Completion of the Studies on Sources of Inoculum, Biology, Epidemiology, and Management of Sour Rot of Stone Fruit in California

Project Leader: Dr. Themis J. Michailides

Cooperators: M. Yaghmour, D. P. Morgan, R. Puckett, H. Reyes, R. Bostock, C. Crisosto, J. E. Adaskaveg

Abstract

The sour rot pathogen of peach and nectarine fruit, *Geotrichum candidum,* can cause significant postharvest losses in California fruit production. Genetic diversity studies and phylogenetic analyses demonstrate that there is considerable genetic diversity within *G. candidum* causing sour rot of stone fruits in California. *Elongation factor1- beta* (*ef1-β*) sequences showed less variation than sequences of *β-tubulin* (*tub*) or ITS regions of ribosomal rDNA among isolates of *G. candidum*. The group comprising the two mating types of *Galactomyces geotrichum* was not congruent among the sequences of the three genes and was monophyletic only in the *ef1-β* and ITS phylogenies. Our results from the mating experiments and analyses of two out of three gene phylogenies suggest that *G. candidum* causing sour rot of peach and nectarine fruit in California does not belong to the same species as *G. geotrichum*.

The sequences obtained from the *ef1-β* gene were used to design a specific primer that is specific to *G. candidum* isolates causing sour rot on stone fruits. The primer was tested against two isolates collected from stone fruit orchards used in this study as well as one of the mating types of *G. geotrichum*, an isolate from the citrus strain, and *Saccharomyces cerevisiae* since the last is closely related to *G. candidum*. Four other fungal species occurring on stone fruit were also used in this test. The primer was specific, detected, and amplified only the DNA of *G. candidum* collected from stone fruit orchards, but did not amplify DNA from *G. geotrichum*, *G. citri-aurantii*, *S. cerevisiae* (baker's yeast), and other fungal genera tested.

Since August 2006, propiconazole has been used as a postharvest treatment to protect peach and nectarine fruit against sour rot. In addition, a high percentage of culled fruits developed sour rot. These fruits had been previously treated with Mentor® (a.i. propiconazole). The mean EC_{50} value (concentration of fungicide resulting in 50% growth inhibition) of 57 isolates of *G. candidum* to propiconazole collected earlier than and during 2006 was 0.072 µg/ml. However, sixty-one isolates from propiconazole-treated, diseased culled fruits collected from 2007 to 2009 had a mean EC_{50} value for mycelial growth of 0.378 µg/ml, a 5-fold shift in mean sensitivity. Comparison of the

sensitivities of the isolates collected from the culled, propiconazole-treated fruit to the reference population revealed a clear shift towards reduced-sensitivity to propiconazole. For the purposes of the present study, 0.1 µg/ml was established to differentiate between the reference and less-sensitive populations of *G. candidum*.

Introduction

Postharvest decay is a significant problem that complicates storage and shipment of produce, resulting in serious economic losses. The ascomycetous fungus *Geotrichum candidum* causes sour rot of fresh-market fruits such as peaches and nectarines (Burton, 1969; Wells, 1977; Michailides et al, 2004), tomato (Eckert and Ogawa, 1988; Butler, 1960), and cantaloupe (Wade and Morris, 1982). Sour rot-like decays may also be caused by other yeasts and possibly other fungi that have not been well-characterized (Michailides et al, 2004; Wells, 1977). The incidence of sour rot decay has increased since 2001 in California, which is considered a semi-arid production area and, thus, is thought to be an atypical and unfavorable climate for this disease.

Sour rot of peaches and nectarines is mainly associated with injured or bruised fruits and fruit with split pits (Adaskaveg and Crisosto, 2006; Michailides, personal communication). Furthermore, the disease mainly occurs on ripe fruit but may also occur on severely injured immature fruit. Symptoms include a dark brown, watery, soft decay with a thin layer of white mycelial growth on the fruit surface (Wells, 1977). The decay may reach the pit and consume the entire fruit (Burton, 1969). Rotted fruit have a characteristic odor, ranging from yeasty to vinegary, and juice may stream from the lesion causing the skin to disintegrate and form furrows (Michailides, 2004).

