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Antagonistic Activity of Fructoplane Yeast Against *Ulocladium* Rot of Papaya

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Abstract

Debaryomyces hanseni Zopf isolated from the fructoplane of apples were found to be effective as biocontrol agent against rot of papaya caused by *Ulocladium. chartarum* (Pr.) Simm. The ability of *D. hansenii* to prevent infection of *U. chartarum* was lost when the antagonist cells were killed by autoclaving. Cell free culture filtrates of antagonist were unable to prevent disease incidence. Efficacy of sodium bicarbonate, sodium chloride, sodium carbonate (0.25%, 0.5% and 1.0%) and calcium chloride ($CaCl_2$ 0.25%, 0.5% and 1.0%) solutions alone or in combination with the application of biocontrol agent *Debaryomyces hansenii* (10^6 and 10^9 CFU ml^{-1}) were simultaneously evaluated for the control of Ulocladium rot of papaya. Fresh cells of biocontrol agent proliferated inside the wounds and their survival was not adversely affected by the presence of residues of calcium chloride salt. Sodium carbonate adversely affected the growth of yeast cells in in-vitro and in-vivo experiments. Sodium bicarbonate and calcium chloride also reduced the percent rot but their integration with biocontrol agent enhanced the activity of antagonist at high levels as compared to the single treatments of salts and *D. hansenii*. The integration of treatments is a promising approach to control the Ulocladium rot of papaya

Keywords: Calcium chloride, *Debaryomyces hansenii*, Papaya, *Ulocladium chartarum.*

INTRODUCTION

Papaya fruits (*Carica papaya* L. cv. Coorg Honey) rich source of vitamin C, are susceptible to postharvest decay caused by several pathogenic fungi which reduce the quality of fruits. Past studies indicate that *Ulocladium chartarum* (Pr.) Simm. is a potential pathogen causing soft rot. Losses due to decay are estimated to be 2-5% when fungicides are used, without fungicidal treatment losses may reach upto 40% or more (Sharma, 1989).

In view of the growing public concerns over environmental and health hazards resulting in cancellation of some of most effective fungicides (Eckert and Ogawa, 1985) and development of resistance in fungal pathogens to chemicals (Holmes and Eckert, 1999). Determined efforts are being made to reduce the use of pesticides (Sharma, 2000). Control of pathogens using microbial antagonists has emerged as one of the most viable option either alone or as part of an integrated control strategy to reduce synthetic fungicide inputs (Tian *et al.*, 2004).

Biological control of postharvest diseases of fruits has met marketable success with peach and apple (Janisiewicz and Roitman, 1988), plum, apricot (Pusey and Wilson, 1984), avocado (Korsten *et al*., 1995), mango (Sharma 2003), citrus (Sharma *et al*., 1996), longan (Jiang, 1977), nectarine (Smilanick *et al.,* 1993), tomato (Sharma, 2000) and strawberry (Tronsmo and Dennis, 1977). Yeast isolated from the fructoplane appear to be promising as they are capable to rapidly colonize the surface wounds and subsequently compete out the pathogens for nutrient and space (Spadaro *et al.,* 2002), they do not produce antibiotics (Sharma, 2003) but get attach tenaciously to fungal cell wall (EI Ghaouth, *et al.,* 2003) suggesting involvement of nutrient and space competition as well as direct parasitism in the mode of action of antagonistic yeasts.

The margin for acceptable performance of biological control is generally lower than that for fungicides. Higher concentrations of antagonists are required to achieve the same control of decay. Also biocontrol agent can not provide by themselves the consistent or broad spectrum control of synthetic fungicides. In general microbial antagonist confer only a protective effect that diminishes as the fruit mature, usually can not eradicate latent infections and do not prevent fungal sporulation. Therefore, primary approaches to improve biocontrol of postharvest diseases are manipulation of the environment (Tian *et al*., 2002) use of mixture of antagonists (Agnihotri *et al.*, 2006), physiological and genetic manipulation of antagonist and integration with other non-biological methods (Usall, *et al.,* 2008).

Improved control of infection by integration of biocontrol agents with other physical or chemical treatments has been proven to provide synergistic or additive inhibitory effects (Obagua and Korsten, 2003). Inorganic salts used in combination with biological methods can be useful tools to manage postharvest decay because in addition to their antimicrobial activity, they are easily available, with minimum risk of injury and above all are inexpensive. Decay caused by fungal pathogens in apples (Conway *et al.,* 1999) and oranges (Palou *et al.,* 2001) was reduced by application of calcium and sodium salts. Calcium attributed resistance to fungal pathogens is due to processes making the cell wall less accessible to cell wall degrading enzymes (Knee, 1978). However carbonic acid salts of sodium although not very persistent fungicides; appear to be fungistatic in nature.

