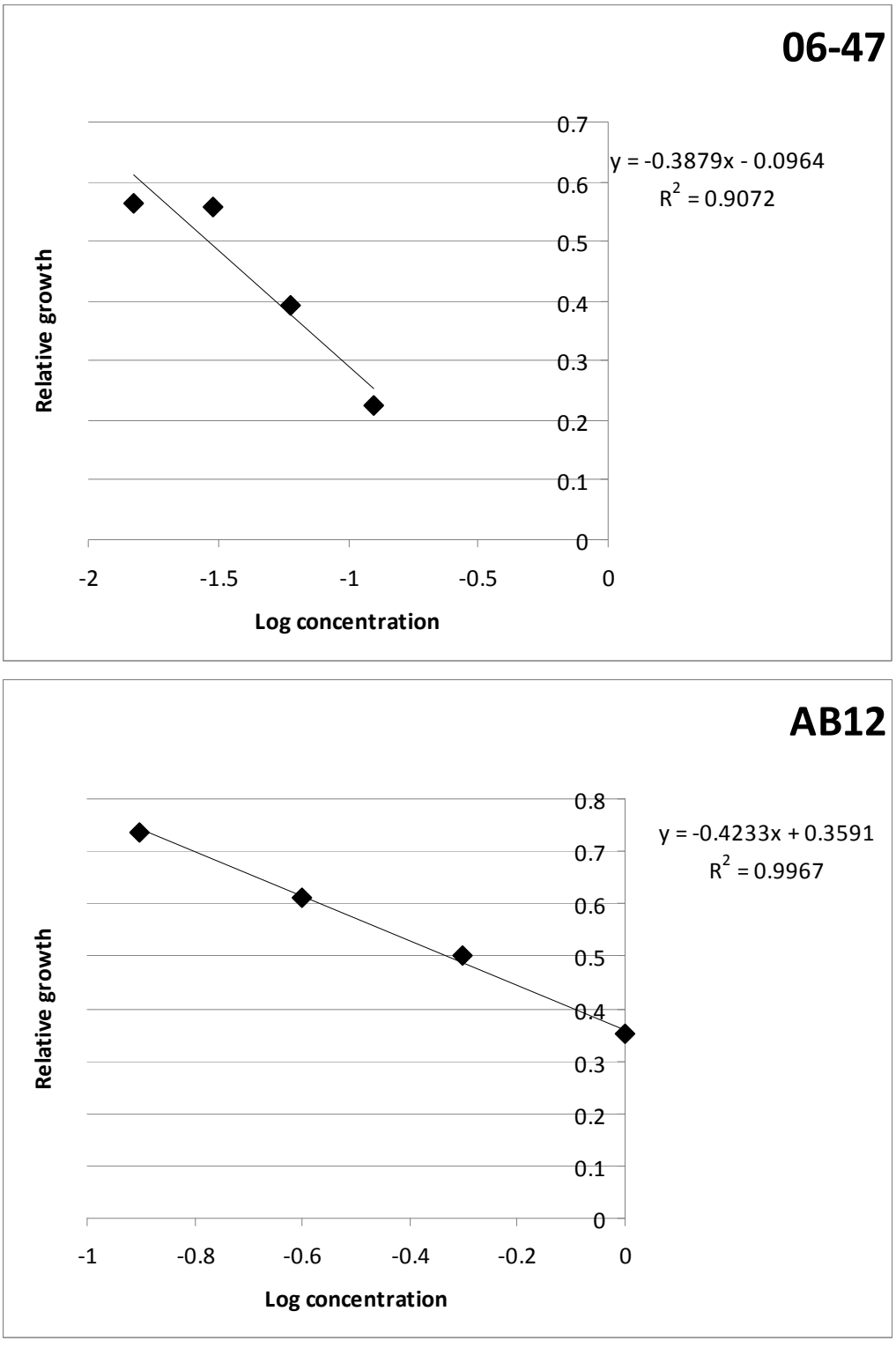

Figure 3. Graphs illustrating the calculations for EC_{50} of sensitive (06-47) and insensitive (AB12) isolates of *Geotrichum candidum.*

