CHAPTER 5

ENVIRONMENTAL CONDITIONS INFLUENCING *IN VITRO* ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST AVOCADO PATHOGENS

1. ABSTRACT

Environmental conditions affect the efficacy of biocontrol agents. Understanding and exploiting conditions required for optimal functioning is essential for the successful implementation of biocontrol systems. The effect of different temperatures on *in vitro* antagonism of *Bacillus subtilis* on avocado postharvest pathogens *Phomopsis perseae*, *Dothiorella aromatica*, *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* were investigated. The *in vitro* effect of 19 carbon sources, 18 amino acids and ammonium chloride on antagonism was also studied. Low temperatures did not support antagonism of *B. subtilis*. However, temperatures higher than 15 ºC increased antagonistic efficacy. Nitrogen sources that increased antagonism without supporting the growth of the tested pathogens included L-glutamic acid, L-glutamine and L-(+)-asparagine, while carbon sources were D-arabinose, D-(+)-mannitol and citrate.

2. INTRODUCTION

A thorough understanding of the impact of environmental conditions on the antagonist is required in order to establish effective preharvest biocontrol systems. Preharvestly low nutrient availability, temperature fluctuations, high levels of UV radiation and dry conditions are the norm (Mari & Guizzardi, 1998; Spadaro & Gullino, 2004). In contrast, biocontrol agents applied postharvestly have an advantage since harvested fruit are kept in areas where the atmosphere is controlled and temperature and humidity are more constant (Wilson & Pusey, 1985; Ippolito & Nigro, 2000). It is well known that environmental conditions affect the survival and efficacy of bacterial biocontrol agents (Morris & Rouse, 1985; Gueldner *et al.*, 1988; Hase *et al.*, 1999; Duffy & Défago, 1999; Knox *et al.*, 2000). Most importantly, the mode of action employed by the antagonist can be triggered, enhanced or even inactivated by environmental conditions.

According to Morris & Rouse (1985), and Hase *et al.* (1999), a microbial community size is limited by the availability of at least one growth-limiting variable. Some nutrients may stimulate infection by the pathogen (Dik, 1991). On the other hand, the normal pathogenic behaviour of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara was found to be suppressed and it developed more saprophytically by adding supplementary nutrients (Blakeman, 1985). Appressoria development was also enhanced by depriving the

pathogen of nutrients (Blakeman & Brodie, 1977; Blakeman, 1985). The involvement of nutrient competition in biocontrol is often cited as a potential mode of action (Brodie & Blakeman, 1976; Blakeman & Brodie, 1977; Dik, 1991; Janisiewicz & Bors, 1995), but is difficult to prove. The competing microorganisms must both require a specific nutrient, which must not be available in abundance (Brodie & Blakeman, 1976; Blakeman & Brodie, 1977). Alternatively, if the growth-limiting nutrient of the biocontrol agent and the pathogen differ, the addition of the nutrient favoured by the biocontrol agent may increase its growth and subsequently its population size. Specific nutrients may also enhance the production of antifungal volatiles (Fiddaman & Rossall, 1994) and antibiotics (Milner *et al.*, 1995). Thus, nutrients incorporated into a biocontrol product can affect its subsequent effectiveness.

Commercial low temperature storage may reduce the effectiveness of the antagonist (Mari & Guizzardi, 1998). On the other hand, antagonists can be selected that are effective at these temperatures (Wilson & Pusey, 1985). The mode of action involved in biocontrol may also be enhanced or inactivated by the reigning temperature. Ohno *et al.* (1995) found temperature enhances the antibiotic production of iturin A and surfactin, with each antibiotic preferentially produced at a specific temperature.