Biocontrol agents, conversely, persist for long periods after application and although they are poor eradicant but can provide persistent protection of the fruit from possible reinfection during postharvest handling and storage. Thus combination of calcium and sodium salts with biocontrol can reduce disease incidence. To the author's knowledge, no efforts have been made to test the efficacy of combination of yeast and salt in controlling decay of papaya caused by *Ulocladium chartarum*. The objective of this study was to determine if the incidence and severity of Ulocladium rot can be reduced by applying the biocontrol agent *Debaryomyces hansenii* strain NS9 to papaya fruits. Further inorganic salts of calcium and sodium were evaluated for their effectiveness, singly and in combination with yeast *Debaryomyces hansenii* Zopf.

MATERIALS AND METHODS

Fruits

Fruits of *Carica papaya* L. cv Coorg Honey at the breaker stage free of blemishes, injury or disease, were selected. They were washed in tap water, air dried, air dried.

Pathogen

Ulocladium chartarum (Pr.) Simm. was isolated from infected papaya fruits (elaborate the isolation process). Cultures were maintained on potato dextrose agar medium at 4°C.Fresh cultures were grown on PDA plates at 25 °C before use. Spore suspensions were prepared from 14 day old culture by washing the culture with 10 ml sterile distilled water (SOW) containing 0.01% (v/v) tween 80. The concentration of spores used for inoculation was 1 x 104 spores ml^{-1} which resulted in at least 80% infection of control wounds after seven days of incubation.

Screening of antagonist

The primary screening was done according to the method given by Sharma *et al*., (2006):

Primary Screening of potential antagonist

The method described by Janisiewicz (1987) was used to select microorganisms capable of reducing disease caused by *Ulocladium chartarum* from a large number of isolates. The minimum criteria used were reduction in incidence of disease by 5 % or more and inhibition of rot diameters by more than 75%. Surface-sterilized papaya was wounded (3 mm) at the stem end at two sites. Then, 20 µl of water suspension of an antagonist were pipetted into the wound, followed by inoculation with 20 µl of an aqueous suspension of *U. chartarum* conidia (104 conidia ml^{-1}). Ten fruit constituted a single replicate and each treatment had three replicate and was repeated three times, lesion diameter were measured after 7 days of incubation at 25±1°C and 85±5% relative humidity.

Potential of different concentrations of antagonist

To determine the minimum effective concentration of *Debaryomyces hansenii* against *U. chartarum*, surface-sterilized fruits were wound with the help cork borer Then 20 µl of aqueous suspensions of *D. hansenii* (1 x 103, 1 x 106 and 1 xl09 CFU ml^{-1}) were applied to each wound. After that, the wounds were inoculated with 20µl of an aqueous suspension of pathogen (104 conidia ml^{-1}) separately. Ten fruits constituted a single replicate and each treatment had three replicates and the experiment was repeated three times. Percent rot was measured after 7 days of incubation, at 25±1°C and 85±5% RH .To observe the relation of increasing concentration of yeast suspension and spore concentration of pathogen a experiment was designed where different concentrations of yeast (1 x 103, 1 x 106 and 1 xl09 CFU ml^{-1}) were applied against different concentrations of pathogen (1 x 102, 1 x 103 and 1 xl04 CFU ml^{-1}).

Effect of inoculation time of antagonist and pathogen

Fresh and ripe papaya fruits were surface sterilized and placed on resting stand. Two wounds of 3 mm in diameter were made on the fruits, 20 microlitre of the antagonist and 20 µl of pathogens conidial suspension (104 conidia ml^{-1}) was inoculated into the wound simultaneously. In the other sets of experiments the pathogen and antagonist were applied on the wound after a time gap of 24 h, 48 h and 72 h for pre inoculation and post inculcation studies. After inoculation the tray was covered with polythene sheet and incubated at 25±1 °C for 7 days. Percent rot was calculated by counting the number of infected sites and the experiment was repeated three times.

Pre-treatment of the antagonistic cell

Killed cells of *D. hansenii* were obtained by autoclaving it. Such autoclaved cell suspension was used to evaluate their efficacy in inhibiting U. chatamum and cell free culture titter was also

used. Fresh and healthy papaya fruits were surface sterilized with ethanol and placed on moist blotting paper in plastic trays. Two wounds (3 mm deep and 3 mm wide) were made and 20 ≒1 retreated antagonist suspension was poured in each wound; allowed to dry and inoculated with spore suspension (104spores ml^{-1}) of the pathogen. The treated fruits were incubated under moist conditions at 25±1°C for 7 days. Sterile water was inoculated to the controls. Percent rot was calculated by counting number of infected sites. The experiment was repeated thrice

In vitro analysis of population dynamics of yeast antagonist

To test the survival of yeast in different concentrations of salts, 0.1 ml of *D. hansenii* suspension was added to 5.9 ml of solution at [0.0, 0.25, 0.50 and 1.0% (w/v)] to give a final count of 1×104 CFU ml^{-1}. Cultures were held under ambient conditions and thoroughly mixed prior to sampling. Aliquots were removed at 0, 16, 20 and 24 h intervals. Samples (0.1 ml) of ten fold serial dilutions were plated on malt yeast extract agar plates (MYEA) and colonies were counted after incubation at 25±1°C.