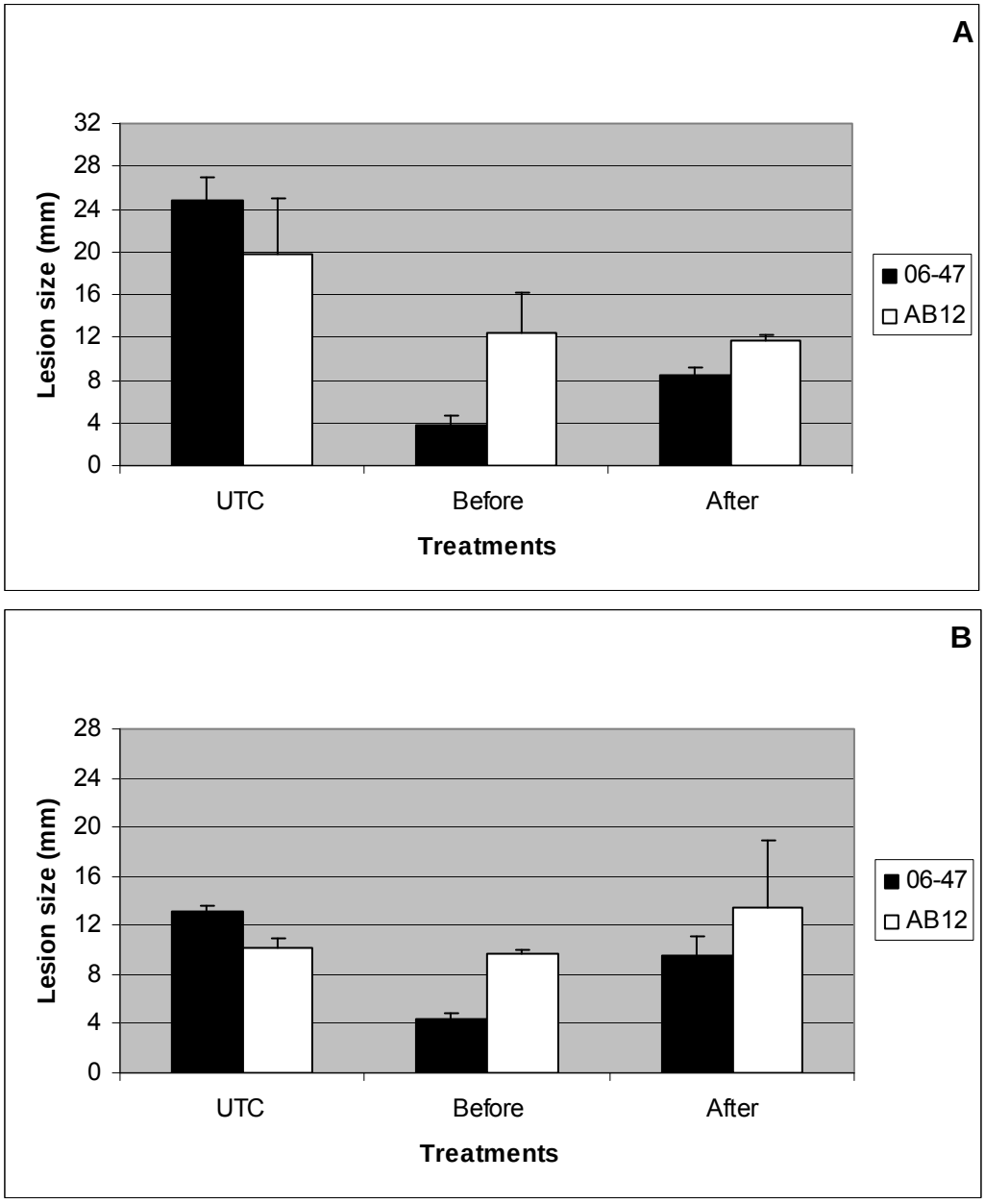

Figure 4. Disease severity of wounded nectarines inoculated with a sensitive (06-47) and an insensitive (AB12) isolates of *Geotrichum candidum*. **A,** Exp. 1 and **B,** Exp. 2.

Table 2. *Geotrichum candidum* population on the surface of leaves, fruits, and soil of commercial orchards in 2006, 2007, and 2008.

