CHAPTER 5

IN VITRO AND *IN VIVO* SCREENING OF NATURAL AND COMMERCIAL ANTAGONISTS AGAINST THE CASHEW POWDERY MILDEW PATHOGEN, *OIDIUM ANACARDII* NOACK

ABSTRACT

Current integrated cashew management strategies for powdery mildew (*Oidium anacardii* Noack) control are based on sanitation, use of tolerant varieties and most importantly recurrent application of fungicides such as sulphur, Anvil and Bayfidan. Global awareness on the negative impact of agrochemicals on the environment and human health as well as build-up of pathogen resistance, associated with limited effectiveness of some fungicides and reluctance of the chemical industry in developing new products, have led to the development of new alternatives for disease control. Biological control is one such option. The aim of this study was therefore to screen *in vitro* and *in vivo*, newly isolated and commercial potential biocontrol agents against cashew powdery mildew. From January to July 2000, natural potential antagonists were isolated from cashew leaves and florets. A total of 72 isolates were obtained and screened *in vivo* alongside three commercial biocontrol agents (*Bacillus subtilis*, *B. licheniformis* and *Candida saitoana*) against *O. anacardii*. Leaf disc and panicle assay techniques were used and a recommended fungicide Bayfidan (triadimenol 25% EC) was used as a positive control. The investigation illustrated that none of the potential antagonists isolated from cashew leaves and florets had significant inhibitory effects on *O. anacardii* spore germination and hyphal growth. All commercial antagonists significantly reduced the length of the germ-tube within 24 hours post inoculation. The biocontrol agent *B. licheniformis* also significantly reduced the rate of branching of the primary hyphae and was thus established as the most promising antagonist for field trials.

INTRODUCTION

Several reasons for the declining production of cashew in eastern Africa have been proposed (Uaciquete, 1997; Mniu, 1998; Shomari, 1998). Of these, powdery mildew disease, caused by the fungus *Oidium anacardii* Noack, was regarded as the most important (Castellani & Casulii, 1981; Intini & Sijaona, 1983; Waller *et al.*, 1992; Shomari, 1996). Losses of between 50 and 70% have been attributed to the disease (Milheiro & Evaristo, 1994), which has become endemic to the region (Nathaniels, 1996). Powdery mildew disease management relies primarily on the application of sulphur dust or new systemic fungicides such as triadimenol EC 250 g a.i./l (Bayfidan), hexaconazole EC 50 g a.i./l (Anvil) and pencanozole EC 100 g a.i./l (Topas) (Waller *et al.*, 1992; Martin *et al.*, 1997; Smith *et al.*, 1997).

Global awareness of the adverse effect of synthetic chemical residues on human health and the environment (Waller *et al.*, 1992; Smith *et al.*, 1997), build-up of resistance by the pathogen (Korsten *et al.*, 1995; Dik *et al.*, 1998), limited control of fungicides and reluctance of chemical industries to invest in development of new products (De Jager, 1999) have necessitated a search for alternative non-chemical methods (Korsten *et al.*, 1995; Dik *et al.*, 1998; De Jager, 1999).

Biocontrol of fruit and leaf diseases through the use of antagonistic microorganisms has recently emerged as a viable disease management strategy (Korsten *et al.*, 1995). In this context, biological control of various biotrophic plant pathogens has been extensively investigated and reviewed (Pusey & Wilson, 1984; Sundheim, 1986; Korsten *et al.*, 1991; Elad *et al.*, 1996; Kiss, 1997; Dik *et al.*, 1998; Koumaki *et al.*, 2000). On mango (*Mangifera indica* L.), screening of bacteria isolated from the phylloplane for antagonism against bacterial black spot (*Xanthomonas campestris pv. mangiferaeindicae* (Patel, Moniz & Kulkarni) Robbs, Ribeiro & Kimura) resulted in identification of two isolates of *Bacillus licheniformis* (Weigmann) Chester (B250 and B251), which in the greenhouse completely inhibited the pathogen (Korsten *et al.*, 1992). In addition, the antagonists reduced powdery mildew (*Oidium mangiferae* Berth.) and anthracnose (*Colletotrichum gloeosporioides* Penz.) in preharvest integrated treatments (De Jager, 1999). Recently, in semi-commercial glasshouse trials, Dik *et al.* (1998) concluded that the yeast-like fungus *Sporothrix flocculosa* Traq. had potential for efficient biocontrol of cucumber powdery mildew caused by *Sphaerotheca fuliginea* (Schlecht.:Fr.) Palacci. Unfortunately, to our knowledge, there is not

much information regarding mycoparasitism or other similar mechanisms of biological control for cashew powdery mildew. The only publication found thus far is the work of Casulii (1979), who speculated that the frequent presence of the hyperparasitic fungus *Cicinnobolus cesatii* De Bary, in association with powdery mildew, could in future constitute a basis for biological control of the disease. The aim of this study was therefore to screen *in vivo* natural and commercial potential antagonists against the cashew powdery mildew pathogen.