In vivo analysis of population dynamics of yeast antagonist

To determine the effect of sodium chloride, sodium carbonate, sodium bicarbonate and calcium chloride on the population of *D. hansenii* in papaya wounds, 20≒1 of water suspension of yeast cells (109 cells ml^{-1}) containing various concentrations of sodium salts or $CaCl_2$ were pipetted into each wound and incubated at ambient temperature under moist conditions in trays with cover. Tissue containing the wound was removed with a cork borer (5 mm in diameter) at 0, 12, 36, 24, 48 and 72 h and homogenized in 5ml of sterile water. Samples were serially diluted and plated on MYEA in order to determine the yeast cell concentration. Three wounds were inoculated for each treatment. MYEA plates were used to determine the population of yeast for each wound. The results were expressed as the mean number (±SEM) of colony forming units (CFU) per wound.

Effect of Inorganic salts on growth of U. *chartarum* (In vitro)

Three sodium salts [at 0.0, 0.25, 0.50 and 1.0% (w/v)] and calcium chloride [$CaCl_2$ at 0.0, 0.25, 0.50 and 1.0% (w/v)] were used in all treatment. Salt solutions were filtered through a 0.45 µl Millipore filter unit before adding them at different concentrations to autoclaved potato dextrose agar (PDA). After the PDA had cooled to 55oC the effect of SBC and $CaCl_2$ on mycelial growth of *U. chartamum* was tested after placing 5 mm diameter disc from periphery of a 10 day old culture in the centre of a 90 mm petri dish containing PDA with appropriate SBC and $CaCl_2$ concentrations. The cultures were incubated at 25±1°C for 7 days. Growth was determined as the average increase in colony diameter following incubation. The experiment was conducted three times with three replications for each fungus. Since the pH of the SBC amended medium was 7.0, control plates were adjusted to pH 7.0 with 1N NaOH. Plates were incubated for 7 days at 25±1oC and colony diameter was measured.

Effect of Inorganic salts on *U. chartamum* in papaya wounds (In vivo)

To determine the effect of sodium salts and $CaCl_2$ on papaya wounds, 20≒1 of salt solutions of different concentrations [0.0, 0.25, 0.50 and 1.0% (w/v)] were pipetted into each wound and incubated at 25±1°C under moist conditions in trays with cover. Percent rot was calculated after 7 days by counting number of infected sites. The experiment was repeated thrice.

Effect of inorganic salts on enhancement of biocontrol activity of antagonist

To test the biocontrol efficacy of *D. hansenii* (106cfu/ml and 109cfu/ml) with sodium salts [at 0.0, 0.25, 0.50 and 1.0% (w/v)] and $CaCl_2$ [at 0.0, 0.25, 0.50 and 1.0% (w/v)], papaya fruits were washed with tap water, dried and uniform wounds were made on the sides of each fruit.

Wounds were inoculated with 20≒1 of yeast suspension in various concentrations of salts. After 72h, 20≒1 of sterile water containing 104spores/ml of pathogen was added to each wound. Percent rot was calculated by counting number of infected sites. The experiment was repeated thrice.

Statistical analysis

The per cent increase/inhibition (decrease) over control was calculated by applying formula
Percent increase/inhibition over control = rc-rt/rc 1 00
Rc = Reading of control
Rt = Reading of treatment

To lest the significant difference between treatment and control student's t test or one way ANOVA was used. Statistical significance was judged at the P< 0.05 levels. Statistical analysis was performed with the help of software Sigma Stat 3.5 Jindal Scientific, 2006 and Microsoft Office Excel worksheets.

RESULTS

From more than 100 microorganisms isolated from fruit surface, 95 were tested in primary screening against *U. chartamum*. More than 60% of the isolated microorganisms showed some antagonistic activity but only 2% of these tested strains reduced the incidence of infected wounds by more than 50% and reduced lesion diameter by more than 75%. The most effective microorganism was the strain NS1, yeast identified as *D. hansenii* by the IMTECH Chandigarh India (Table- I).

D. hansenii strongly inhibited development of *U. chartamum* at 25±1°C. Reduced rots were observed on fruits treated with any of the three concentrations 1×10^3, 1×10^6 and 1×10^9 CFU ml^{-1} of the *D. hansenii* against 104 conidia ml^{-1} of pathogen. At concentration of 1×10^6 and 1×10^9 CFUml^{-1}, *D. hansenii* reduced the infection of pathogen. There was direct relation between the concentrations of yeast required to inhibit the increasing concentration of pathogen spore suspension (Fig. 1).