In a previous study, the commercial product, Avogreen® (Stimuplant CC, Pretoria, South Africa), with *B. subtilis* as the active ingredient, performed well on a semi-commercial scale (van Dyk *et al.*, 1997). However, in one postharvest trial, the incidence of decay increased when compared with the untreated control (Korsten *et al.*, 1998). The ineffectiveness of Avogreen® in that case was ascribed to product formulation which might have contained nutrients that could stimulate pathogen growth (Korsten *et al.*, 1998). A subsequent study on the role of various nutrients in the *in vitro* interaction between *B. subtilis* and the anthracnose pathogen, *Colletotrichum gloeosporioides* Penzig., showed that some nutrients enhance the antagonist efficacy (Havenga *et al.*, 1999). The nutrients that enhanced the antagonist activity were subsequently incorporated into a new powder and liquid formulation (Personal communication, Prof P.L. Steyn, Stimuplant CC, 2003). Previously, Korsten & Cook (1996) found that the best growth temperature for *B. subtilis*, biocontrol agent of avocado fruit disease, was between 30 and 37 °C. However, after treatment of avocado fruit in the packinghouse, fruit are immediately placed in cold storage at 5.5 to 7 °C (Personal communication, Derek Donkin, South African Avocado Growers' Association, South Africa, 2004). This may also have contributed to the lowered efficacy of Avogreen® in the trial.

In this study, the effect of specific nutrients and temperatures on *in vitro* antagonism of *B. subtilis* against stem-end rot (*Phomopsis perseae* Zerova, *Dothiorella aromatica* (Sacc.) Petrak & Sydow and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.) and anthracnose (*C. gloeosporioides*) pathogens were investigated.

3. MATERIALS AND METHODS

3.1. Isolates

Bacillus subtilis subculture MI-14 (Chapter 3) was used throughout this study. Stock cultures of the antagonist were stored in 30 % glycerol with Ringer's (Merck, Johannesburg, S.A.) solution at –70 ºC. Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at 25 – 28 ºC for 24 h before use.

Fungal pathogens isolated and identified in Chapter 3 (*C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae*) were used throughout this study. All fungi were maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as keeping mycelium-containing plugs in sterile water at room temperature. An agar disk (5 mm) containing the fungus was placed on PDA and incubated at 25 – 28 ºC for three days prior to use.

3.2. Effect of different temperatures on the in vitro *inhibitory action of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The dual culture technique described in Chapter 3 was used to compare the effect of temperature on *in vitro* activity of *B. subtilis* on avocado postharvest pathogens. The antagonist-pathogen combinations were placed on PDA and incubated at 4, 10, 15, 20, 25, 30, 37 and 42 ºC. Duplicate plates were used and the experiment was repeated three times. Petridishes containing either *B. subtilis* or one of the test fungi incubated at the specified temperatures served as controls. Radial growth of the pathogens was measured after seven days' incubation at the specified temperature and recorded. The percentage inhibition was determined as described in Chapter 3. Data was analysed using the statistical program GenStat (2000). Fisher`s protected t-test least significant difference was used to test for differences between means per day. Significance was obtained at the 5 % level of significance (Snedecor & Cochran, 1980).