In previous seasons, studies on sour rot biology indicated that *G. candidum* is present in soil, which provides the main source of inoculum in California orchards. Spores can reach the canopy, where they are deposited on fruit and leaf surfaces. In addition, packing lines can be contaminated with *G. candidum*-infested orchard soil and debris carried on harvest bins, and from spores present on non-symptomatic leaves and fruit or on diseased fruit (Yaghmour et al., 2007; Yaghmour et al., 2009).

Significant progress was made in understanding the various factors affecting the disease in California stone fruit orchards as well in the packing houses, including varietal susceptibility, disease transmission, and characterization of *G. candidum*, the main causal agent of sour rot. Specifically, the objectives in 2010 were:

Objectives

1. Complete the studies on genetic diversity and design and test species-specific primers for the purpose of using them as a molecular diagnostic tool for isolates of *Geotrichum candidum* causing sour rot.

2. Complete survey and identify resistance to propiconazole *G. candidum* isolates in the field and packinghouse.

Methods, Results, and Discussion

1. **Complete the studies on genetic diversity and design and test species-specific primers for the purpose of using them as a molecular diagnostic tool for isolates of *Geotrichum candidum* causing sour rot.**

Previously, we assessed the mating behavior of *G. candidum* with *Galactomyces geotrichm* and we found that none of *G. candidum* isolates mated with either of the mating types of *G. geotrichum*. We also studied the differences and genetic diversity among the isolates of *G. candidum* from soil, canopy, and stone fruit using molecular procedures and their relationship to *G. geotrichum*. *Ga. geotrichum* is the teleomorph, sexual state, of *G. candidum*. ITS region (ITS1, 5.8S, and ITS2) and partial region including intron of the beta tubulin gene 1 (*tub*) were sequenced and analyzed. Isolates representing the citrus strain (Galactomyces citri-aurantii), and the two mating types of *G. geotrichum* were also included in the analysis.

In 2010, we completed this study by completing the sequence of a third gene *Elongation factor1- beta* (*ef1-β*). In total sixty-three isolates of *G. candidum* collected from decayed fruit, fruit surface, leaves, soil, insects, and packinghouse equipment in three counties in the central valley of California were used in the current study.

Analysis of *elongation factor1- beta* (*ef1-β*) sequence did not show as much variation as sequences of *β-tubulin* (*tub*) and ITS among *G. candidum*. Sequence analyses of the three loci demonstrated considerable genetic variability among isolates of *G. candidum* causing sour rot in California. There was incongruence between the phylogenies of the three loci. This was especially evident in the grouping of the mating type testers of *G. geotrichum*. These two isolates formed a distinct group in the *ef1-b* phylogeny, but clustered more closely to the majority of *G. candidum* isolates from California in the ITS phylogeny. Additionally, these two isolates grouped differently in the *tub* gene phylogeny.

Results from mating experiments as well as analysis of the three gene phylogeny suggest that *G. candidum* causing sour rot of peach and nectarine fruit in California does not belong to the species *G. geotrichum*. However, more data are needed to resolve the phylogenetic history of *G. candidum*.

The sequences obtained from the *ef1-β* gene were used to design a specific primer that is specific to *G. candidum* isolates causing sour rot on stone fruits. The primer was tested against two isolates collected from stone fruit orchards used in this study as well as one of the mating types of *G. geotrichum*, an isolate from the citrus strain, and

Saccharomyces cerevisiae since it is closely related to *Geotrichum candidum*. Four other genera of fungi were also used in this test. These isolates were *Aspergillus* sp., *Alternaria* sp., *Stemphylium* sp., and *Cladosporium* sp. The primer was specific, detected, and amplified only the DNA of *G. candidum* collected from stone fruit orchards, but did not amplify DNA from *G. geotrichum*, *G. citri-aurantii*, and *S. cerevisiae* (baker's yeast) (**Fig. 1**). This primer did not also amplify other fungal genera tested.