The time of application of the antagonist and pathogen on fruit surface clearly affects disease development. Conidial suspension of U.chartamum was applied 24h, 48h and 72h before inoculation of *D. hansenii* at 1×10^3, 1×10^6 and 1×10^9 CFU ml^{-1} and 71%, 63%, 55%, 49%, 39%, 35%, 24%, 21% and 19% rots were recorded.

In another set of experiments, *D. hansenii* at 1×10^3, 1×10^6 and 1×10^9 CFU ml^{-1} was applied on wounds prior to inoculation of *U. chartamum* and pathogen was inoculated after 24h, 48h and 72h. Percent rots were found 52%, 44%, 42%, 39%, 26%, 21%, 16% 8.0 and 6.0% respectively. Ninety two percent wounds were found infected with U.chartamum in control fruits. At 72h pre and post treatment by *D. hansenii* 109 CFU ml^{-1} produced 8 and 19% of rot respectively. The results suggest that pretreatment with *D.hansenii* gave better protection as compared to post treatments (Fig. 2)

Effect of pre-treatment of *D. hansenii* on its biocontrol efficacy was investigated. Both the autoclaved cell suspension and the culture filtrate, sterilized by filtration, failed to provide any protection and 92.0% infection was recorded in both the treatments against the *U. chartamum*. Application of live cells of *D. hansenii* on wounded sites showed maximum inhibition of infection caused by *U. chartamum*. Two types (Distilled water and sterile medium) of control were made and 92.0% and 95.0% infection was recorded in papaya fruits against *U. chartamum*. These observations suggest that *D. hansenii* was antagonizing the pathogen not through the production of antibiotics (Table 2).

The survival of *D. hansenii* at three concentrations; 0.25, 0.50 and 1% of salts at an initial concentration of Log_{10} 4.0 was observed. The population of *D. hansenii* increased at different concentrations (0.25%, 0.5% and 1.0%) of calcium chloride. In 0.25%, 0.5% and 1.0% concentration of calcium chloride population of antagonist was raised to Log105.9, Log106.0 and Log106.5 after 24hr incubation. However in SBC at 0.25%, 0.5% and 1.0% the population of *D. hansenii* in-

creased slightly to Log105.5, Log105.5and Log105.7 but it was significantly lower than survival of *D. hansenii* in calcium chloride. The greatest survival was observed in sodium chloride that was even greater than calcium chloride. But sodium carbonate was not supporting the growth of yeast antagonist. Both salt solutions did not alter cell morphology, size of vacuole and viability of *D. hansenii* (Fig. 3).

Population dynamics of *D. hansenii* within wounds was evaluated. Papaya fruits were inoculated with *D. hansenii* in combination with SBC or calcium chloride solution and stored at 25±1°C for 7 days. The wound population was quantified at interval of 0, 12, 36, 24, 48 and 72h. The population of *D. hansenii* with calcium chloride (0.25, 0.50 and 1%) increased progressively to reach maximum population (Log_{10}8.0, Log_{10}8.2 and Log_{10}8.4) after 72 h of storage as compare to initial population (log_{10}5.6) but after 72h population of antagonist was lower in SBC (0.25, 0.50 and 1%) treated wounds (Log_{10}7.0, Log_{10}7.1 and Log_{10}7.3) and was lowest in sodium carbonate than population of antagonist in control fruits (Log_{10}8.4) (Fig. 4).

In vitro treatments of SC and SBC and $CaCl_2$ significantly decreased radial growth of fungus but sodium chloride failed to inhibit the growth of pathogen. The test fungus *U. chartamum* was grown on (at 0.25%, 0.50% and 1%) sodium salts and CaCl2-amended PDA medium and recorded lowest in radial growth (12.0mm) in $CaCl_2$ (1%) amended PDA medium as compared to control (74.0mm). The radial growth (25mm) of pathogen was also restricted at 1% SBC -amended PDA medium. Lower concentration (0.25%) of both the salts was not able to reduce the radial growth of *U. chartamum* (Table 3).

Papaya fruits were treated with 0.25%, 0.5% and 1% $CaCl_2$ and then counter inoculated with spore suspension of *U. chartamum* and percent rot was recorded. 0.5% and 1% of calcium salt were found to inhibit the rot development (67% and 55%) as compared to control (95.0%). Papaya fruits treated with 0.25%, 0.5% and 1% sodium salts were also evaluated for disease development.75% and 60% fruits were recorded infected with pathogen when treated with 0.5% and 1% $NaHCO_3$, as compared to control (95.0%). Sodium chloride was totally ineffective in reducing infection and at all concentrations the lesions developed were more aggressive than control. Calcium chloride at 1% was found best in reducing infection among the tested different concentrations of both the salt. One percent SBC treatments were found inferior to reduce percent rot in papaya. Low concentration (0.25%) of sodium bicarbonate and calcium chloride were not so effective in reducing percent rot caused by *U. chartamum* .