Field	Leaf surface (propagules/ leaf)	Fruit surface (propagules /fruit)	Soil (propagules /gram)	Leaf surface (propagules/ leaf)	Fruit surface (propagules/ fruit)	Soil (propagules/ gram)	Leaf surface (propagules/ leaf)	Fruit surface (propagules/ fruit)	Soil (propagules/ gram)
		2006		2007				2008	
5	100	0	244.4	0	-	0	-	-	5.6
6	0	0	0	1.64	-	16.7	0	-	
7	0	0	44.4	0	0	1766.7	0	5.6	161.1
8	0	0	0	0	0	0	0	0	1188.9
9	0	0	0	0	0	77.8	-	0	-
10	0	0	0	0	-	0	0	66.7	138.9
19	0	0	0	0	0	0	0	0	5.6
20	0	0	0	0	0	0	0	5.6	5.6
21	0	0	44.4	0	0	0	0	5.6	27.8
22	0	0	0	0	-	0	0	0	0
23	0	0	0	0	-	0	0	0	11.1
25	0	0	0	0	-	0	11.1	0	5.6
27	0	0	0	0	0	0	0	0	1494.4
28	256	0	0	0	-	105.6	0	177.8	2294.4
29	0	0	88.9	0	0	0	-	-	-
31	5	67	377.8	0	0	0	0	50	688.9
32	0	0	155.6	0	-	0	0	0	0
33	0	0	0	0	-	0	0	0	0
34	0	0	0	0	-	0	0	0	0
35	0	0	200	0	-	0	0	5.6	188.9
36	0	0	1200	0	0	0	0	1200	455.6
38	0	0	0	0	0	255.6	0	22.2	2450
39	0	0	555.6	0	0	55.6	0	22.2	16.7
41	0	0	0	0	0	0	0	0	216.7
42	0	0	66.7	0	0	44.4	0	5.6	0

43	0	0	133.3	0	0	27.7	0	11.1	150
44	0	0	22.2	0	-	1344.4	0	0	22.2
45	0	0	22.2	0	0	27.8	-	-	-
46	0	0	0	0	0	27.8	116.7	0	100
47	0	0	333.3	0	0	16.6	44.4	144.4	244.4
48	0	0	2155.5	0	0	1177.8	72.2	0	116.7
49	0	0	866.7	0	0	0	0	0	2305.6
50	0	0	0	0	15	22.2	0	0	461.1
51	0	0	17111.1	0	0	0	0	-	0
52	0	0	0	0	0	0	0	-	11.1
53	0	0	0	0	0	1127.8	-	-	16.7
54	323	593	22.2	0	0	0	0	0	5.6
56	18	0	66.7	0	0	194.4	0	-	155.6
57	116	0	133.3	-	0	-	-	-	-
59	0	0	3333.3	48	0	2383.3	-	-	-
60	8	0	111.1	0	0	0	-	-	-
62	-	-	-	3.3	-	455.6	50	-	1688.9
63	-	-	-	0	-	44.4	0	-	11.1
64	-	-	-	0	-	0	-	-	-