We don't expect that this primer to amplify other fungi since it did not amplify the baker's yeast and other fungal genera tested. To validate that the absence of bands from fungi other than *G. candidum* was due to the specificity of the primer and not due to poor quality of DNA, we tested the DNA from the same isolates using a universal primer that is expected to amplify DNA from all the isolates used. For that purpose we used primers to amplify the ITS region. The ITS region was amplified from all the fungi tested (**Fig. 2**). In conclusion the specific primer designed based on sequences obtained from the *ef1-β* gene can specifically amplify DNA from *G. candidum* causing sour rot of peaches and nectarines in California.

2. **Survey and identify resistant *G. candidum* isolates to Mentor® in the field and packinghouse.**

Previously, we identified that the packing line is a major source of contamination of fruit with *G. candidum*. In addition, high percentage of culled fruits developed sour rot. These fruits had been previously treated with Mentor® (a.i. propiconazole) as a postharvest treatment. In 2007 to 2009, a total of 61 isolates were collected and marked as propiconazole-less-sensitive and were collected from cull fruit or decaying commercial fruit treated with propiconazole. Fifty two isolates were collected from decaying cull fruit with sour rot after fruit were incubated at 78°F and 90% RH for 5 days. Nine more isolates were collected from decaying commercial fruit in 2009. The sensitivities of these isolates were compared to 57 isolates marked as reference isolates. These reference isolates were collected from isolations made from soil, leaf and fruit surfaces, as well as from packinghouse equipment and decayed fruit. The reference population was 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 detect any shift in sensitivity towards less-sensitivity. It is very important for the stone fruit industry to be aware of any shifts in sensitivity and be aware of possible emergence and risk of resistance development to Mentor,® and thus stay one step ahead in disease management.

Sensitivity of mycelial growth of *G. candidum* to propiconazole was assessed for the reference isolates collected before 2006 (before the postharvest registration of propiconazole) and isolates collected from 2007 to 2009. Isolates were retrieved from stock spore suspensions and allowed to grow on PDA for 48 hours. An agar cube (3 mm × 3 mm × 3 mm) of the culture was transferred to a fresh 9-cm PDA plate and spread

across the surface of the plate. After 5 days at 25°C mycelial disks (5 mm in diameter) were removed from the margins of the colony and were transferred to PDA amended with 0.015, 0.03, 0.06, or 0.125 µg/ml of propiconazole for the reference population and 0.125, 0.250, 0.5, or 1.0 µg/ml of propiconazole for the less sensitive population of isolates.

The EC_{50} values for the effect of propiconazole on mycelial growth on 57 isolates of *G. candidum* collected earlier than and during 2006 from different substrates ranged from 0.013 to 0.444 µg/ml. The calculated mean EC_{50} was 0.072 µg/ml. Approximately 86% of the isolates had an $EC_{50} < 0.072$ µg/ml, while only 14% of the isolates in this population had an $EC_{50} > 0.125$ µg/ml. In contrast, the EC_{50} values for 61 isolates collected during 2007 to 2009 from decayed cull fruit and fruit that had been treated with the fungicide ranged from 0.013 to 0.566 µg/ml, with a mean of 0.378 µg/ml. Approximately, 10% of the isolates collected during 2007 to 2009 showed an $EC_{50} < 0.06$ µg/ml, while 90% of these isolates had an $EC_{50} > 0.125$ µg/ml. The EC_{50} frequency distributions for all isolates are presented in **Fig. 3**.