The results indicated that sodium chloride was supporting the propagation of yeast antagonist but it failed in alone treatment to control the disease at any level. Rather than inhibition it was supporting the pathogen to grow in PDA amended plates and in wounds of papaya. Sodium carbonate was although inhibiting the pathogen in alone treatment but it was not supporting the growth of yeast antagonist. On the basis of these observations sodium bicarbonate and calcium chloride were selected for further experiments.

$CaCl_2$ results in the enhancement of control with *D. hansenii* as shown in results. Percent inhibition of rot on papaya fruit was significantly lowered at 1% $CaCl_2$ with antagonist, compared to alone treatments with *D. hansenii*, $CaCl_2$ and the water. Enhancement of the activity of *D. hansenii* with addition of $CaCl_2$ was recorded in the present study. 5.7% wounds were found infected when *D. hansenii* (at 1×10^9 CFU ml^{-1}) was applied with 0.5% $CaCl_2$ while 1% $CaCl_2$ solution was used with *D. hansenii* (at 1×10^9 CFU ml^{-1}) 2.0% wounds were infected up to seven days. $CaCl_2$ concentration (1.0%) enhanced the biocontrol activity of *D. hansenii* making even the lower concentration (at 1×10^6 CFU ml^{-1}) of antagonist better than alone treatment of *D. hansenii* at that concentration but lesser than combination of $CaCl_2$ with higher concentration of *D. hansenii* (at 1×10^6 CFU ml^{-1}). SBC (0.25%, 0.5% and 1%) in combination with *D. hansenii* (at 1×10^6 CFUml^{-1} or 1×10^9 CFU ml^{-1}) was significantly lower than efficacy of combined treatment of $CaCl_2$ with *D. hansenii* (at 1×10^6CFU ml^{-1} or 1×10^9 CFU ml^{-1}) (Fig. 5).

DISCUSSION

Papaya (*Carica papaya* L.) is one of the most important fruit grown in India. Its production has been hampered due to the diseases which happen to cause heavy losses. Ulocladium rot of papaya is among the unattempted disease. So, the present investigation was focusing on the control of *U. chartamum*, the causal agent of Ulocladium rot of papaya (Sharma, 1981).

An important attribute of a successful biocontrol agent is the ability to be efficient at low concentrations (Wisniewski and Wilson, 1992). *D.hansenii* confirmed to this prerequisite by being effective against *U. chartamum* on papaya at a concentration of 1×10^9 CFU ml^{-1}. Almost similar results have been reported by Janisiewicz (1988) using a species of Pseudomonas applied at 3×10^8 CFU ml^{-1} while Mari *et al.,* (1996) recommended the application of 1×10^8 CFU ml^{-1} for *Erwinia sp*. and *Bacillus sp*.

The time of application of the antagonist appears to be an important factor in yielding desired level of disease inhibition. Application of *D.hansenii* to the wound 72 h prior to the inoculation of wound with fungal pathogen did not allow the infestation of wound with the pathogen. Reducing the time gap between the application of Biocontrol agent and pathogen increases disease incidence. Similar results were obtained in post inoculation experiments, with the increase in time gap between the pathogen inoculation and application of *D.hansenii* the effectiveness of *D.hansenii* was reduced. These results are in accordance with those of Chalutz and Wilson (1990) and Droby *et al.,* (1993). The inhibitory effect due to competition for nutrients and space is considerably lower when yeast was applied after the pathogen is already established and actively growing within the infection site in fruit. Because of this disadvantage in the competition many antagonistic microbes, mainly yeast whose mode of action is not antibiosis or parasitism, are poor eradicants of pathogens (Spadaro and Gullino, 2004). It appears that time required for establishment of the biocontrol agent over the fruit surface increasing time gap facilitate better colonization of the fruit surface by the biocontrol agent. However, when pathogen is applied prior to biocontrol agent, the scope of establishment of biocontrol is hampered and the biocontrol efficacy is reduced. It may be therefore suggested that the treatment with biocontrol agent should be practiced during their harvest in the fields.

The ability of *D.hansenii* to prevent infection of *U. chartamum* was lost when the antagonist cells were killed by autoclaving. Cell free culture filtrates of antagonist were unable to prevent disease incidence. Only live cells of *D.hansenii* were effective in protecting infection of wounds against *U. chartamum*. According to these finding, it may be assumed that *D.hansenii* did not inhibited the disease by production of antibiotic (Droby, 1991).Its possible mode of action is competition for nutrients and space as the population studies in wounds indicated continuous rise in Log10 population of antagonist and inhibition of decay of papaya fruits.

