# Sources of Inoculum, Biology, Epidemiology and Management of Sour Rot in Stone Fruit Orchards

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## Abstract

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 showed an unusual decay and were brought to our laboratory for diagnosis. Symptoms of decaying lesions originated close to the stylar end with leaking juice streamed from the lesions. When the lesion was at the stem end of the fruit and the fruit touched the cardboard box, it developed a ring-shape decay of 0.5 to 2.0 cm inner and 1.0 to 3.0 cm outer diameter. This ring was the result of the leaking juice that dissolved the cuticle, the epidermis, and the outer layers of the flesh. Similarly, the leaking juice created distinct furrows in the fruit surface. These symptoms, described earlier, were observed several times during 2001-2009 and repeated isolations were made from decaying fruit.

Isolations from these fruit consistently yielded the yeast *Geotrichum candidum* along with two other yeasts, which were identified as *Issatchenkia scutulata* and *Kloeckera apiculata. G. candidum,* however, was isolated more frequently than the other two yeasts. Pathogenicity tests were performed with all three organisms, and we concluded that each of these yeasts by itself and in combination with one or both of the others was able to cause sour rot decay on stone fruit (Michailides et al., 2004). Because *G. candidum* was shown to be more aggressive than the other yeasts and was the most frequently isolated microorganism, all further inoculation and transmission experiments were done using *G. candidum* isolates.

In previous seasons, significant progress was made in understanding the biology of the main cause of sour rot (*G. candidum*) and the various factors affecting the disease in California stone fruit orchards as well in the packing houses, including sources of inoculum, varietal susceptibility, disease transmission, and characterization of the main causal agent of sour rot.

## OBJECTIVES

1. Complete studies on pathogenicity and determine difference in polygalacturonase production by *G. candidum* as compared to *G. citri-aurantii,* corresponding to various levels of virulence.

2. Complete the study on the effects of relative humidity on spore germination of *G. candidum* and disease development.

3. Survey and identify 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, RESULTS AND DISCUSSION

**1. Determine Differences in Pathogenicity**

In 2007, we observed that in a major packing house they were packing citrus fruits near the end of the stone fruit harvesting season and there was an overlap when both commodities were packed simultaneously. That raised the question whether the citrus strain, which causes sour rot of lemons, can also infect stone fruit and cause sour rot. In 2008, we tested the pathogenicity of 8 isolates isolated from citrus fruits from two different packing houses in Fresno and Tulare counties. The citrus strain *Geotrichum citri-aurantii* was found to be pathogenic on nectarine fruit and caused a lesion comparable to lesions caused by *G. candidum*. Morphologically and microscopically, *G. citri-aurantii* looks similar to *G. candidum;* thus, molecular tests were needed to make a definite characterization. We used molecular tools to confirm that these isolates are indeed the citrus strain and not the stone fruit strain of *Geotrichum*. We extracted DNA from the 8 isolates and amplified the internal transcribed region (ITS). The amplified DNA was sequenced at UC Davis Biological Sciences Sequencing Facility and compared to the sequences of *G. citri-aurantii* at the Gene Bank. The comparison of the sequences confirmed that seven isolates were the citrus strain and thus we confirmed that the citrus strain can infect nectarine fruits and reproduce symptoms of sour rot similar to those caused by *G. candidum.* The 8th isolate could not be confirmed as *G. citri-aurantii.* The results suggest that extra caution needs to be taken when processing stone fruits and citrus fruits using the same packing line at the end of the stone fruit packing season and the beginning of the citrus fruit season. Also when the culled fruit is disposed, they should not be returned in stone fruit orchards or dumped in roads between orchards to prevent any possible introduction of the citrus strain into stone fruit orchards.

Previously, we looked at differences and genetic diversity among the isolates of *G. candidum* from soil, canopy, and stone fruit using molecular procedures using sequence analysis. ITS region and partial region including intron of the beta tubulin gene 1 were sequenced and analyzed. One isolate of the citrus strain was also used in this analysis. ITS region sequence analysis showed high genetic diversity while sequences obtained from beta tubulin gene 1 were more conserved. This analysis also showed that *G. candidum* is genetically distinct from *G. citri-aurantii*. These data were also used as the foundation for the design of a specific primer to be used in molecular tools that can detect isolates of *G. candidum* from soil, fruits, and packinghouse.

***Genetic Diversity of G. Candidum -*** In 2009, we looked at a third gene to come to a definite conclusion regarding the genetic diversity of *G. candidum* collected from different substrates in California. With the sequencing of the third gene, so far we found that the two mating type isolates and a citrus strain isolate had about 20 extra base pairs than the isolates of *Geotrichum* collected from stone fruit causing sour rot. We still have to finish the sequence of the third gene for all the isolates before making final conclusions. The results from the third gene analysis and the results of various groups of *Geotrichum* isolates not being able to mate with the two mating testers acquired from the Phaff Collection of UC Davis, could support the conclusion that the isolates from different groups are genetically different and answer the question about the species concept of *G. candidum* causing sour rot on peaches and nectarines.

The sequences obtained from the β-tubulin gene were used to design a primer that is specific to *Geotrichum candidum* isolates causing sour rot on stone fruits. The primer was tested against isolates collected from stone fruit orchards used in this study as well an isolate from tomato, the citrus strain, and *Saccharomyces cerevisiae* since it is closely related to *Geotrichum candidum*. The primer was specific, detected, and amplified only the DNA of *Geotrichum* from tomato, stone fruit and citrus fruit, but not that of *Saccharomyces cerevisiae* (baker's yeast) **(Fig. 1).** We don't expect that this primer to amplify other fungi since it did not amplify the baker's yeast, but we may need to test this primer against fungi of major postharvest diseases to confirm that they will not be amplified. (This primer is being tested as a molecular diagnostic tool on tomatoes by a professor working on sour rot of tomatoes.) If the stone fruit industry needs to design a specific primer for stone fruit isolates and separate them from citrus strains and the mating types, then a primer will be designed based on the sequences of the third gene which is showing differences between the stone fruit isolates and the mating types and the citrus strain isolates.

**2. Evaluate the Effects of Relative Humidity on Spore Germination of *G. Candidum* and Disease Development**

In 2009, we repeated the experiment from 2008 (**Fig. 2A)** and looked at the effect of different relative humidity levels on spore survival by testing the germination of arthroconidia of *G. candidum*. We adapted a protocol described by Smilanick and Mansour, 2007 after minor modifications. A suspension of $10^6$ of arthroconidia were placed on a 0.4µmWhatman filter paper and allowed to dry in a sterile transfer hood. Then the filter paper was placed in chambers containing different relative humidity levels developed by various salt concentrations as described in the literature (Winston & Bates, 1960). The salts used were sodium chloride, ammonium sulfate, potassium chloride, and potassium sulfate. By changing the ratio of these salts in solutions, one can obtain different levels of humidity in closed chambers. Subsequently, the chambers were placed in a 25˚C incubator. Spore germination was assayed at four time points, 0, 24, 48, and 72 hours by re-suspending the conidia by vortexing for 1 minute the filter paper into a solution containing sterile water and 2% Silwet (a wetting agent). An aliquot of the spore suspension was then plated on the surface of a semi selective medium in Petri plates. The plates were incubated at 25˚C for 24-48 hours and spore germination was recorded over time as colony forming unit (cfu) and the data were expressed as percentage of the spores germinated at time 0.