3.3. *Effect of nutrients on the* in vitro *inhibitory action of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The minimum salt-based medium of Janisiewicz *et al.* (1992) was used. The medium consisted of the following: 13 mM $Na_2HPO_4.7H_2O$ (Sigma, Johannesburg, S.A.), 20.4 mM KH_2PO_4 (Saarchem, Merck), 2 % v/v Hutner's vitamin free mineral base, 1.5 % w/v bacteriological agar (Biolab, Merck) in double distilled water. The pH of the medium was adjusted to 6.8 before autoclaving. Hutner's vitamin-free mineral base consisted of the following: 52.3 mM nitrilotriacetic acid (dissolved and neutralized with KOH to pH 7), 0.0075 mM $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.4 mM $FeSO_4.7H_2O$ (all from Sigma), 58.6 mM $MgSO_4.7H_2O$ (Saarchem), 22.7 mM $CaCl_2.2H_2O$ (Fluka, Sigma-Aldrich, Johannesburg, S.A.), and 5 % v/v Metals 44. The mineral base was supplemented with 0.000001 % w/v D-(+)-biotin (Fluka), 0.0001 % w/v nicotinic acid, and 0.00005 % w/v thiamine hydrochloride (both from Sigma). The pH of the Hutner's mineral base was adjusted to 6.6 – 6.8. Metals 44 consisted of the following: 8.6 mM ethylenediaminetetraacetic acid, 1.8 mM $FeSO_4.7H_2O$, 0.09 mM $Co(NO_3)_2.6H_2O$ and 0.05 mM $Na_2B_4O_7.10H_2O$ (all from Sigma), as well as 3.8 mM $ZnSO_4.7H_2O$ (AnalaR, British Drug Houses (BDH)), 0.9 mM $MnSO_4.H_2O$ (Saarchem) and 0.2mM $CuSO_4.5H_2O$ (Pro Analysi, Merck). Precipitation was prevented by adding of a 6 N H_2SO_4 (uniLAB, Saarchem) solution. All nitrogen and carbon sources were filter sterilized and added to the medium after autoclaving. Where the effect of different carbon sources was investigated, glucose was replaced by the carbon source to be tested, added at a concentration of 10 % w/v. The following carbon sources were used: acetate, benzoate, citrate, D-gluconic acid, D-(+)-glucose, glycerol, D-(-)-lyxose, L-(-)-malic acid, D-(-)-mannitol, pectin, peptone, pyruvate, L-(-)-rhamnose, D-(-)-sorbitol, starch, D-(+)-trehalose, L-(+)-xylose (all from Sigma), and D- and L-arabinose (Merck). Ammonium chloride (NH_4Cl (Sigma)) was replaced with the following amino acids as nitrogen source at a concentration of 1 % w/v: L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cystein, L-isoleucine, L-leucine, L-methionine, L-serine, L-tyrosine, L-valine (all from Merck), L-glutamine, L-glutamic acid, L-phenylalanine, L-proline (all from Sigma), glycine (Saarchem), L-histidine (BDH), and L-lysine (Fluka). The dual culture technique described in Chapter 3 was used. The antagonist-pathogen combinations were placed on the various nutrient combinations. Duplicate plates were used and the experiment was repeated three times. Plates were maintained at 25 °C. Petridishes containing either *B. subtilis* or the fungus on its own were also incubated on the nutrient combination plates and served as controls. Radial growth of the pathogens was measured after seven days and recorded. Percentage inhibition was determined as described in Chapter 3 and statistically analysed as described for 3.2.

4. RESULTS

4.1. Effect of different temperatures on the in vitro *inhibitory activity of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The *in vitro* activity of *B. subtilis* at 10, 15, 20, 25 and 30 ºC is depicted in Figure 5.1 to 5.4. No pathogen growth was observed after seven days at 37 and 42 ºC. The optimal radial growth temperature of *P. perseae* and *D. aromatica* was 25 ºC, while the radial growth of *C. gloeosporioides* was best at 30 ºC. The radial growth of *L. theobromae* was supported equally at 15, 20, 25 and 30 ºC. However, Petridishes allowed only for measurements up to 40 mm and *L. theobromae* grew faster than the other fungi. In the experiment conducted at 4 ºC, no bacterial growth was observed after seven days and growth was restricted at 10 ºC. The best inhibition against *P. perseae* was observed at 25 ºC. Inhibition of *C. gloeosporioides* was best at 30 ºC. There was no significant difference between inhibition at 15, 20, 25 and 30 ºC against *D. aromatica*. Antagonism against *L. theobromae* was greatest at 15 and 20 ºC after seven days' incubation. In general, 15 ºC supported the best inhibition of all pathogens.

4.2. Effect of nutrients on the in vitro *inhibitory activity of* Bacillus subtilis *on avocado fungal postharvest pathogens*

Antagonism of *B. subtilis* against *P. perseae* was most effective when evaluated on the following amino acid base mediums: L-arginine, L-(+)-asparagine, L-cysteine, L-glutamic acid, L-glutamine, L-methionine and L-serine (Table 5.1; Figure 5.5). However, of these, L-methionine supported the growth of *P. perseae* most effectively. Growth of *P. perseae* was the least on L-aspartic acid and L-(+)-glutamic acid. The amino acids L-isoleucine and L-lysine did not support antagonism at all. Of the carbon sources tested, acetate, D-(-)- and L-(-)-arabinose, citrate, glycerol, peptone, pectin and D-(+)-trehalose supported antagonism most effectively (Table 5.2; Figure 5.6). However, pathogen growth was supported by L-(-)-arabinose, glycerol, peptone and D-(+)-trehalose, while the least growth was observed on D-(-)-arabinose, citrate, D-(+)-glucose and D-(+)-mannitol. Antagonism was not supported by benzoate, D-gluconic acid, pyruvate, L-(-)-malic acid, L-(-)-rhamnose and L-(+)-xylose.