The survival of *D.hansenii* in sodium bicarbonate and calcium chloride at an initial concentration was also observed. Population increased consistently in the sodium bicarbonate and calcium chloride solutions. In one percent sodium bicarbonate coating population initially decline but thereafter increased to 10^6 CFU ml^{-1} but lesser than the population of *D. hansenii* in calcium chloride. Similar results were observed by Mc Guire (2000). The population of *D. hansenii* increased consistently in calcium chloride up to 72h. These results also confirm with the work done by Potjewijd *et al.,* (1995)

In vitro studies conducted using inorganic chemicals indicated their efficacy in controlling fungal infection of fruits. Sodium bicarbonate and calcium chloride significantly reduced the radial growth of *U. chartamum*. These observations are in accordance with Mc Laughlin (1990). *In vitro* studies conducted using exposure to sodium bicarbonate reduced mycelial growth of *U. chartamum*. One percent sodium bicarbonate and Calcium chloride were able to control percent rot when applied to wounds in papaya fruits. The direct and indirect effects of bicarbonate salts on microorganisms have previously been noted (Punja and Grogon, 1982; Depasquale and Montville, 1990). Bicarbonate salts have broad-spectrum antimicrobial properties and are generally regarded as safe (GRAS) compounds, which do not require expensive testing and validation by regulatory agencies

(Aharoni *et al.,* 1997).

The inhibitory effect of bicarbonate salt on *U. chartamum* was probably due to the reduction in fungal cell turgescence pressure which resulted in collapse and shrinkage of hyphae and conidia, and consequent inability of fungi to sporulate (Fallik *et al.,* 1997, Correll *et al.,* 1988). Similarly treatment of papaya with 1% $CaCl^2$ was found to reduce percent rot during storage and its efficacy was significantly higher than sodium salt. The mechanism by which increased tissue Ca reduces decay and maintains firmness may be related to calcium ions in the cell wall. Calcium induced resistance to fungal pathogens is attributed to processes making the cell wall less accessible to fruit softening enzymes produced by fungal pathogens (Conway *et al.,* 1989).

Application of *D. hansenii* along the $CaCl_2$ significantly increased the biocontrol efficacy of *D. hansenii*. The inclusion of $CaCl_2$ with a cell suspension of *D. hansenii* resulted in a marked improvement in antagonist activity when *D. hansenii* was applied on papaya fruits. Such beneficial effects on biocontrol activity achieved by the addition of $CaCl_2$, were also observed by Mc Laughlin *et al.,* (1990) using the yeast Pichia guilliermondi as a postharvest biocontrol agent. Calcium alone was shown to reduce the disease incidence therefore increased efficacy of *D. hansenii* in presence of calcium may be taken as additive or synergistic effect. Application of calcium may increase the cytosolic calcium levels. This increase in calcium level may lead to increased expression of defense related genes like those responsible for PR protein, glucanases and chitinases. The increased calcium level may also strength the existing cell wall and also those formed in response to the fungal infection. Therefore, supplementation biocontrol agents with calcium may have important implications for the future use of *D. hansenii* on a commercial scale for the control of postharvest diseases of fruit. Calcium as part of the formulation for these biocontrol agents will improve efficacy and may help in replacing the current requirement for addition of low concentrations of fungicides to ensure consistent performance of antagonist under large scale and commercial conditions (Glenn and Poovaiah, 1990, Droby *et al.,* 1993). As reported by Droby *et al.,* (1989) effective biocontrol activity of antagonists depends on the number of cells present in wound site. The addition of $CaCl_2$ to the suspension of antagonist would also help in by lowering the concentration of antagonist required for effective biocontrol activity. Similar observations were obtained in the present study when even low concentration of *D. hansenii* (1×10^6 $CFUml^{-1}$) with calcium chloride (1.0%) resulted in only 10% rot as compared to control (95%).

Compatibility of *D. hansenii* with calcium chloride shall make possible to exploit both the eliciting property of calcium chloride and the biological activity of the antagonist. The results from the present study confirm that the combination of *D. hansenii* and 1% calcium chloride offers better control of Ulocladium rot of papaya fruits than *D. hansenii* and calcium chloride alone. Such enhanced biological activity has been attributed to additive and synergistic activity between the additives and the biocontrol agents and the increased effectiveness of the combination of *D. hansenii* with 1% calcium chloride may be due to the interplay of the biological activity of *D. hansenii* and the eliciting property of 1% calcium chloride.

In conclusion, combining antagonistic yeast with calcium chloride can be expected to provide better control of decay then the use of biocontrol agents alone. This is in accordance with the previous results that combination of treatments was more effective than alone treatments (Teixidó *et al.,* 2001, Torres *et al.,* 2007).This combination could be useful as part of strategy to reduce losses caused by pathogens isolates resistant to currently used postharvest fungicides (Eckert and Ogawa, 1985). It may be therefore concluded that application of biocontrol agent followed by an incubation period required for effective colonization of fruit surface will hampered the establishment of pathogens during storage and will help transit of fruit without any sign of decay.

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Tables

Table 1- ANOVA of effect of plant density and nitrogen on the measured traits.