Survival of arthroconidia decreased with decreased relative humidity except when incubated at 100%. In both years, only the 100% RH favored germination and survival of propagules (**Fig. 2 A-B)**.

This may suggest that the spores might have actually germinated and the mycelium produced more arthrospores at 100% RH, which resulted in a population increase. This also suggests that the high relative humidity during storage and preconditioning may contribute in survival and reproduction of *G. candidum* on stone fruit surfaces.

### 3. Survey and Identify Resistant *G. Candidum* Isolates to Mentor® in the Field and Packinghouse

In previous research, we identified that the packing line is a major source of contamination of fruit with *G. candidum*. In addition, high levels of sour rot developed in the culled fruit that had been previously treated with Mentor® as a postharvest treatment. In 2007 and 2008, we collected a number of isolates of *G. candidum* from these culled fruit after incubating them at 68ºF and >90% relative humidity to test them for their sensitivity to propiconazole, the active ingredient in Mentor®. Sensitivity of these isolates to Mentor® was assessed and also sensitivity of isolates collected in and earlier than 2006 (before the registration and use of Mentor® as a postharvest treatment). The sensitivity to propiconazole of *G. candidum* isolates in the two groups (those collected before 2006 and those collected after 2006) was assessed to compare any selection of isolates insensitive to propiconazole. It is essential and very important for the stone fruit industry to be aware of any possible emergence of resistance to Mentor® and thus stay one step ahead in disease management. If a resistant population is selected in a packinghouse, then it is likely that a resistant population can be established in the fields through dumping of contaminated culled fruit back into these fields.

*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 as illustrated in (**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.

In 2008 we tested a sensitive and an insensitive isolate for their pathogenicity and disease severity after and before treatment with propiconazole on Summer Fire nectarines. This year, we conducted another experiment and inoculated Ryan Sun peaches with a sensitive and an "insensitive" isolate to test if there is any difference in the pathogenicity and disease severity between the two isolates. The $EC_{50}$ values for these two isolates were 0.03 and 0.47 ppm, respectively (**Fig. 3**). Fruits were surface sterilized and subsequently wound-inoculated with the two isolates. The fruit were either treated with 135 ppm of propiconazole before wound inoculation or treated 18-20 hours after wound inoculation by dipping the fruits in the fungicide solution for 30 seconds. Wound-inoculated fruits without fungicide treatment were used as control; the experiment was done twice.

***Stability of Resistant Isolates*** - To determine whether propiconazole resistance is stable or not, isolates classified as insensitive and sensitive were transferred 10 sequential times to PDA plates free of propiconazole at 7 days interval. The $EC_{50}$ to propiconazole was determined as described by Avenot and Michailides, 2007.

From 42 isolates collected from culled stone fruit treated with Mentor®, we found that 88% of the isolates were insensitive to propiconazole and their $EC_{50}$ averaged 0.43 ppm. These insensitive isolates are expected to grow at a concentration of 2 ppm according to $EC_{90}$ (**Fig. 4, 5**). The

insensitive isolates have been compared to 53 sensitive isolates used previously in pathogenicity tests and it was determined that the $EC_{50}$ averaged 0.07 ppm for the sensitive isolates. The average $EC_{50}$ of the insensitive isolates is approximately 6 times the average $EC_{50}$ of the sensitive isolates. This indicates that a shift in sensitivity to propiconazole has developed on culled fruits that are still being dumped back into stone fruit orchards. Currently we finished repeating this experiment as well finished collecting data for 10 more isolates that are insensitive to bring the total number of insensitive isolates examined to 52.

Last year, we inoculated nectarines with a sensitive and an insensitive isolate and treated the fruit with 135 ppm of propiconazole for 30 seconds (**Figs. 6A**, and **6B**). In untreated control fruits, the sensitive isolate caused a smaller lesion in the first experiment and a bigger lesion in the second experiment (**Figs. 6C** and **6D**). The insensitive isolate (AB12) showed a trend in causing larger lesions after treating the fruits with propiconazole before or after inoculation as compared to the sensitive (06-47) isolate (**Figs. 6C** and **6D**), although these differences were not significant. The results of the second experiment had the same trend as that of the first experiment. It is clear that isolates of *G. candidum* growing on culled fruits are less sensitive to propiconazole than isolates collected from stone fruit fields and fruits in 2006 and before. This result followed the same trend as the experiment using Summer Fire nectarines.

Results to look at the isolate stability to fungicide after transferring the isolates weekly for 10 consecutive weeks (starting with no transfer, T0; and finishing with the 10th transfer, T10) are shown in **Figs. 7A**, and **7B**. There were no significant differences between T0 and T10, when we averaged $EC_{50}$ of all sensitive isolates and insensitive isolates and then compared T0 with T10. This suggests that insensitivity and $EC_{50}$ are stable and are not affected by multiple transferring of the isolates. This implies that the insensitive isolates can produce insensitive populations for multiple generations under natural conditions in stone fruit orchards.

## 4. Identify Sanitation Practices in Harvest Equipment and the Field which can Reduce Sour Rot Decay

### a. Soil Population in Relation to Organic Matter

In 2007 and 2008 we looked at the possible relationship between soil population and percentage of organic matter in the soil. After the population was enumerated in soil samples, a composite soil sample from 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. We found no significant correlation between the organic matter content and the number of propagules of *G. candidum* in soil in both years **(Fig. 8**).

### b. 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 fruit decayed with sour rot. The spore suspension was prepared by macerating 30 sour rot decayed fruit with 3 liters of water in a blender after removing the pit, adding the mixture and mixing it with 25 kg of soil. The resulting

soil paste was distributed in wire baskets which were buried at 4 and 8 inches below the soil surface (Michailides and Ogawa, 1987). Change in the levels of *G. candidum* propagules at the two depths in comparison to the initial sampling was monitored twice a month for 12 months. Soil samples were air dried and a spore suspension was prepared as described earlier. Using a Spiral Gradient Plater, 50 µl of the soil suspension was plated on semi selective media and colony forming units were counted and calculated per gram of soil; data were expressed as percentages of the initial soil sample (time zero).

At 4 inches depth, the spore population declined to 19.5% after 2 weeks; then increased to 137% after 1 month, kept decreasing with time, and reached 1.8% after 2 months before the population increased again to 43.4%. In contrast, at the 8-inch depth, *G. candidum* population decreased to 52.7% after 2 weeks, decreased further to 0.3% after 1 month, and was only 1.5, 0.5, and 1% during the following sampling periods (**Fig. 9**). At both the 4- and 8-inch depths, the population remained very low after March and declined even more (<2%) until the last sampling point which was on 21 August (**Fig. 9**).