Comparison of the sensitivities of the isolates collected from the culled, propiconazole-treated fruit to the reference population revealed a clear shift towards less-sensitivity to propiconazole. Although only 14% of the isolates in the reference population had EC_{50} values over 0.125 µg/ml, 90% of the isolates obtained from the culled, propiconazole-treated fruit had EC_{50} values above this dose. The shift in sensitivity is further illustrated with the latter population, where 85% of these isolates had an EC_{90} over 2 µg/ml (**Fig. 4**). Thus, these isolates would be expected to grow on medium amended with 2 µg/ml propiconazole. The maximum residue limits for propiconazole for stone fruit are 2 µg/g (EPA) or 1 µg/g (International), suggesting that as a general rule residues on fruit should not exceed 1µg/g (Adaskaveg et al., 2007). However, the concentration of fungicide is expected to be considerably higher than 2 µg/g on the fruit surface, as residue levels are determined on the basis of whole fruit analyses. Thus, even with the shift towards reduced sensitivity observed in this study, propiconazole at current accepted label rates would be expected to continue to protect fruits against infection by *G. candidum*.

The data generated from the sensitivity experiments was used to determine a discriminatory dose to differentiate between the two populations. The discriminatory dose was determined as described by Wong and Midland (2007) and Putman et al (2010). For this, two discriminatory doses, 0.1 and 0.125 µg/ml were tested to differentiate between the reference and the less-sensitive populations, Log EC_{50} values for propiconazole-sensitive and -less-sensitive populations of *G. candidum* were regressed against calculated RG values of each of 57 sensitive and 61 less-sensitive isolates at each discriminatory dose. The dose with the highest coefficient of determination (r^2) was selected as a discriminatory dose (Putman et al., 2010).

The relationship between relative growth and log EC_{50} at 0.1 µg/ml is presented in **Fig. 5**. Based on regression analysis of relative growth versus calculated log EC_{50} values in

both sensitive and less-sensitive populations, a concentration of 0.1 µg/ml was determined to be the most accurate propiconazole concentration for a single discriminatory dose to screen for sensitivity shifts between the sensitive and less-sensitive isolates tested in this study. The r^2 was higher with 0.1 µg/ml as a discriminatory dose than using the 0.125 µg/ml.

The discriminatory dose is an important tool to monitor shifts in fungicide sensitivity in pathogen populations, providing a threshold to differentiate sensitive and less sensitive or resistant isolates of a pathogen (Russell, 2004). Russell (2004) defines a resistant isolate as "one with growth of 50% or more in the presence of fungicide" at the discriminatory dose. For the purposes of the present study, 0.1 µg/ml was established to differentiate between the reference and less-sensitive populations of *G. candidum*. However, to establish a discriminatory dose that differentiates between baseline (i.e., never exposed to propiconazole) and resistant isolates associated with a control failure, a more comprehensive analysis of field isolates will be needed. For example, a discriminatory dose of 0.3 µg/ml was established to monitor resistance to propiconazole of *M. fructicola*, which was 10-fold of the baseline population (Zehr et al., 1999; Cox et al., 2007).

Conclusions

1. The genetic diversity study showed that there is variation among *G. candidum* causing sour rot of peaches and nectarines. Results from the genetic diversity study and previous mating studies suggest that *Geotrichum candidum* causing sour rot does not belong to *Galactomyces geotrichum*.

2. The designed specific-primer was able to successfully amplify DNA from *G. candidum* causing sour rot but did not amplify DNA from *G. geotrichum* and *G. citrii aurantii*. Thus, it is specific to *G. candidum*.

3. Studies on *G. candidum* sensitivity to propiconazole revealed that there is a shift in propiconazole sensitivity among isolates collected from cull fruits and that cull fruits can carry less-sensitive isolates to propiconazole. Less-sensitive isolates to propiconazole were also detected on commercial fruits. Thus, cull fruit should not be returned and discarded into stone fruit fields. A 0.1 ppm of propiconazole was identified as a discriminatory dose to differentiate between less-sensitive population and a reference population.