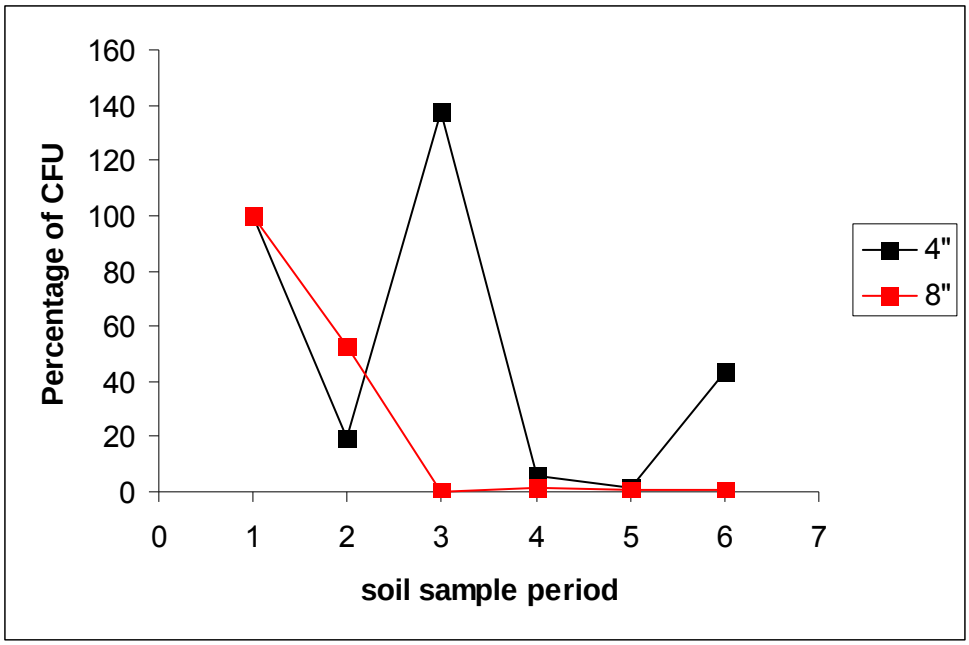

Figure 5. Survival of *Geotrichum candidum* propagules at two different soil depths.

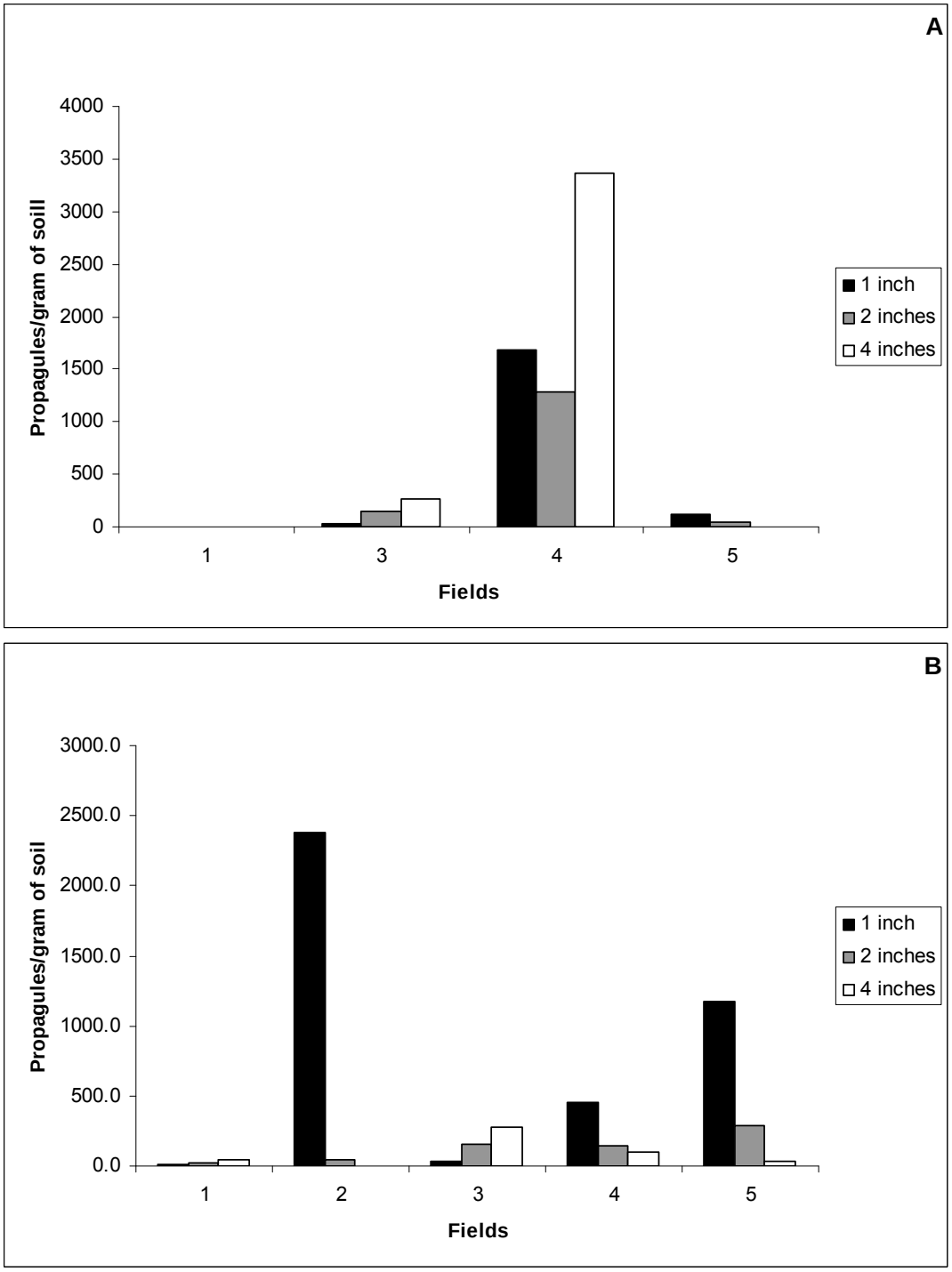

Figure 6. Populations of *Geotrichum candidum* propagules at different depths in 2008 (**A**), and 2007 (**B**). In 2008, orchard #2 was not sampled because stone fruit trees were pulled out and the orchard was not available.