Burying the spores at the 8-inch depth in the soil decreased the population by approximately 99% and the results were more consistent at the 8-inch depth as compared to the 4-inch depth burial. This suggests that burying fruit with *G. candidum* spores at 8 inches will significantly reduce the survival of the spores of *G. candidum* at this depth.

**c. Harvest Equipment and Preconditioning Rooms**

In 2009, we did look at the population of the sour rot pathogen associated with harvest equipment. We sampled three different packing houses. One packing house used plastic “totes and bins”, the second packing house used cardboard “totes”, and the third packing house used plastic bins. All samples were taken after the plastic harvest equipment was washed except the cardboard “totes”, which are not washed. Samples were taken by sampling randomly the surfaces of the harvest equipment using Rodac plates containing potato dextrose agar amended with novobiocin (Nov-PDA) and supplemented with fludioxonil. The harvest equipment was sampled 10 times during the stone fruit season. We found viable *G. candidum* spore on the surface of harvest equipment that comes in contact with fruit surfaces. Propagules were detected 4 times during the season on card board totes and plastic bins, while it was detected 7 times during the season on plastic totes (**Fig. 10**). This finding and other findings regarding sources of inoculum stress the importance of sanitation of harvest equipment and the packing line.

This year we also used an Anderson spore trap in the ripening room of a packing house operation to see if *Geotrichum* spores become airborne in the ripening room. The first time we ran the trap for 15 min and we could not detect any *Geotrichum* spores. The second and third attempt was conducted for 2 hours each time, and we could not detect any spores either. These findings suggest that the spores of *G. candidum* do not become airborne in the ripening rooms and decay on the fruit occurs because either the spores (propagules) of the pathogen are present on the fruit surface as fruit is placed in the ripening room, or are deposited on the fruit surface as the fruit comes in contact with contaminated surfaces of harvest equipment. This also supports the contention that contamination of fruit can occur with the harvesting equipment to

some extent and also in the packing line as fruit comes in contact with the surface of contaminated packing lines.

## CONCLUSIONS

1. *Geotrichum* isolated from decayed citrus fruits is as pathogenic as *G. candidum* causing sour rot of peaches and nectarines.

2. In general, survival of *G. candidum* spores decreases as relative humidity (RH) decreases from 100% to 88, 77, and 67% respectively. At 100% RH, the colony forming units increased; the importance of this study is to monitor relative humidity during postharvest practices (i.e. preconditioning) and keep humidity to the recommended levels. However, even the recommended levels could be conducive to sour rot development, if propagules of the pathogen were present on the fruit surface.

3. *G. candidum* that grew on culled fruit treated with Scholar® and Mentor® was the result of contamination from the packing line with *G. candidum* propagules. We detected a shift in sensitivity to propiconazole among isolates of *G. candidum* collected from culled fruits. Disposal of culled fruit back in stone fruit orchards or on roads between orchards, may introduce *G. candidum* inoculum back in the field. In the same way, propiconazole insensitive isolates could be introduced in stone fruit orchards.

4. A comparison of effective fungicide concentration to inhibit 50% of mycelium growth ($EC_{50}$) for sensitive and insensitive isolates showed development of insensitivity among the isolates of *G. candidum*. These insensitive isolates are expected to grow at a concentration of 2 ppm according to $EC_{90}$.

5. An insensitive isolate to propiconazole caused somewhat larger lesions on treated fruit than those caused by a sensitive isolate after treating the fruit with propiconazole.

6. Insensitivity to propiconazole is stable as shown with the experiment of the multiple transfers of *G. candidum* colonies.

7. There is no significant correlation between the soil organic matter and soil population of *G. candidum* which suggests that there are other factors other than organic matter in the soil that may affect spore population and survival.

8. *G. candidum* population decreased significantly by approximately 99% when buried at 8 inches depth as compared to the 4-inch depth burial. Therefore, burying culled fruits deep in the soil will decrease the percentage of viable spores of the pathogen.

9. Sampling the harvest equipment during the stone fruit packing season showed that *G. candidum* propagules can contaminate the bins and totes used during harvest. Good sanitation practices of this equipment can reduce pathogen's population and may decrease the incidence of sour rot.

10. *G. candidum* spores do not become airborne in preconditioning rooms. However, recommended conditions (temperature and relative humidity) are conducive to sour rot development.

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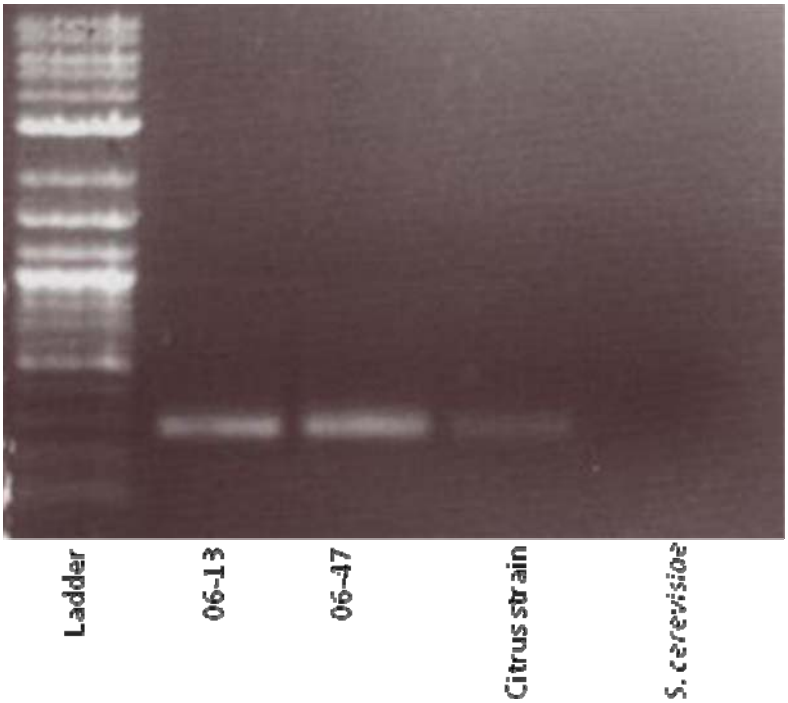
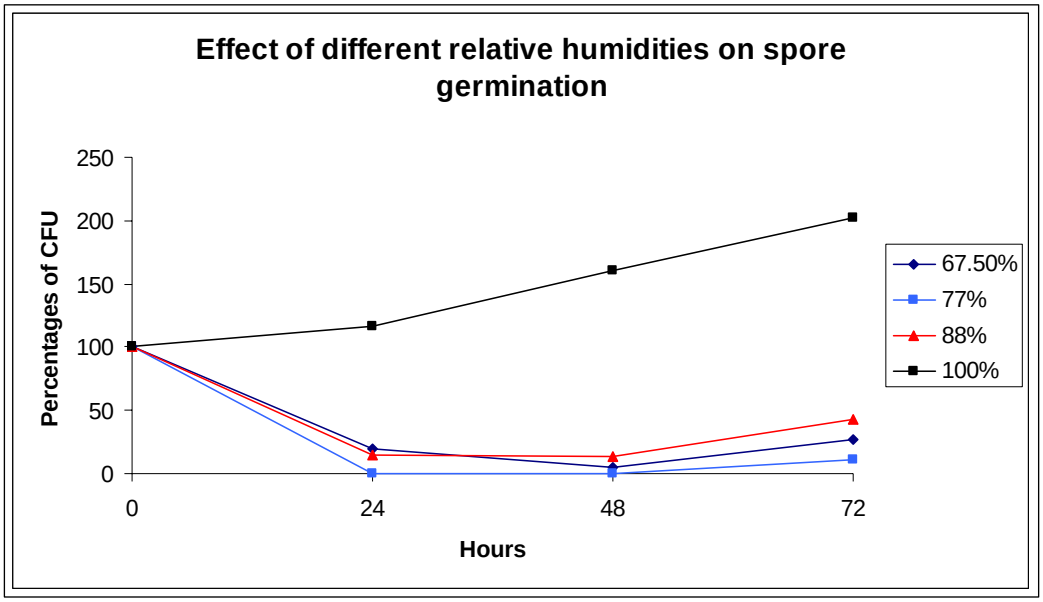