All the amino acids evaluated supported antagonism of *B. subtilis* against *C. gloeosporioides*, except for L-cysteine, L-isoleucine and L-lysine (Figure 5.7; Table 5.1). The inorganic nitrogen source, NH_4Cl, supported antagonism most effectively. Amino acids that did not support radial growth of *C. gloeosporioides* represented L-alanine, L-(+)-asparagine, L-aspartic acid, L-arginine, L-cysteine, L-glutamic acid, L-

glutamine, L-isoleucine, L-lysine and L-methionine. Antagonism was most effective on D-(+)-glucose and D-(+)-trehalose carbon base medium and to a lesser extent L-(-)-arabinose, glycerol, D-(+)-mannitol, pectin, peptone, pyruvate, D-(-)-sorbitol and starch (Figure 5.8; Table 5.2). Antagonism was not evident on citrate-amended medium. Peptone and pectin supported the growth of the pathogen most effectively, while acetate, D-(-)- and L-(-)-arabinose, benzoate, citrate, D-gluconic acid, L-(-)-malic acid, D-(+)-mannitol, pyruvate, D-(-)-sorbitol, starch and D-(+)-trehalose did not.

The amino acid bases that supported *in vitro* antagonism of *D. aromatica* by *B. subtilis* most effectively was NH_4Cl, L-alanine, L-arginine, L-(+)-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid and L-proline (Figure 5.9; Table 5.1). However, radial growth of *D. aromatica* was supported by all amino acids, with L-aspartic acid, L-cysteine, glycine and L-lysine being the most effective. The highest percentage inhibition was measured on D-(+)-glucose, D-(+)-mannitol, pectin, peptone, pyruvate, D-(-)-sorbitol, starch and D-(+)-trehalose as carbon source (Figure 5.10; Table 5.2). Of these, peptone, pyruvate and D-(+)-mannitol did not support radial growth of the pathogen effectively.

The most effective nitrogen sources for supporting antagonism of *B. subtilis* against the four pathogens are listed in Table 5.1. For each pathogen, the sources supporting the growth of the other three pathogens were marked. The remaining sources are those that do not support growth of any of the pathogens. The nitrogen sources that support antagonism of all four pathogens without enhancing growth of any of the pathogens tested, are L-(+)-asparagine, L-glutamic acid and L-glutamine. Table 5.2 lists all carbon sources that supported high levels of inhibitory activity of *B. subtilis* against the four pathogens tested. Antagonism against both *P. perseae* and *L. theobromae* were effectively supported by D-(-)-arabinose and citrate while they were not as conductive to antagonism against *C. gloeosporioides* or *D. aromatica*. However, neither carbon source enhanced the growth of any of the pathogens tested. D-(+)-mannitol supports inhibition of *C. gloeosporioides*, *D. aromatica* and *L. theobromae* effectively, and *P. perseae* to a lesser extent. However, it does not support the growth of any of the pathogens tested.