MATERIALS AND METHODS

Acquisition and preservation of antagonists: Epiphytic microorganisms were isolated from cashew leaves and florets (when available) of five randomly selected trees at the National Agronomic Institute, Mozambique. Samples were collected once each month, from January until July 2000. Ten powdery mildew infected leaves of approximately the same size and ten florets were picked at five points at eye level representing north, south, east and west and within the tree canopy. Thus, two sample units of each type (leaf or floret) were collected from each side of the tree. Leaves were handled by the petiole and florets by the pedicel, placed into sterile paper bags, transported to the laboratory in a cooler box and processed on the same day (Korsten *et al.*, 1995). Following the methodology described by Koomen and Jeffries (1993) entire leaves were manually shaken in 10 ml sterile distilled water for approximately 10 min. About 10 florets were directly vortexed in 10 ml sterile distilled water, followed by a dilution series. Sub-samples were spread onto either nutrient agar (NA, Oxoid) for isolation of bacteria or malt extract agar (MEA, Oxoid) with penicillin (30 mg/l) and streptomycin (50 mg/l) for isolation of yeasts and filamentous fungi. Bacterial single colonies were subcultured for purification and preservation. Fungal cultures were isolated by aseptically removing 0.5 cm^2 plugs from the edge of the actively growing colonies. All isolates were numerically coded, purified and stored at 4-5°C on respective growth media. Commercial biocontrol agents include *Bacillus licheniformis* previously isolated from mango leaves (lot 16, 27/7/2000, Korsten, L., Department of Microbiology and Plant Pathology, University of Pretoria), *Candida saitoana* Nakase & M. Suzuki from citrus fruit (lot CS-T2, 01/2000, Anchor Yeast, S.A), and *B. subtilis* (Ehrenberg) Cohn from avocado leaves (lot 3, Avogreen, 5/6/2000, Stimuplant CC, S.A.).

The host and inoculation of antagonists: Two approaches were followed to screen the effect of antagonists on powdery mildew disease development, namely the panicle and leaf disc assays. In the panicle assay, powdery mildew free panicles were cut just beneath the third leaf and transported in a cooler box to the laboratory where they were immediately placed in test tubes filled with sterile sorbitol (2% w/v). The cut section of each individual panicle was submerged to at least 10 cm deep. All test tubes were placed vertically in a wet sand-bed in the greenhouse. The sand-bed was watered to runoff at 9h00 and 16h00 to maintain approximately 90% relative humidity at temperatures of 25-28°C. Temperature and relative humidity in the greenhouse were monitored by a thermohygrometer (Type 252 44T 7d, Wilh. Lambrecht Gmbh, Goffingen). A completely randomised block design (Gomez & Gomez, 1984) was adopted. For each of the eigth experimental sets, 10 treatments were used in three replicates. Individual replicates consisted of three panicles in different test tubes. Treatments consisted of testing nine isolates separately as potential antagonists. A water treatment was included as a negative control. A total of 72 cashew isolates were tested as potential antagonists against cashew powdery mildew using the panicle assay technique.

Natural isolates were grown on NA or MEA media for 2 d (bacteria) and 7 d (fungi) respectively at 25-28°C before being scraped off the surface and suspended in sterile distilled water for immediate use. A day before inoculation with the pathogen, panicles were sprayed to runoff with either bacterial cells or fungal spores calibrated to concentrations of 10^7 cells and 10^6 spores or colony forming units per ml respectively (Koomen & Jeffries, 1993). The commercial antagonist *C. saitoana* was provided in granular formulation, thus 20 g of the granules were rehydrated in 316 ml of water, activated at 35°C for 20 min with no stirring and then poured into 9 l of water and stirred. A hand sprayer (500 ml) was used to apply either natural or commercial antagonists at recommended rates to each panicle.