**Figure 1.** Specific primer to identify *Geotrichum candidum*. Presence of a band confirms the amplification and detection of *Geotrichum*. The primer detected the tomato (06-13), the stone fruit (06-47), and a citrus strain, but could not detect *Saccharomyces. cerevisiae* (baker's yeast).

A

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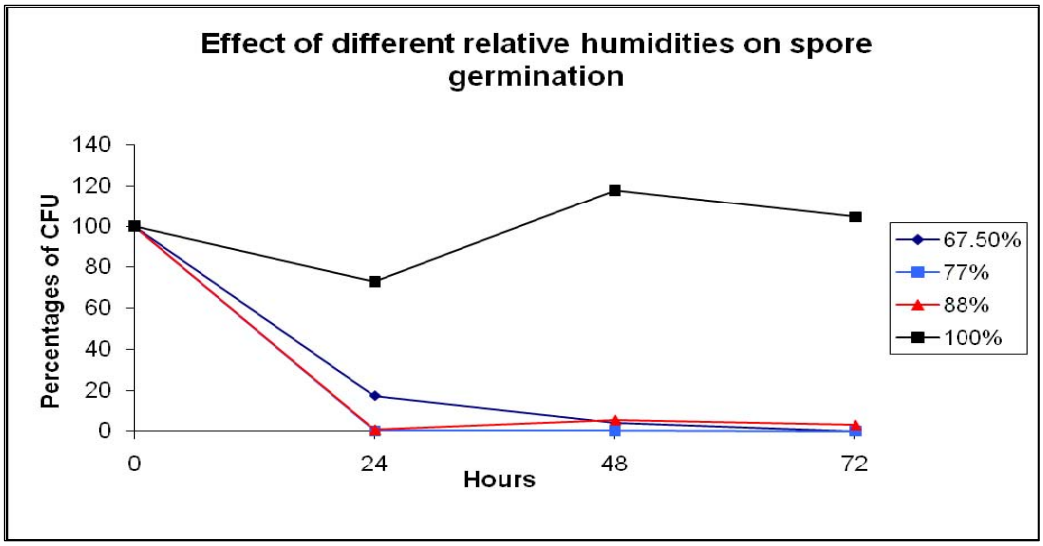
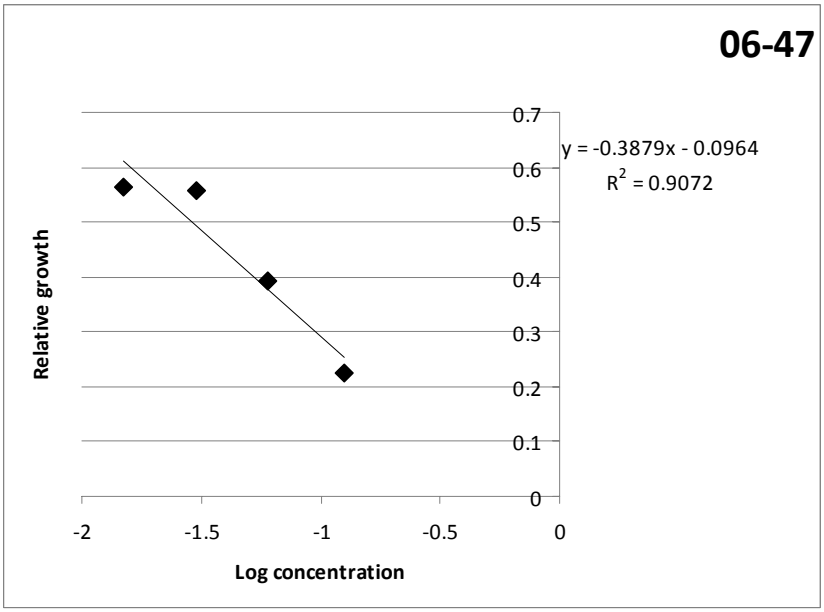



**Figure 2.** Effect of different levels of relative humidity on spore germination and viability of *Geotrichum candidum* arthroconidia. **A,** 2008 and **B,** 2009 experiments**.**



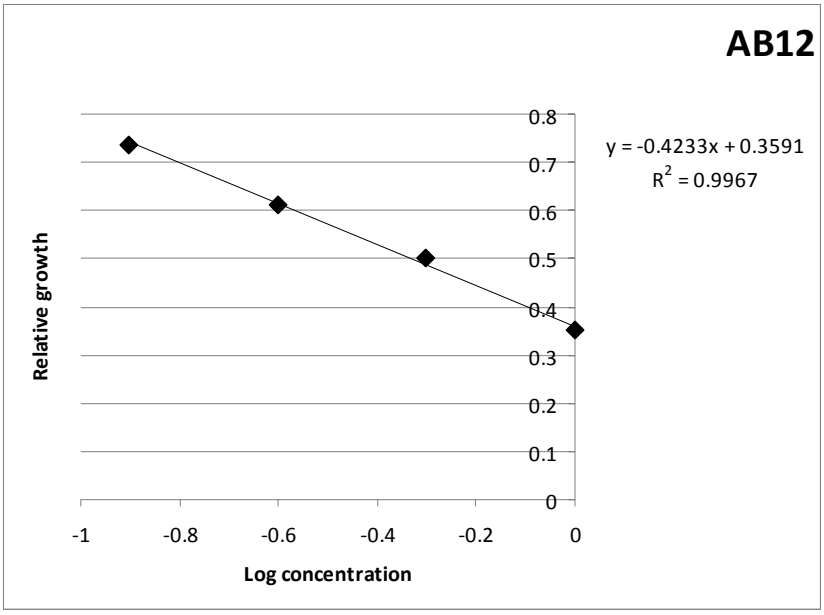




**Figure 3.** Graphs illustrating the calculations for $EC_{50}$ of sensitive and insensitive isolates of *Geotrichum candidum*. 06-47 is sensitive and AB12 is the insensitive isolate.