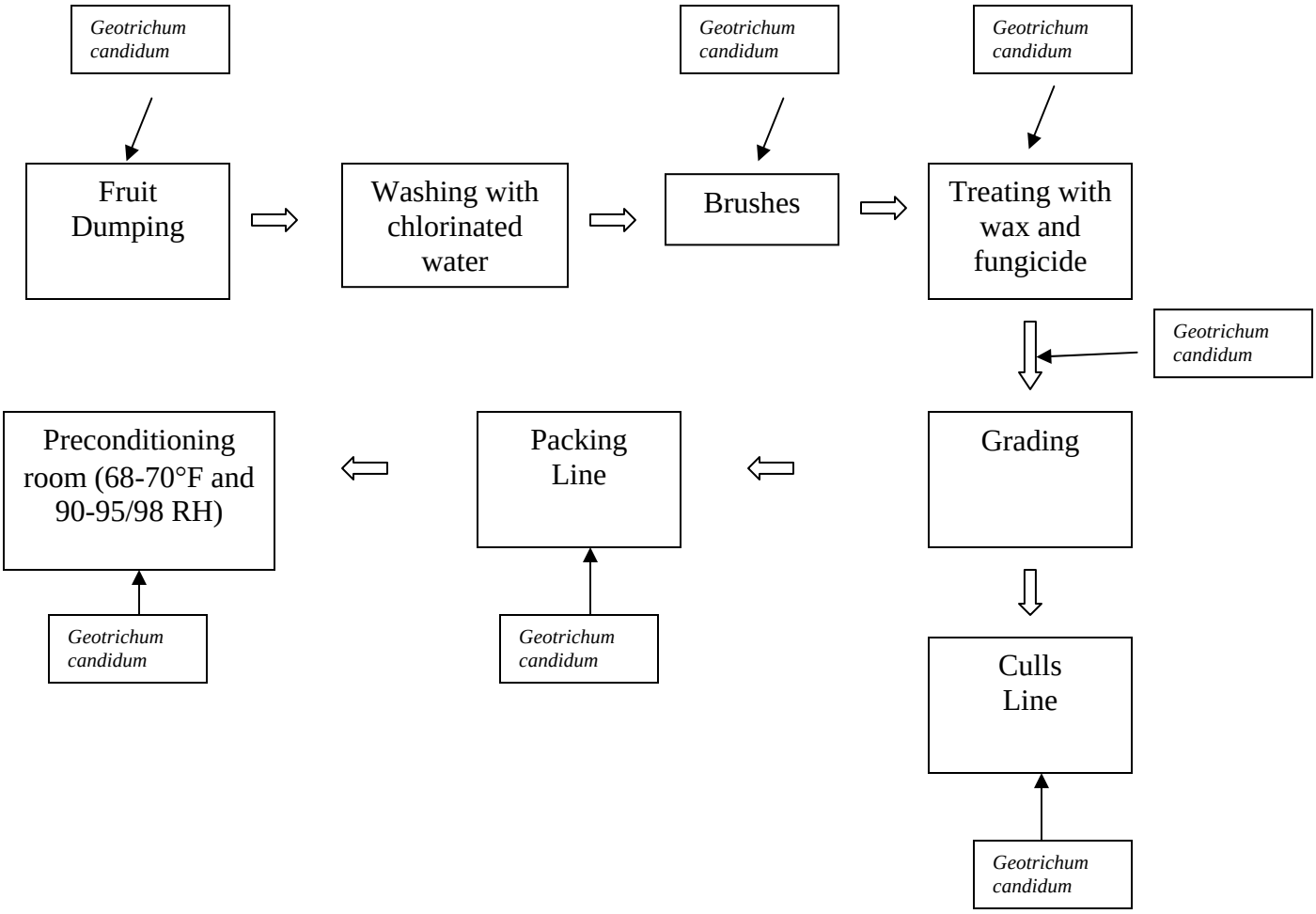

Figure 7. A diagram showing the areas of a packing line in a packinghouse where *Geotrichum candidum* propagules was recovered.