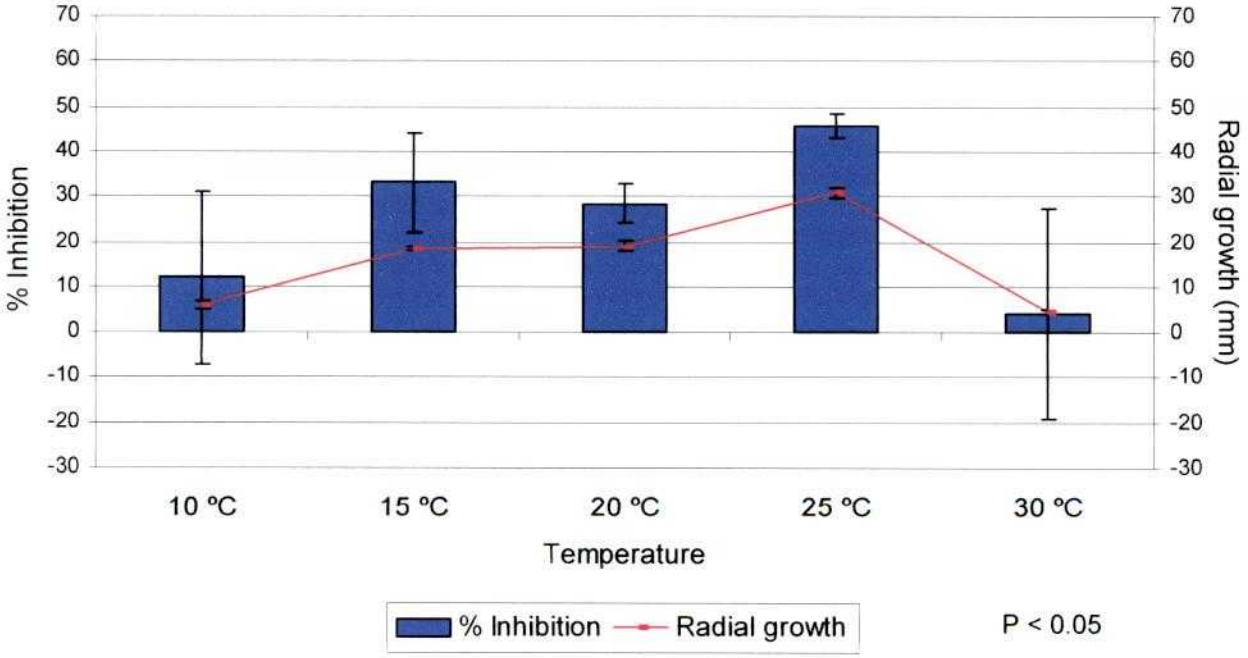

Figure 5.1: Effect of temperature on *Bacillus subtilis* antagonism of *Phomopsis perseae* at respective temperatures.