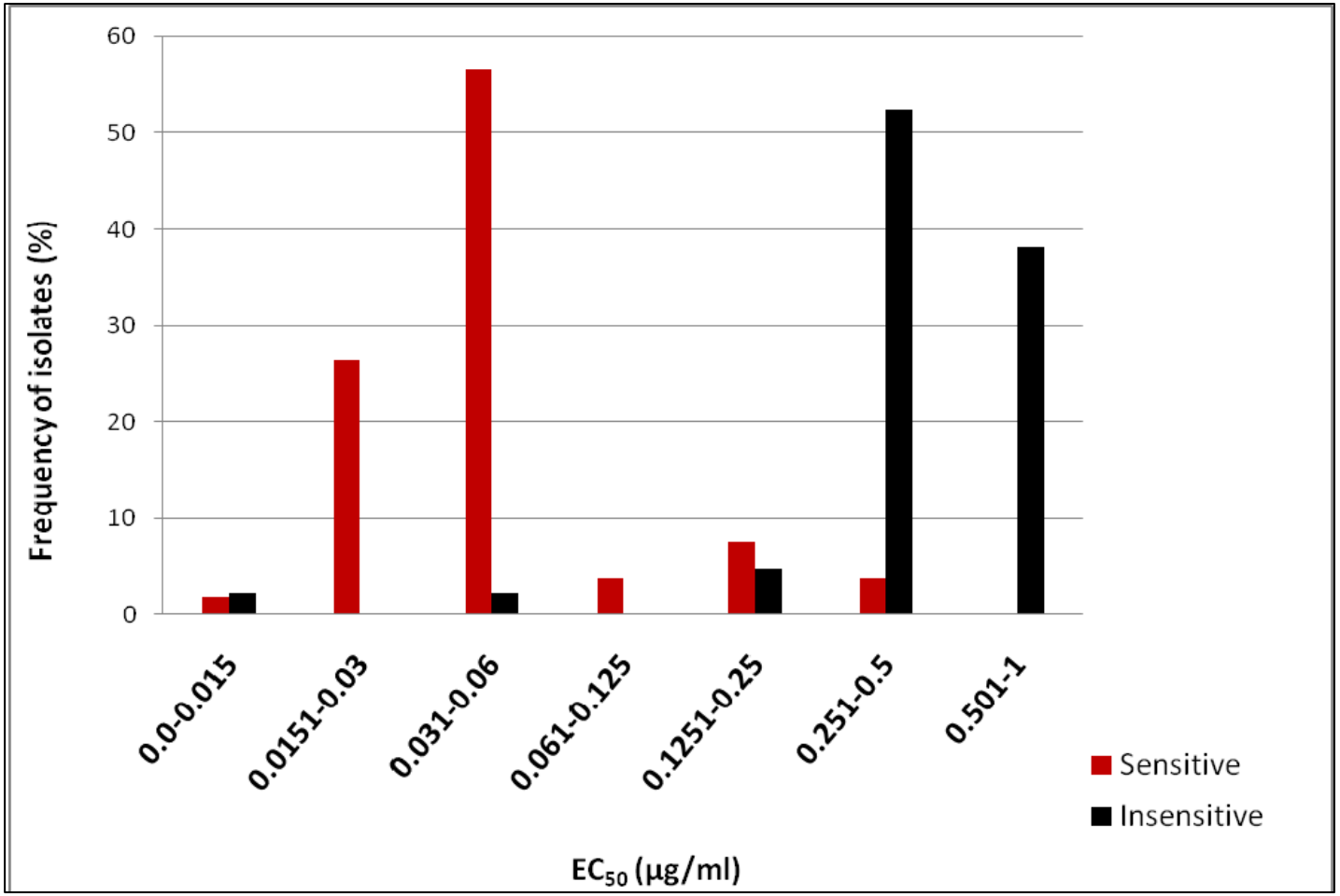



**Figure 4.** Frequencies distributions of $EC_{50}$ values to propiconazole for 53 sensitive isolates of *Geotrichum candidum* collected from different substrates before 2006 (red bars) and 42 isolates of *G. candidum* collected from culled fruits treated with propiconazole (black bars).

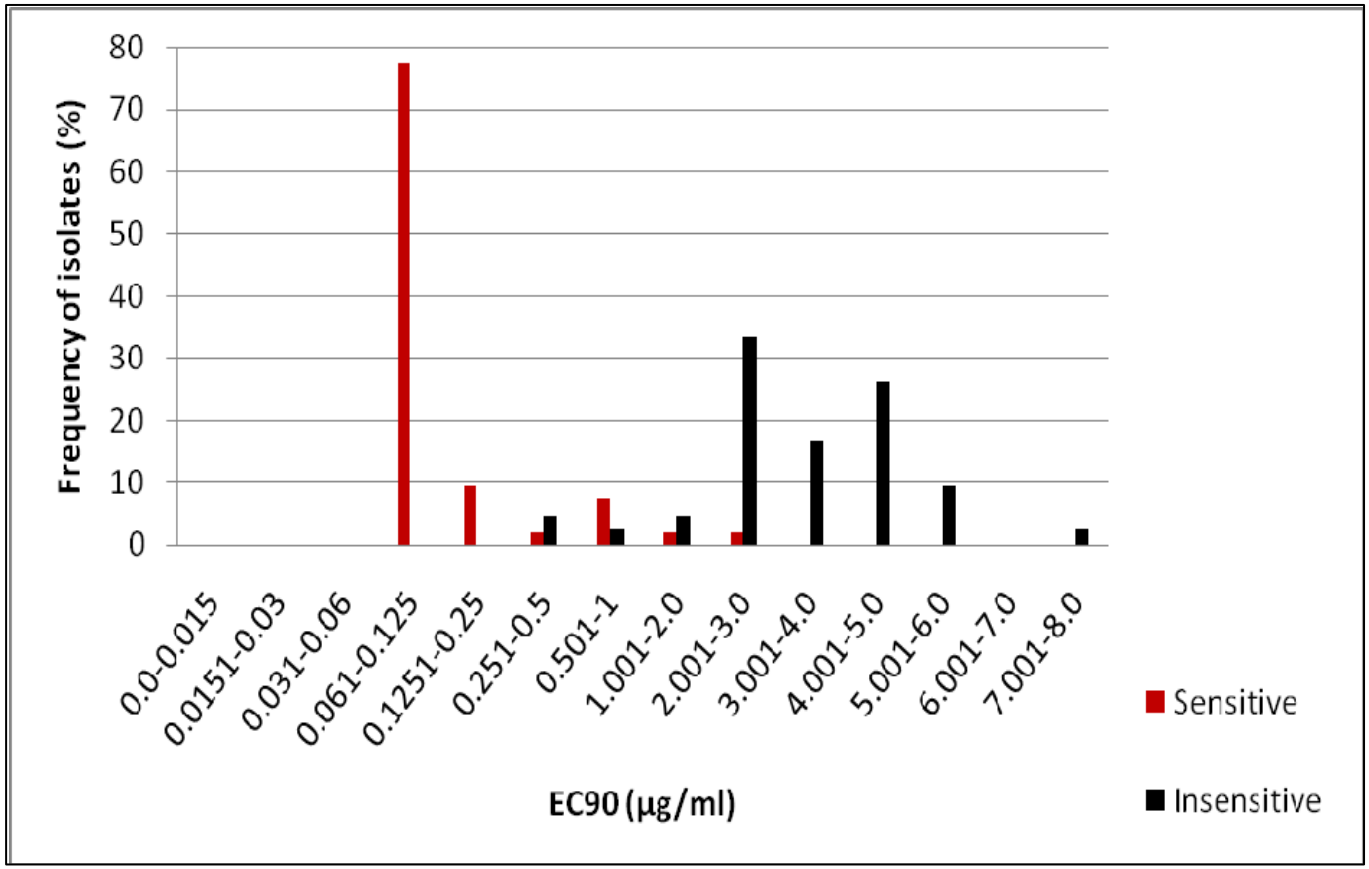



**Figure 5.** Frequency distributions of $EC_{90}$ values to propiconazole for 53 sensitive isolates of *Geotrichum candidum* collected from different substrates before 2006 (red bars) and 42 isolates of *G. candidum* collected from culled fruits treated with propiconazole (black bars).