The disc assay technique was adopted when disease free panicles became scarce in the field (November-April) and temperatures rose to above 40°C in the greenhouse. Under these circumstances, panicles could not be maintained surviving in sorbitol filled test tubes. Thus, reddish leaves from the second or third nodes of young healthy looking shoots were collected and superficially sterilised by immersion in 70% ethanol for 1 min (Pruvost & Luisetti, 1991). Leaf discs were excised with a 20 mm diameter cork borer which had been surface sterilised in a 0.5% sodium hypochlorite solution for 1 min, rinsed in sterile distilled water and air dried in the laminar flow (Celio & Hausbeck, 1997). The discs were placed on sterile Whatman

no.1 filter paper saturated with sorbitol (aqueous concentration 2% w/v) (Nathaniels *et al.*, 1993). A completely randomised block design with six treatments in three replicates was used. Each replicate consisted of three Petri dishes with three leaf discs in each. The treatments consisted of triadimenol 25% EC (Bayfidan) and bromuconozole 20 EC (Granit) at final concentrations of 15 ml/l and 0.15 ml/l respectively as positive controls, three commercial antagonists *B. licheniformis, C. saitoana* and *B. subtilis* and a negative water control. Commercial antagonists were prepared as described for the panicle assay technique and sprayed onto pathogen inoculated discs. The pathogen was inoculated on the 24 h pre-treated discs by releasing a single pipette drop of the inoculum on the disc surface.

Pathogen inoculation: Cashew foliage / panicles with natural mildew infection were selected 24 h before each inoculation date and shaken to dislodge old spores and encourage production of fresh spores overnight. Using the camelhair brush method (Shomari, 1996), inoculum was transferred from the source surface into 10 ml of sterile distilled water in order to standardise concentration. Thus, the concentration of the conidia was adjusted to 10^6 spores/ml using a haemacytometer. A single 5 ml pipette drop was released onto the surface of each disc. The plates were then positioned at an angle to allow a smooth absorption of excessive water by the filter paper beneath. All inoculated plates were incubated at room temperature with daylight for 24 h (Shomari & Kennedy, 1999). In the test tube trials, panicles were sprayed with a suspension of individual potential antagonists until runoff.

Observations and data analysis: Disease development on panicles was monitored daily and severity scores taken 10 d after pathogen inoculation when the untreated control reached maximum disease severity levels. In order to assess levels of the disease on individual panicles (panicle assay), a zero to six cashew blossom disease severity scale (Nathaniels, 1996; Masawe *et al.*, 1997) was used. Since the blossom disease severity scale could not be used for the leaf disc method, spores were removed 24 h after pathogen inoculation, using the cellotape technique (Nathaniels *et al.*, 1993). Cellotape was firmly pressed onto the inoculated side, then removed and mounted on a dry microscope slide. Three light microscope fields corresponding to one leaf disc were examined and at least 50 conidia per field were observed. A total of nine fields per treatment, per replicate were surveyed. From each slide, the following growth parameters at 200x magnification were noted: Total conidia observed, total germinated conidia, total conidia with two or more hyphae and the length (µm) of primary hyphae (Nathaniels *et al.*, 1993). To assist in continuous counting of spores, a

counting tool (Ferrari Statitest, Berlin, Germany) was used. Conidia germination was considered to have occurred when a new developing hypha, could be seen emerging from the spore (Isaac, 1998) with its length equal to at least half the width of the conidium (Celio & Hausbeck, 1997).

All data were transformed into percentages except for the length of the germ-tube. To transform data into percentages, the following equation was used: Total number of germinated conidia or total number of conidia with two or more hyphae, divided by total number of conidia observed times hundred. The percentages of powdery mildew on panicles were derived from Nathaniels (1996) scoring system as indicated before.

Overall scores on panicles and growth parameters were estimated as percentages and angular transformation (Arcsine) was carried out (Gomez & Gomez, 1984; Masawe *et al.*, 1997) for analysis of variance, except for the data on length of the germ-tube. Finally, all data were analysed using the ANOVA test in the statistical software MSTAT version 1.41, University of Michigan, U.S.A. Wherever justifiable, Duncan's multiple range test at $p \leq 0.05$ was used to compare and rank treatment means accordingly.