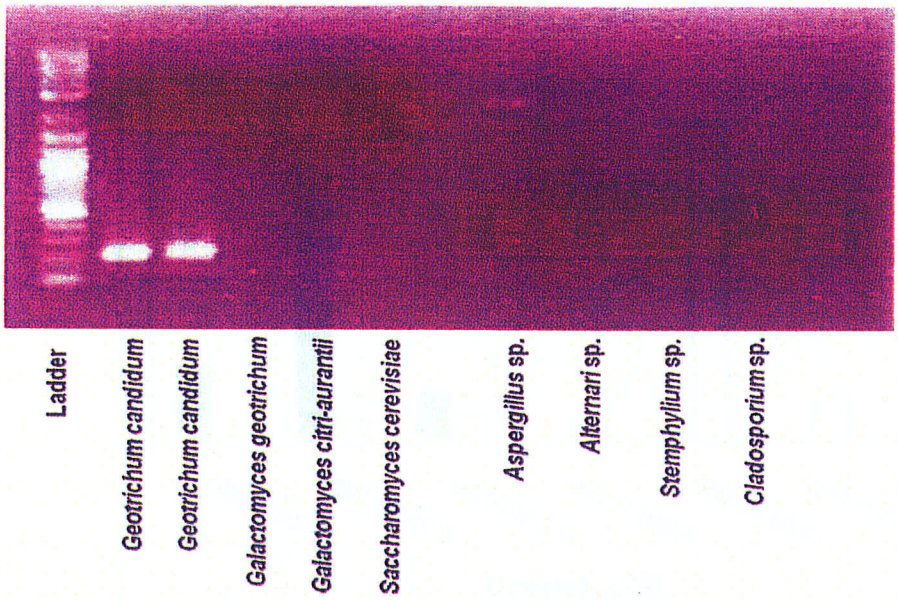

Figure 1. Specific primer to identify *Geotrichum candidum*. Presence of a band confirms the amplification and detection of *Geotrichum candidum*. The primer detected isolates from stone fruit, but did not detect the mating type, a citrus strain, *Saccharomyces cerevisiae* (baker's yeast), and four other genera of fungi.

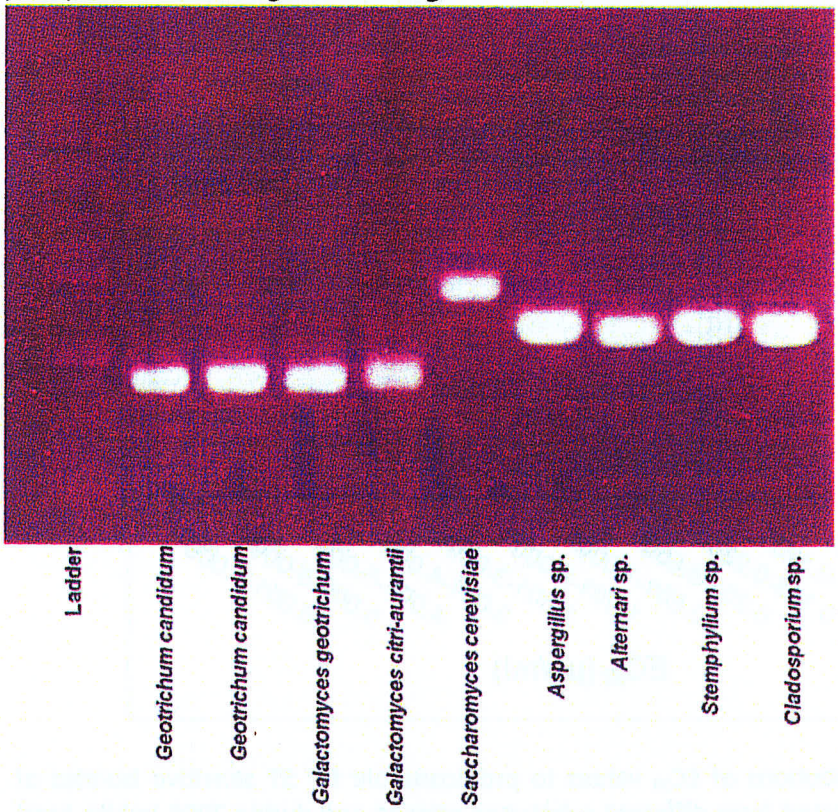

Figure 2. Amplification of the ITS region from *Geotrichum candidum* from stone fruit, *G. geotrichum* (mating type), a citrus strain, *Saccharomyces cerevisiae* (baker's yeast), and four other genera of fungi. Presence of a band confirms the amplification of ITS.