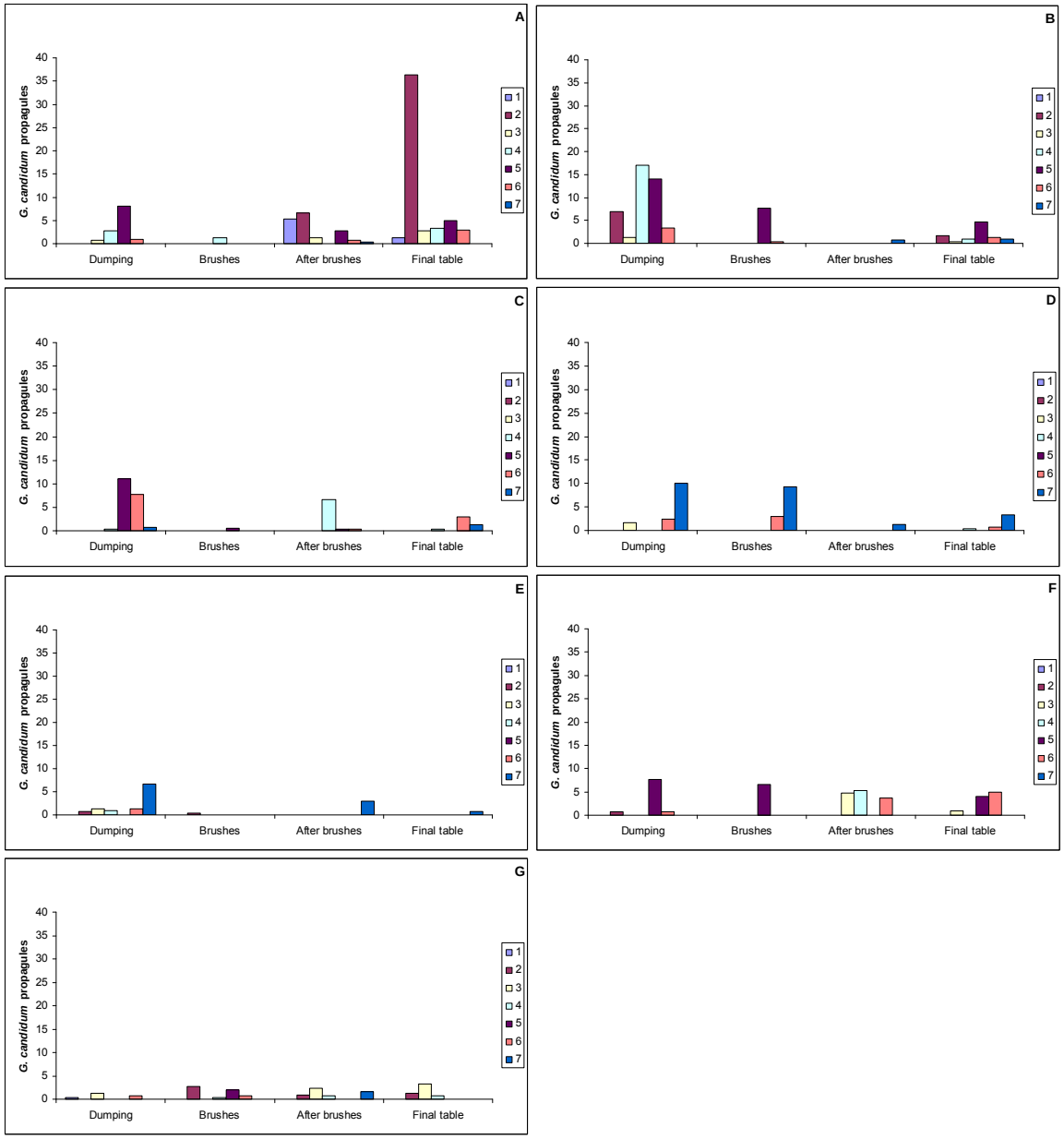

Figure 8. Propagules of *Geotrichum candidum* per plate after sampling seven times from various locations along the packing line in each packinghouse (letters indicate the different packing houses).

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