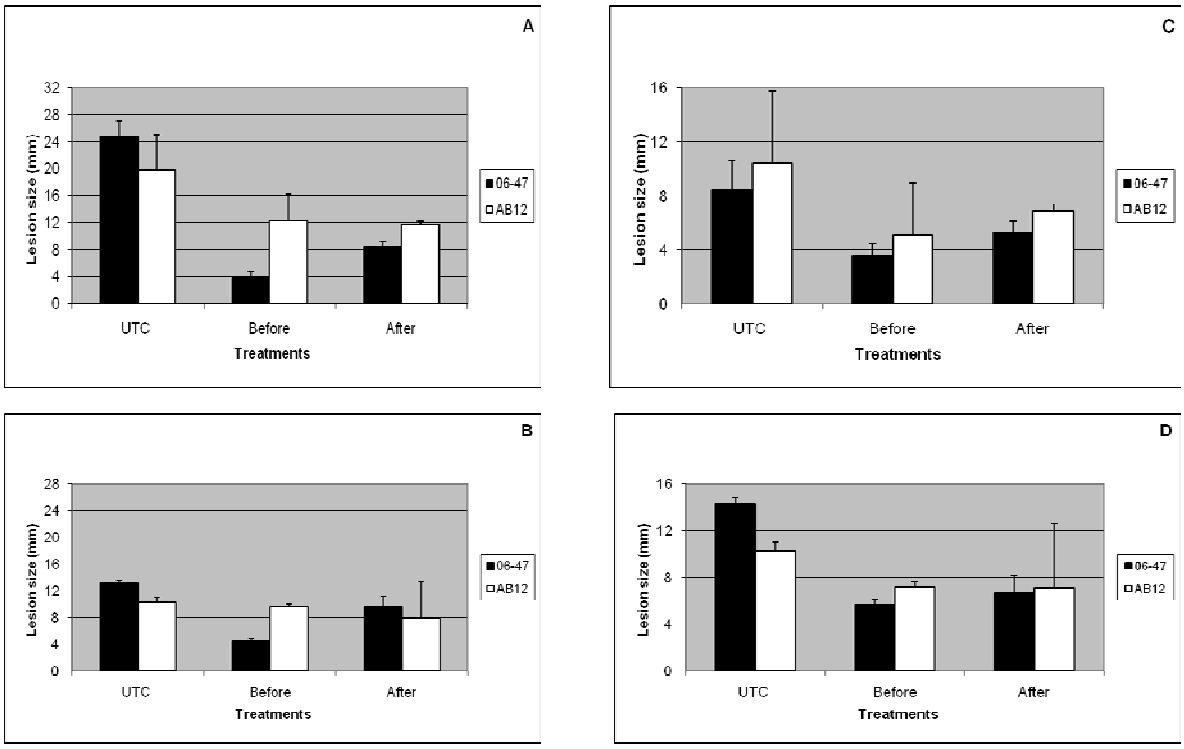
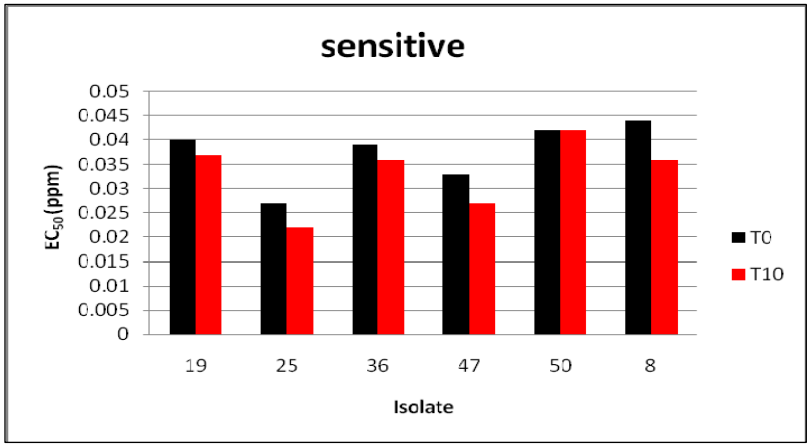



**Figure 6.** Disease severity of wound inoculated Summer Fire nectarine fruits with sensitive (06-47) and insensitive (AB12) isolates of *Geotrichum candidum* before and after fruit treatment with 135 ppm of propiconazole. **A,** Experiment 1*;* and **B,** Experiment 2 (repeat of Exp. 1). **C,** Experiment 1*;* and **D,** Experiment 2 of inoculated Ryan Sun peaches**.**

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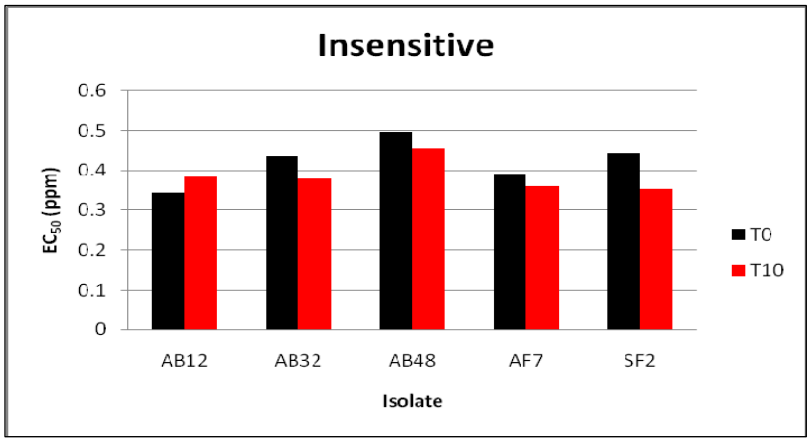