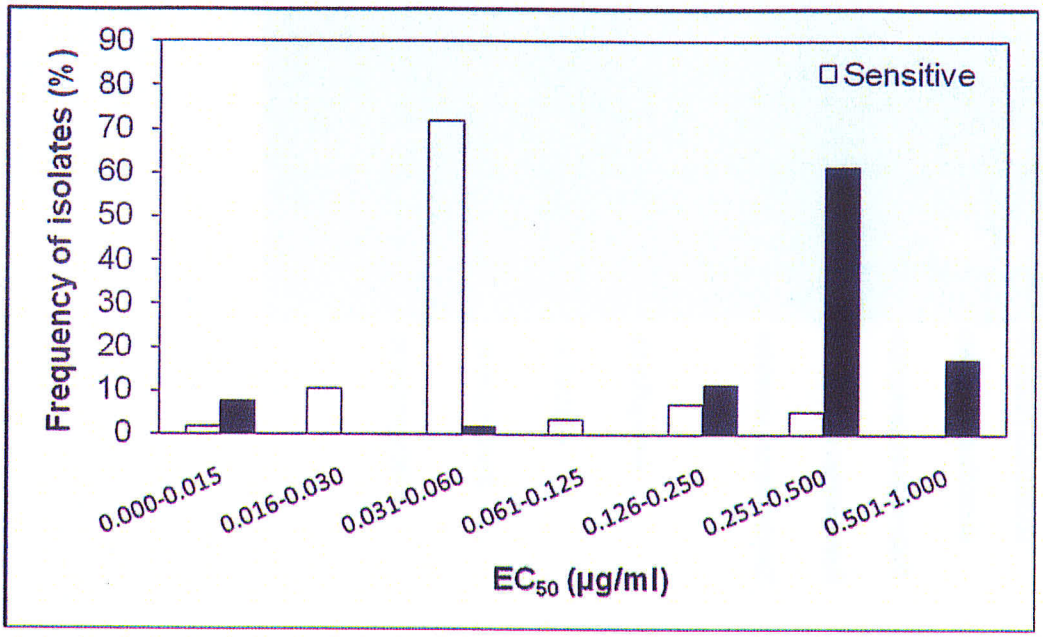

Figure 3. Frequency distributions of EC_{50} values to propiconazole for 57 sensitive isolates of *Geotrichum candidum* collected from different substrates prior to and during 2006 (white bars) and 61 isolates collected from propiconazole-treated cull fruit from 2007 to 2009 (black bars).