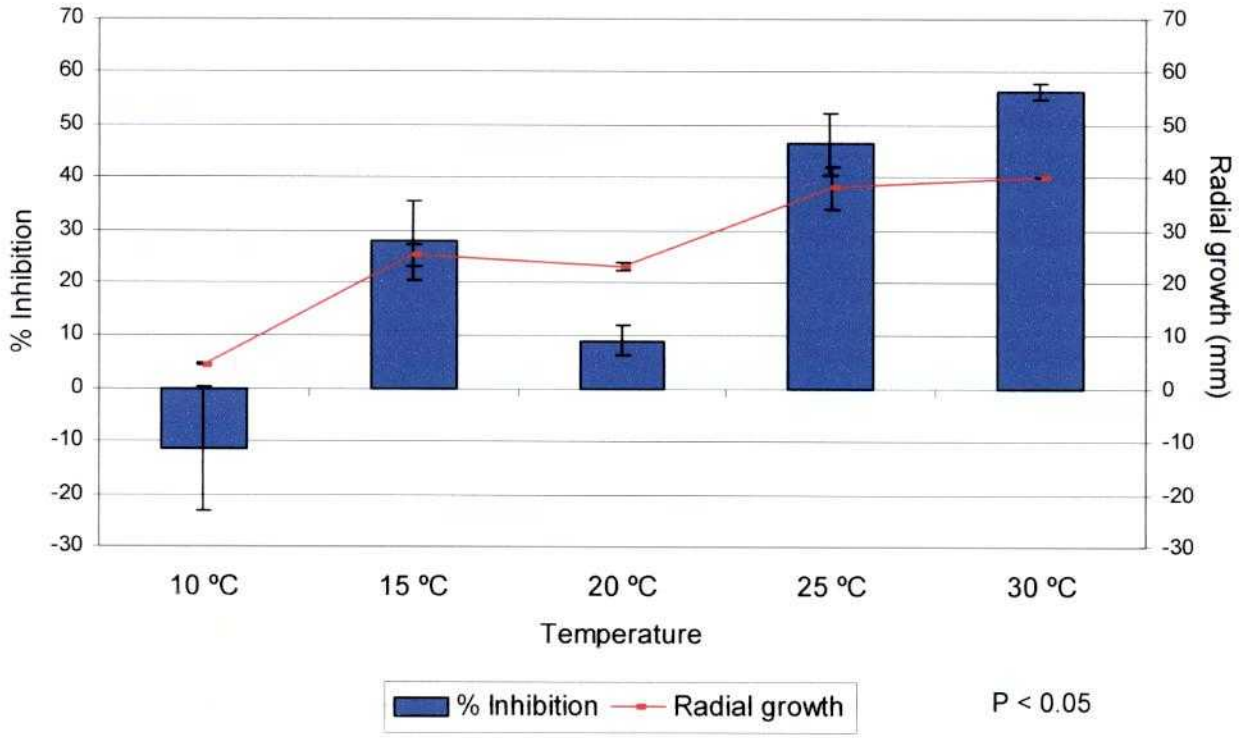

Figure 5.2: Effect of temperature on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides* at respective temperatures.

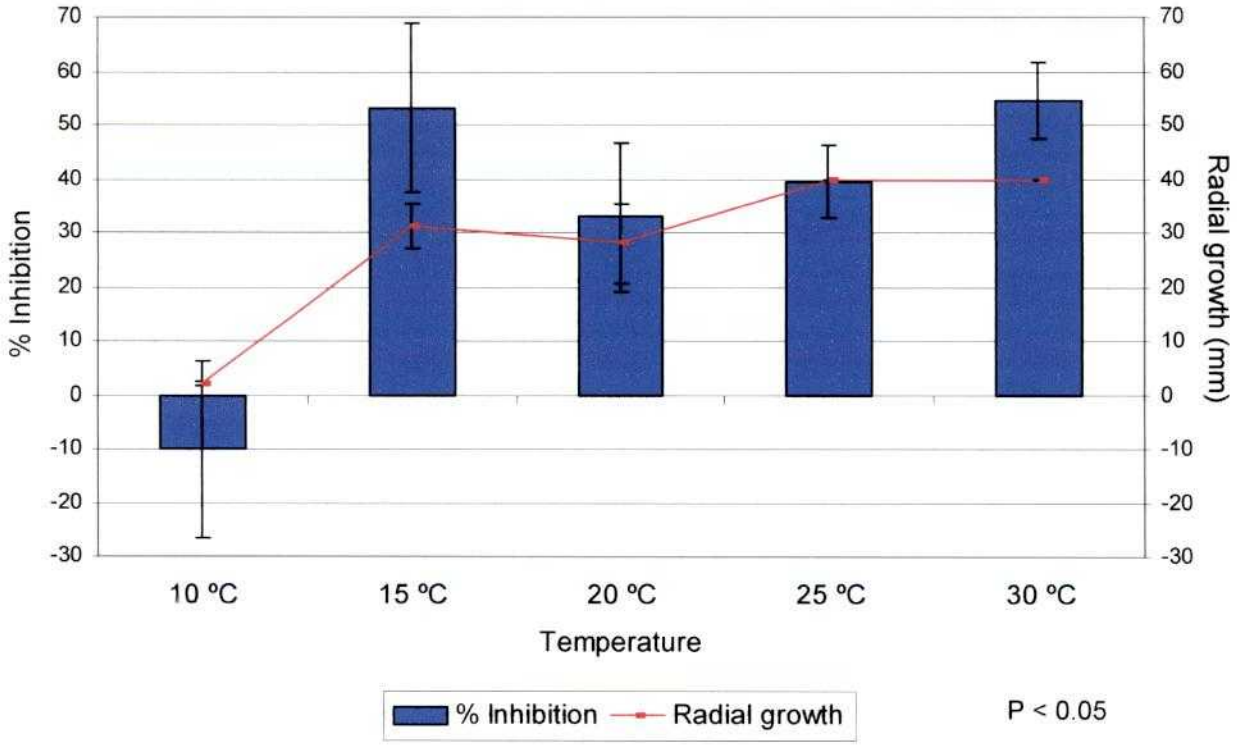

Figure 5.3: Effect of temperature on *Bacillus subtilis* antagonism of *Dothiorella aromatica* at respective temperatures.