Yeast	Source	Infection (%)
Rhodotorula mucilaginosa	Grape fruit	13
Debaryomyces hansenii	Tomato	57
Candida utilis	Citrus	50
Control	-	90

Table 2. Effect of cell suspension, cell free culture-filtrate and autoclaved cells of *D.hansenii* applied on wounds of papaya fruits, on the growth of *Ulocladium* rot.

S. No.	Treatments	Percent Infection[v]
1	Distilled water (Control)	92.00 (74.36)[b]
2	Sterile medium (Control)	95.00 (79.78)[a]
3	Cell suspension of *D.hansenii*	08.30 (05.29)[c]
4	Autoclaved cells of *D.hansenii*	92.00 (74.36)[b]
5	Cell free culture-filtrate	92.00 (74.36)[b]

[v]Data in parenthesis are Arcsine transformed value. Different superscript letters in the same column indicate significant differences between mean values using Duncan's Multiple Range Test (P<0.05). The values followed by same letters are statistically not significant.

Table 3. Inhibition of radial growth (in vitro) and percent rot (in vivo) of *Ulocladium chartamum* at different salts concentrations.

Salt	Concentration (%)	Radial Growth (mm)	Percent rot
$NaHCO_3$	0.0	75.0[a]±0.250	95.0[a] ±3.00
	0.25	75.0[a]±0.500	90.0[a] ±1.51
	0.50	70.0[b]±1.155	75.0[d]±1.54
	1.0	25.0[c]±0.500	60.0[f]±1.00
Na_2CO_3	0.0	75.0[a]±0.250	95.0[a] ±3.00
	0.25	72.0[a]±1.100	89.0[b]±1.50
	0.50	65.0[b]±1.100	70.0[c]±1.50
	1.0	20.0[c]±0.100	57.0[f]±1.50
NaCl	0.0	75.0[a]±0.250	95.0[a] ±3.00
	0.25	75.0[a]±1.150	95.0[a] ±.50
	0.50	77.0[b]±0.500	95.0[a] ±1.00
	1.0	79.0[c]±0.100	97.0[a] ±1.00
$CaCl_2$	0.0	74.0[a]±1.155	95.0[a] ±3.53
	0.25	73.3[a]±1.764	81.0[c]±1.00
	0.50	46.7[b]±8.000	67.0[c]±1.55
	1.0	12.0[c]±0.000	55.0[f]±1.70

Values in the same column followed by different superscript's latter are significantly different (P<0.05) according to Duncan's Multiple Range Test. Each value is mean of three replicates and ±SE are given along the mean values.

Figures

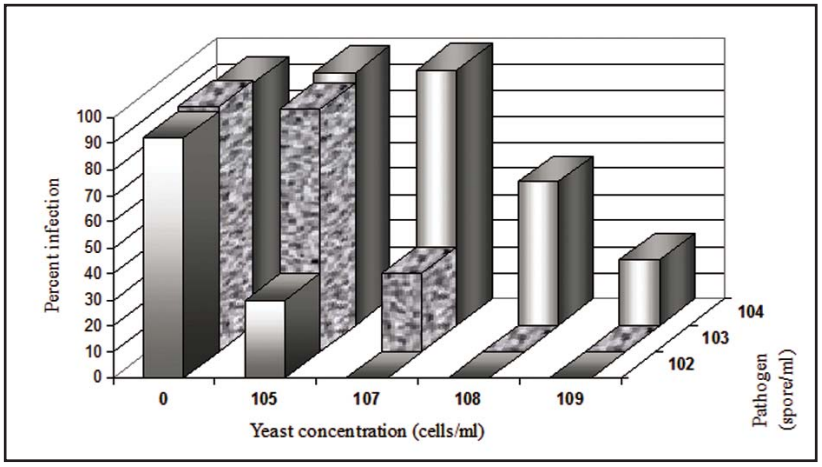

Fig. 1. The relationship between concentration of the pathogen spore suspension and the antagonist cells in the inhibition of Ulocladium decay of papaya fruit by *Debaryomyces hansenii*.

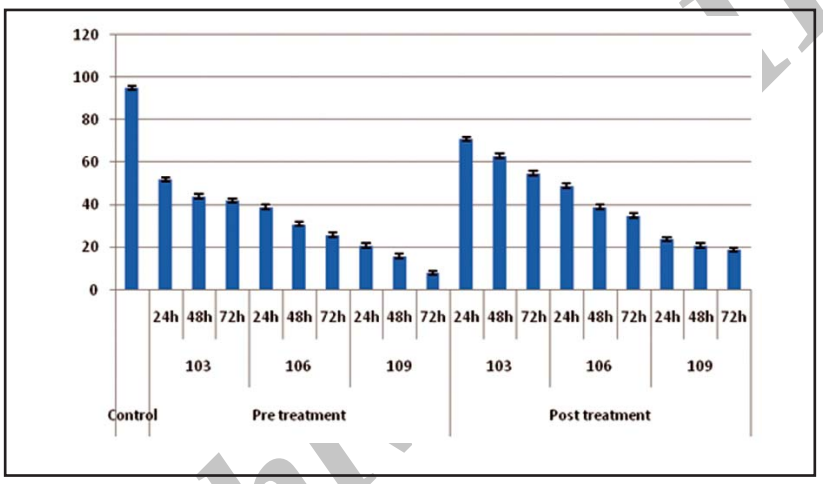

Fig. 2. Percent rot of papaya fruits treated with different concentrations of yeast antagonist.