RESULTS

In total, 72 isolates (40 bacteria and 32 fungi) from cashew leaves and florets were tested as antagonists against cashew powdery mildew. None of these newly isolated potential antagonists were significantly effective in inhibiting disease development on panicles ($p = 0.3414$). Data from a 10 treatment experimental set are presented in Fig. 5.1.

On leaf discs, none of the commercial potential antagonists effectively prevented conidial germination in contrast to the standard fungicides, which significantly ($p = 0.0025$) reduced the percentage of germinated conidia (Fig. 5.2). *Bacillus licheniformis* and *B. subtilis* were as effective as the standard and recommended fungicide (triadimenol) in reducing the length of the primary hyphae ($p = 0.0253$) (Fig. 5.3). The yeast *C. saitoana* was less effective than the others but better than the negative control (water) (Fig. 5.3). However, all commercial biocontrol agents were significantly more effective in reducing the percentage of conidia with two or more hyphae as compared to the negative control (water) ($p = 0.0269$) (Fig. 5.4).

DISCUSSION

Previous studies reported the recovery of numerous isolates from the phylloplane of different plant species (Dickinson, 1976; Koomen & Jeffries, 1993; Korsten *et al.*, 1995; De Jager, 1999). The variation in types and numbers isolated is reflected in the technique used which varies between sampling unit, frequency in terms of time and space and more importantly the host and isolation technique chosen (Warren, 1976; Jacques & Morris, 1995). Through this investigation we recovered 72 isolates including bacteria and fungi, from cashew leaf and florets. The procedure was restricted to washable and culturable microorganisms which had been associated with the host / pathogen system during the sampling period. The aim of this strategy was to specially select potential hyperparasites since Casuli (1979) observed that *C. cesatii* was frequently associated with powdery mildew and he speculated that this hyperparasite could be a source of potential antagonism. However, in this study hyperparasitism was not investigated. This is the first report in which microbial isolates from cashew leaves and florets were isolated and evaluated against powdery mildew.

An important attribute of a successful biocontrol agent is the ability to be efficient at low concentrations (Korsten *et al.*, 1995). Thus, various concentrations ranging from 10^3 to 10^9 cells/ml have been used in evaluating potential antagonists against bacterial and / or fungal plant pathogens (Pusey & Wilson, 1984; Pusey, 1989; Korsten *et al.*, 1991; Korsten *et al.*, 1995; Dik *et al.*, 1998). Koumaki *et al.* (2000) succeeded in controlling cucumber powdery mildew (*S. fuliginea*) with bacteria and fungi at a concentration of 10^9 cells/ml while Dik *et al.* (1998) used only 10^6 cells/ml to reduce the level of the disease. Various authors working with bacterial antagonists commonly apply them at 10^7 cells/ml (Pusey, 1989; Pruvost & Luisetti, 1991; Korsten *et al.*, 1992). In our study, a concentration of 10^6 cells/ml of fungal antagonists and 10^7 cells/ml for bacterial antagonists were used, therefore within the commonly used range to effect.

A longer stabilisation period for the potential antagonist within the host plant organ is important for its preemptive colonisation and subsequent effectiveness against the pathogen (Fokkema, 1976). Our potential antagonists were added to the host only one day before inoculation with the pathogen. This takes into consideration the fact that production of susceptible tissue is continuous and prolonged for cashew (Shomari & Kennedy, 1999) and

the pathogen is polycyclic (Agrios, 1988). Therefore, simultaneous host colonisation is almost certain under field conditions. Thus, any promising antagonist must be a fast coloniser to be successful against powdery mildew. This may explain why bioagents were not as effective as chemical molecules in our experiment.

None of the potential biocontrol agents tested significantly reduced the percentage of conidia germination as compared to classic fungicides. But the initial growth stages of hyphae such as elongation and branching, were significantly reduced within 24 hours post inoculation with *C. saitoana, B. licheniformis* and *B. subtilis*. A comparable study where grape powdery mildew (*Uncinula necator* (Schwein)) was challenged with a chemical fungicide (pencanazole) at low concentrations also resulted in no inhibition of conidial germination but prevented hyphal development (Leinhos *et al*., 1997). This finding could be attributed to the fact that germ-tubes are more susceptible to environmental changes than conidia (Nathaniels *et al*., 1993). In conclusion, the above biocontrol agents could be established *in vivo* as potential antagonists of *O. anacardii*. Amongst these, *B. licheniformis* appears to be the most promising. It reduces both the growth in length and the branching capabilities of the primary hyphae. *B. subtilis* has successfully been used for field applications on various crops against various diseases (Korsten *et al*., 1997), but the fact that *B. licheniformis* isolates originally were from mango, which is a taxonomically close relative to cashew and has previously shown potential against mango mildew, may have contributed to its success in this experiment. However, growth and survival on cashew blossoms and leaves will require further testing under field conditions. Since endophytes live in an environment protected against sudden weather changes and radiation and are especially important for biological control (Tronsmo, 1992) further isolation work will be needed and perhaps semi-commercial screening.