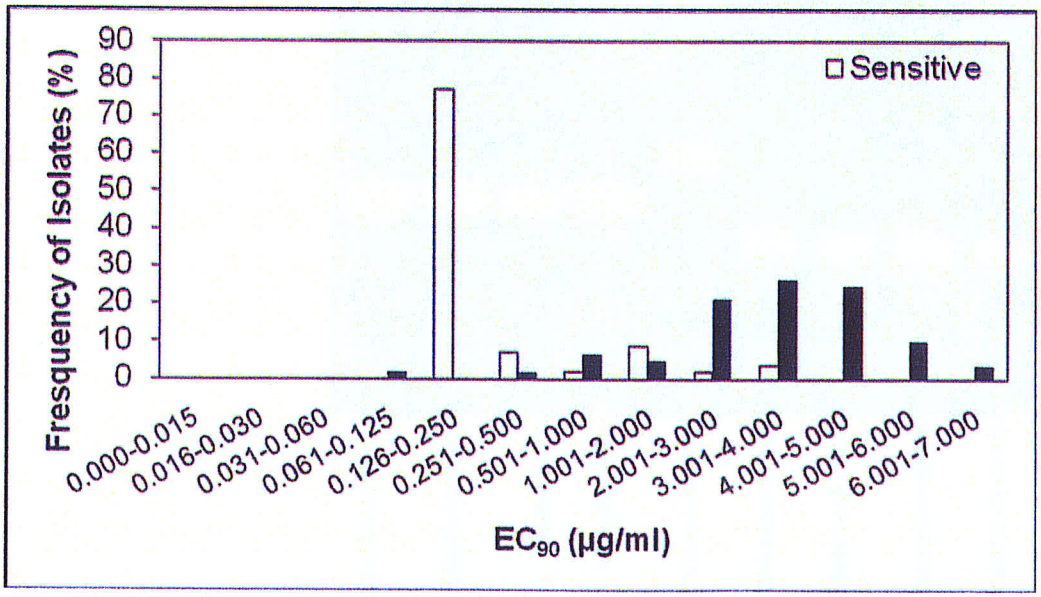

Figure 4. Frequency distributions of EC_{90} values to propiconazole for 57 sensitive isolates of *Geotrichum candidum* collected from different substrates prior to and during 2006 (white bars) and 61 isolates collected from propiconazole-treated cull fruit from 2007 to 2009 (black bars).

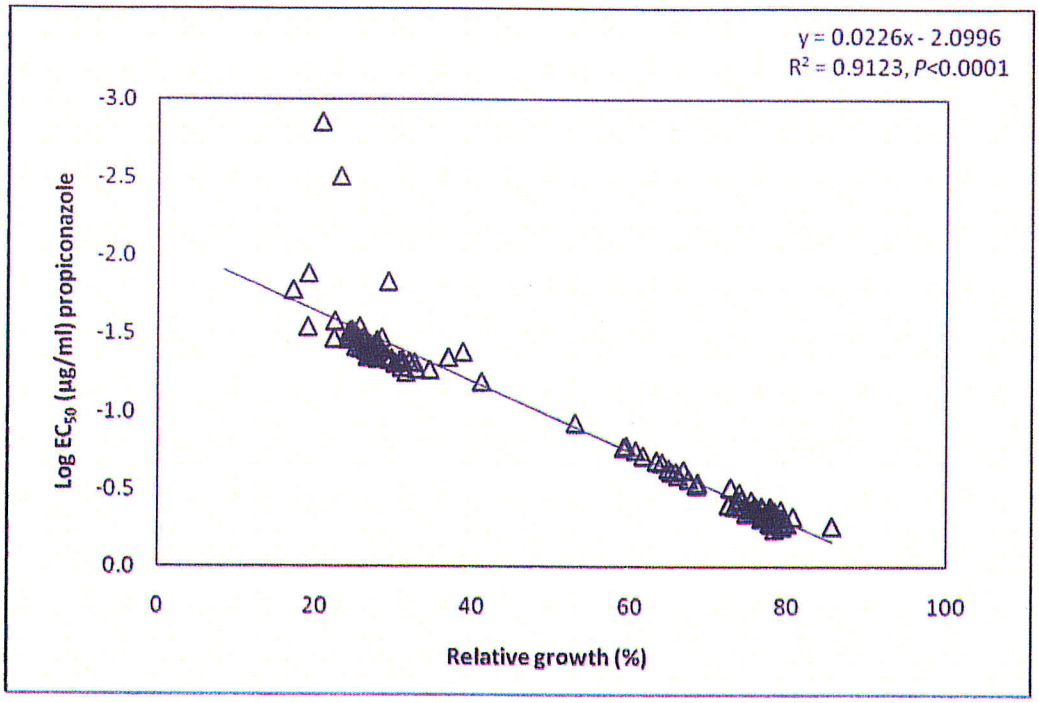

Figure 5. Regression analysis between percent relative growth and calculated EC_{50} values of 57 sensitive and 61 less-sensitive populations of *Geotrichum candidum* at a discriminatory dose of 0.1 µg/ml propiconazole.