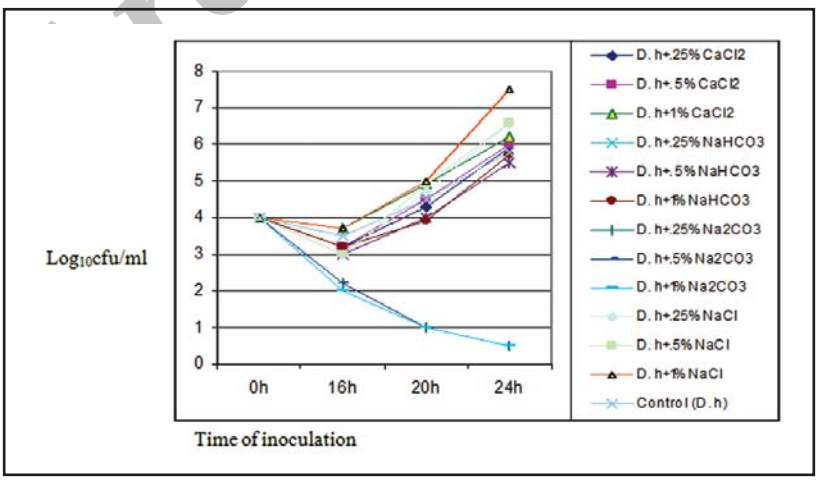

Fig. 3. Population studies of yeast antagonists with different concentrations of salts *(in vitro)*

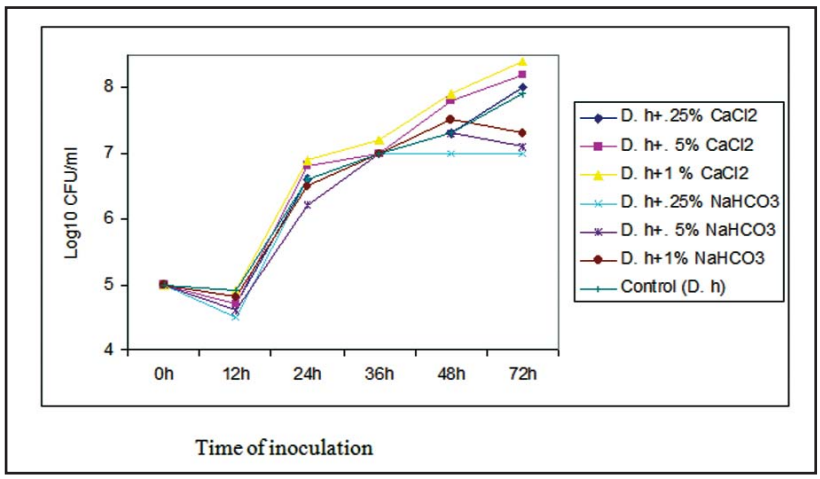

Fig. 4. Population studies of yeast antagonists in papaya fruit wounds and surface with different concentrations of salts.

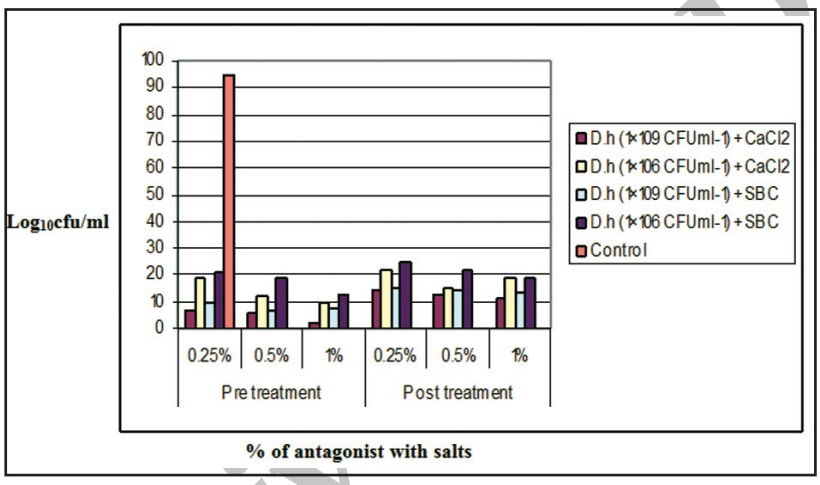

Fig. 5. Percent rot of papaya fruits treated with yeast antagonist and SBC/$CaCl_2$.