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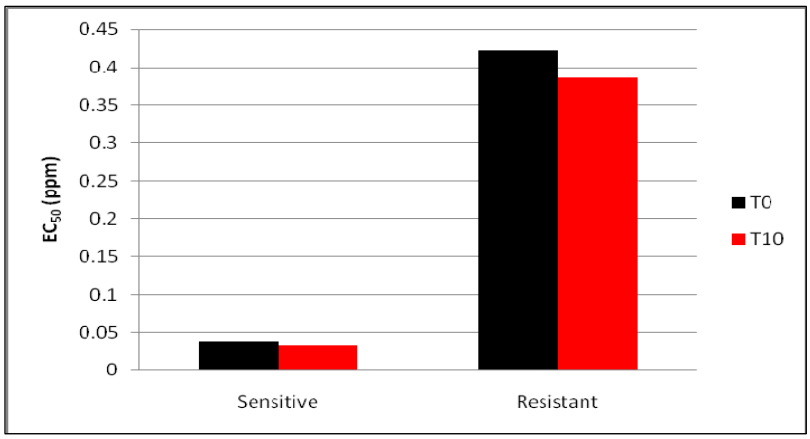
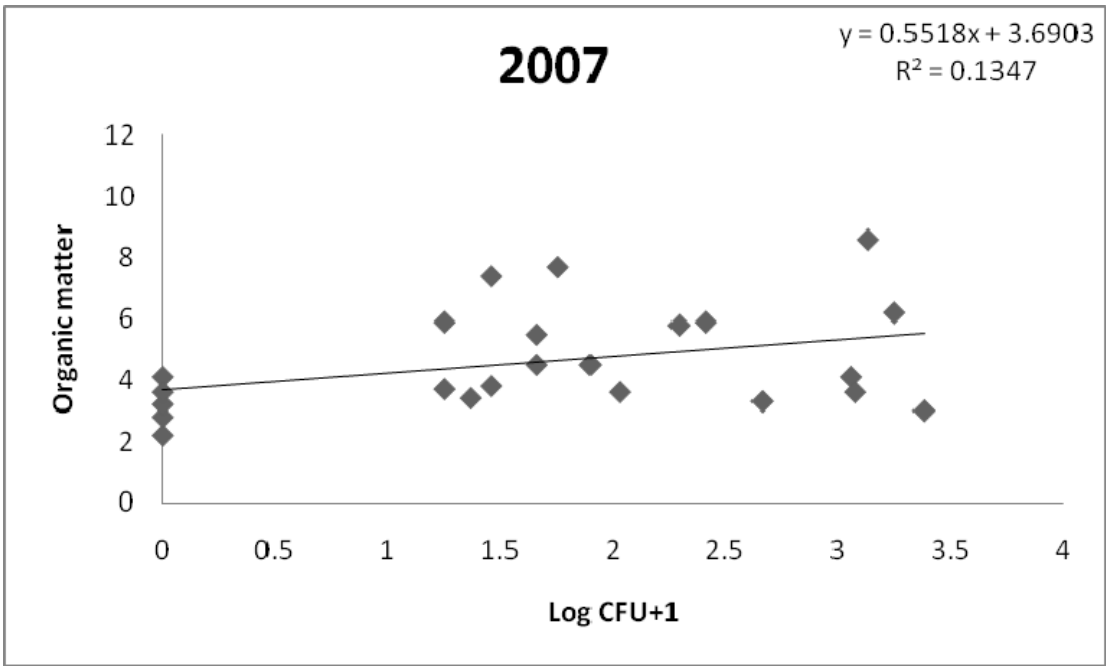



C

**Figure 7.** Stability of sensitive and insensitive isolates of *Geotrichum candidum* by measuring $EC_{50}$ of six sensitive isolates (**A**), and five insensitive isolates (**B**). (**C**) shows the average $EC_{50}$ of sensitive and insensitive isolates at T0 and T10 transfers.