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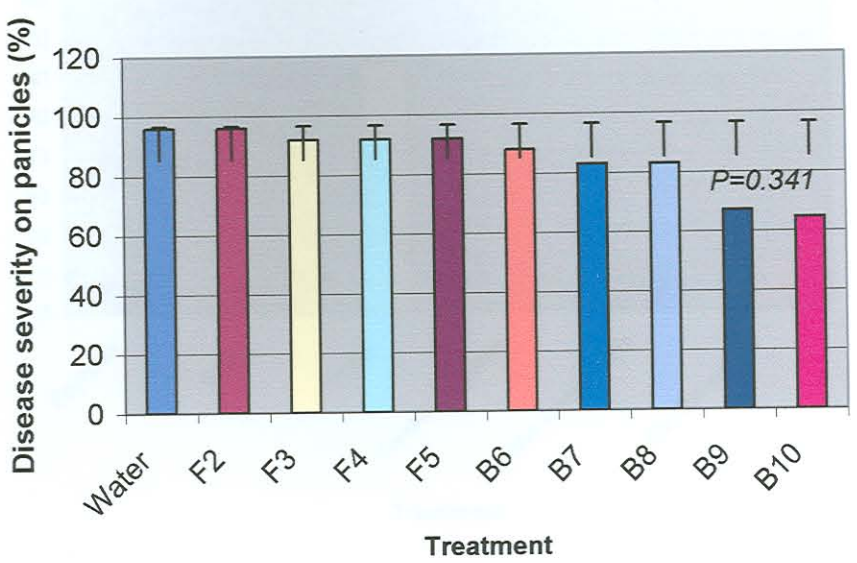

Figure 5.1 Powdery mildew severity means (in %) on panicles, 10 d after inoculation and 24 h later challenged with nine potential antagonists isolated from cashew trees and a negative water control. F = fungal antagonist, B = bacterial antagonist. Analysis of variance of angular (arcsin) transformed data, indicated $p = 0.3414$.

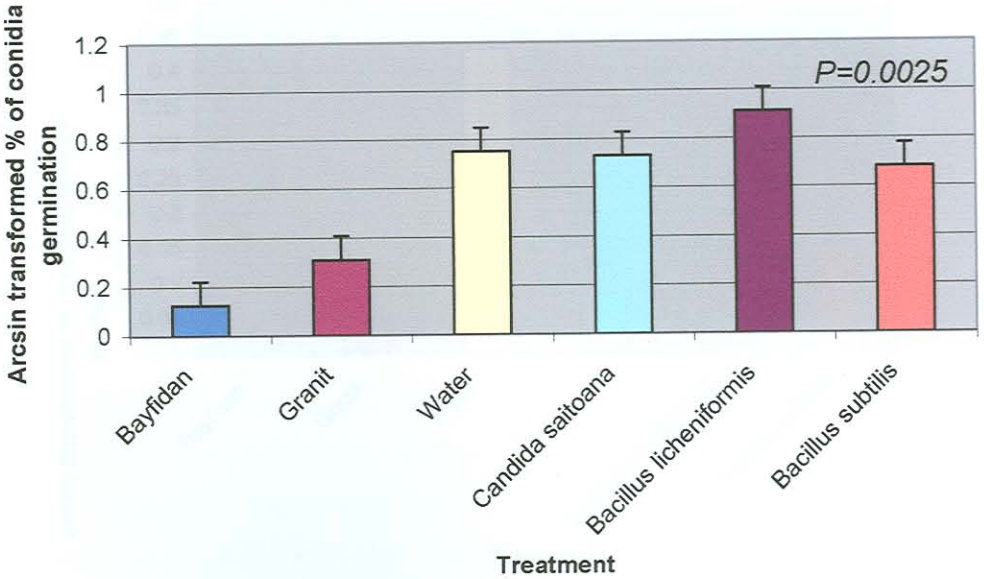

Figure 5.2 Effect of different treatments on angular arcsin transformed % of germinated conidia of *Oidium anacardii* Noack, applied 24 h prior to the pathogen inoculated on cashew leaf discs preserved on sorbitol 2% (w/v). Error bars represent the standard deviation of arcsin transformed % mean.

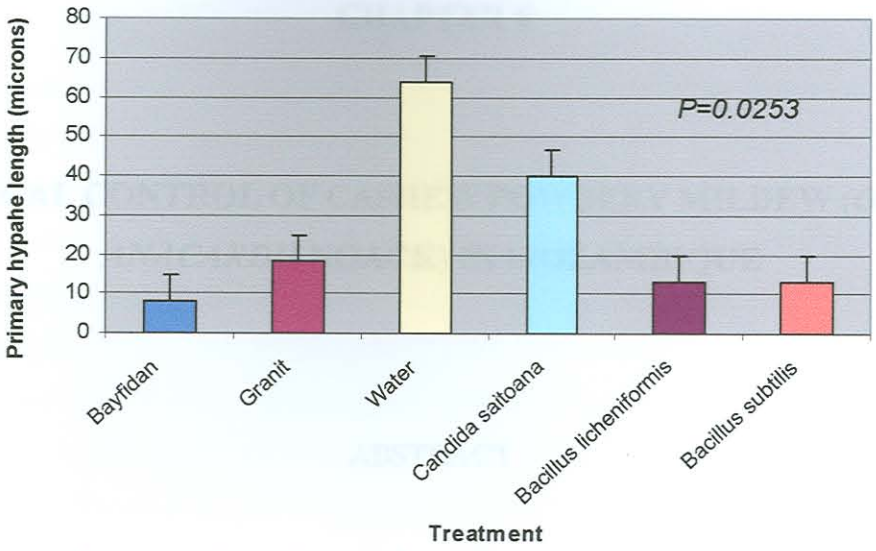

Figure 5.3 Effect of different treatments on the length at 200x magnification of the primary hyphae of *Oidium anacardii* Noack applied 24 h prior to the pathogen inoculated on cashew leaf discs preserved on sorbitol 2% (w/v). Error bars represent the standard deviation of mean lengths.

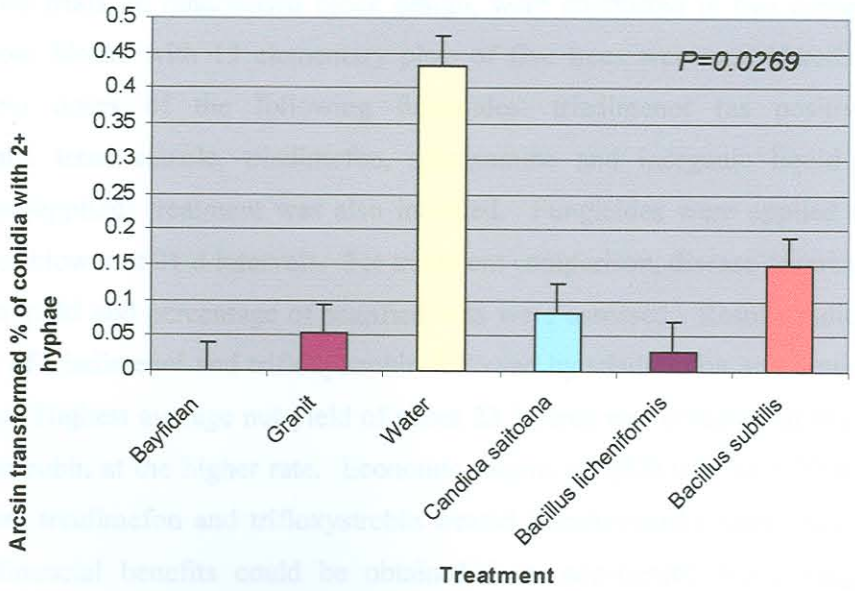

Figure 5.4 Effect of different treatments on angular (arcsin) transformed % of germinated conidia of *Oidium anacardii* Noack with two or more hyphae, applied 24 h prior to the pathogen which was inoculated on cashew leaf discs preserved on sorbitol 2% (w/v). Error bars represent the standard deviation of arcsin transformed % mean.