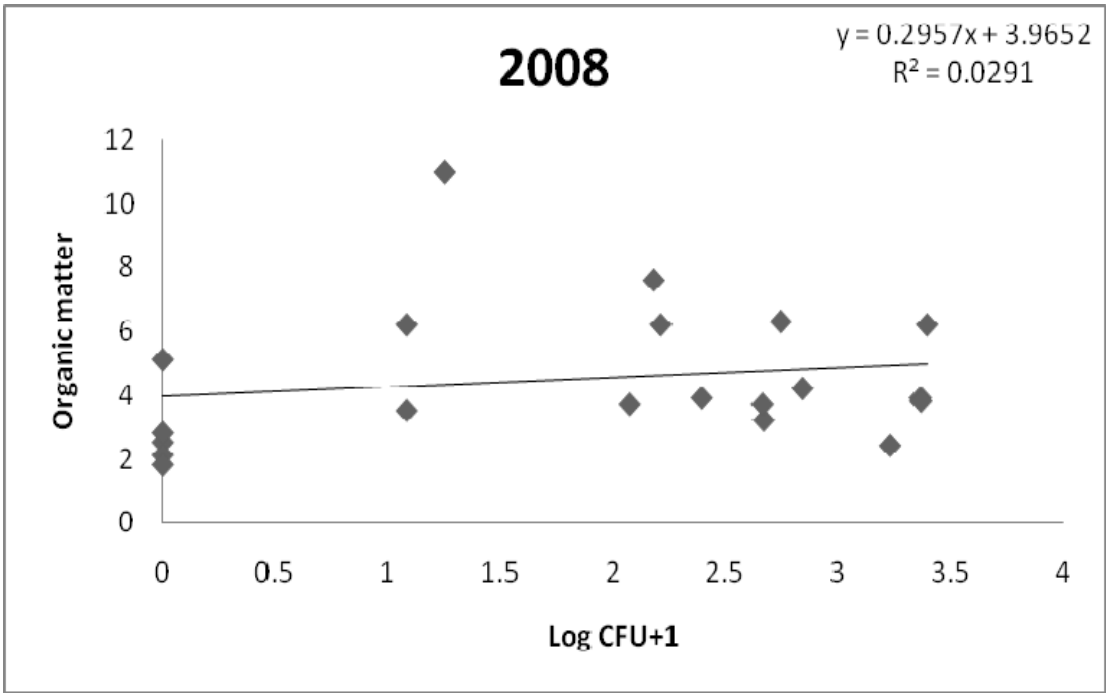




**Figure 8.** Relationship of soil organic matter and populations of *Geotrichum candidum* in 2007 and 2008.

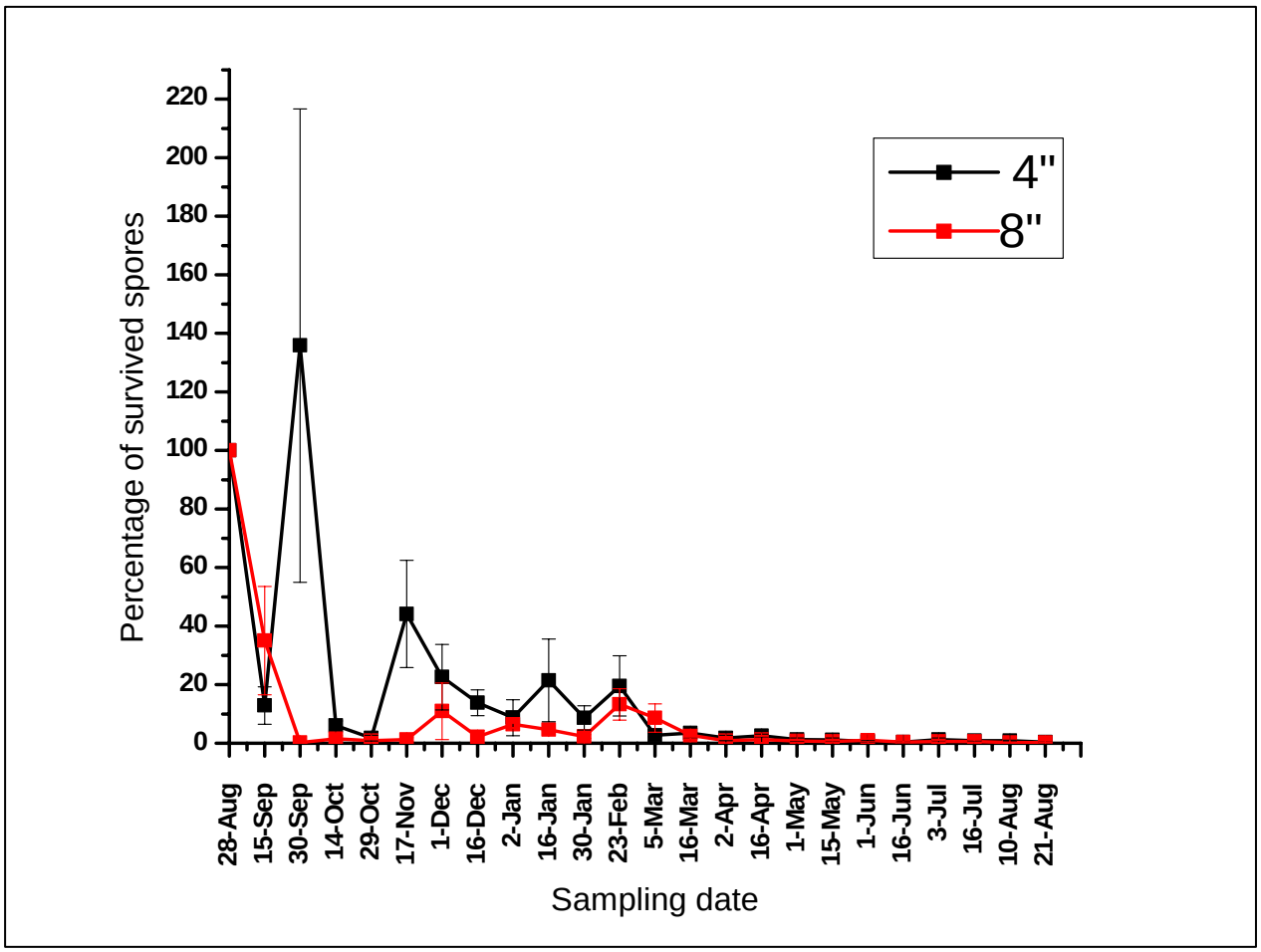



**Figure 9.** Survival of *Geotrichum. candidum* propagules at 4 and 8 inches depths. Experiment started on 28 August 2008 and ended on 21 August 2009. Bars indicate standard errors.

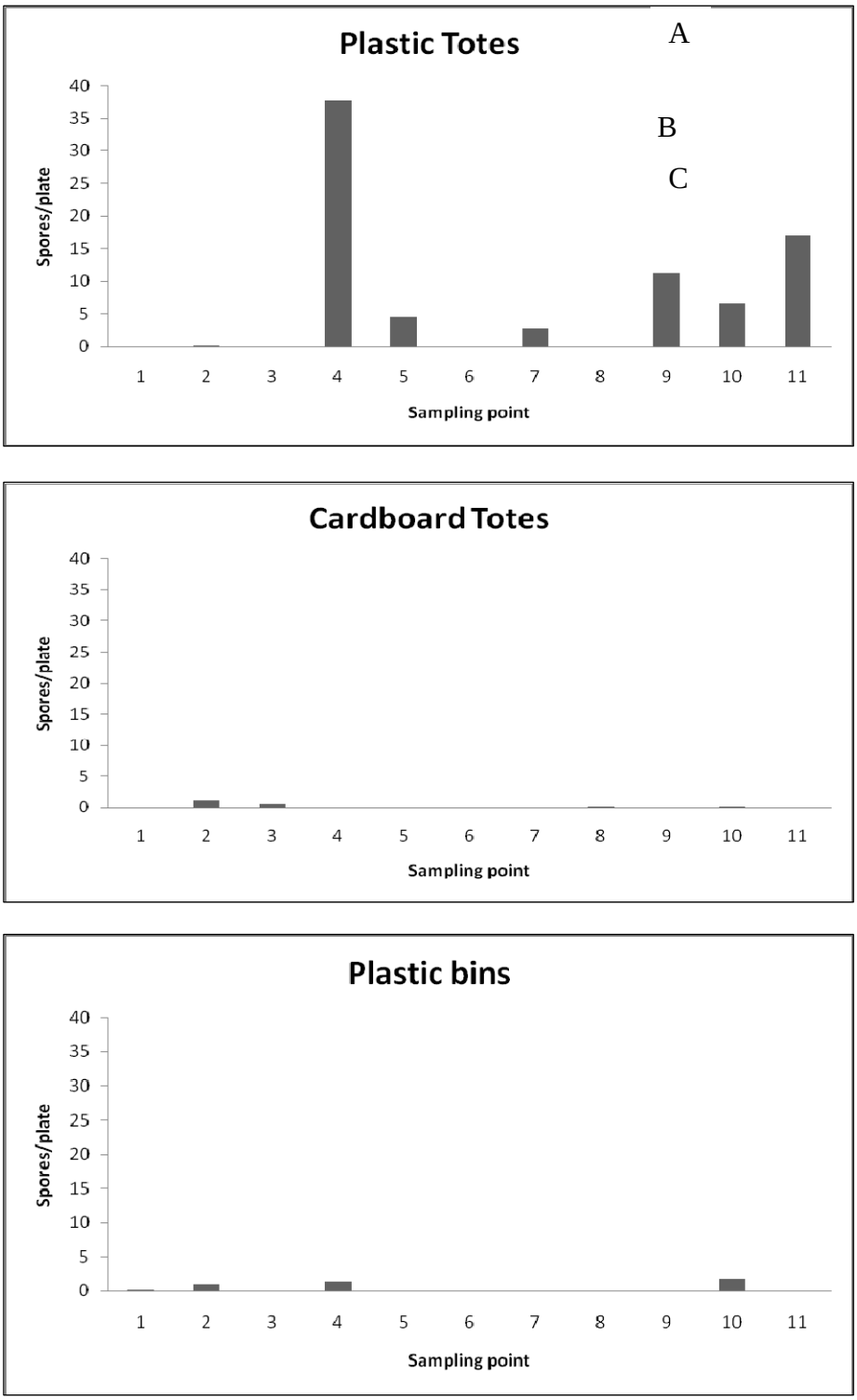



**Figure 10.** *Geotrichum candidum* propagules per plate after sampling 10 times harvest equipment from three packing houses.