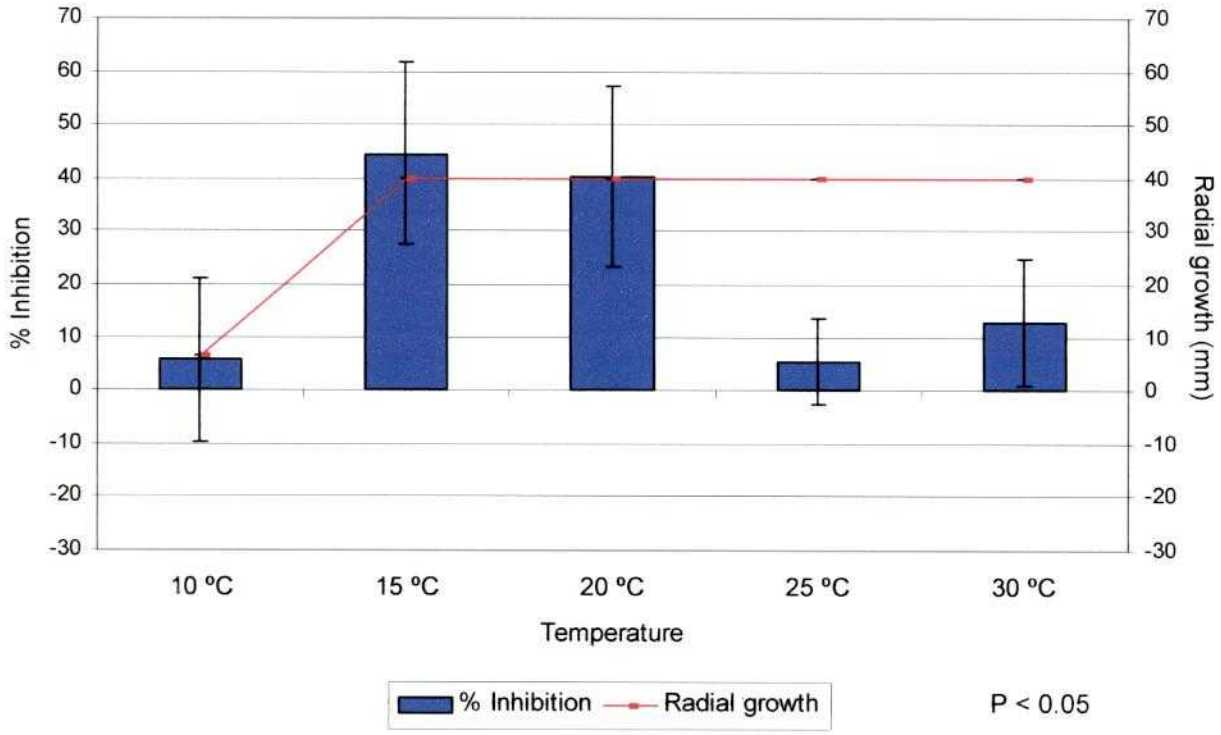

Figure 5.4: Effect of temperature on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae* at the respective temperatures.

5. DISCUSSION

In this study, prevailing temperatures and available nutrients impacted on the effectiveness of antagonism between *B. subtilis* and the fungal avocado pathogens causing stem-end rot and anthracnose. Temperatures negatively affected *in vitro* antagonism of *B. subtilis* against a range of fungal avocado pathogens. Most of the fungal pathogens tested, were able to grow at temperatures of 10 to 15 ºC. Of the pathogens evaluated, *P. perseae* grew the slowest and the least uniform, making radial growth measurement difficult and resulting in increased variability. The least variability was observed on day seven. Since *L. theobromae* was fast growing, less significant differences were observed. At 10 ºC, little or no antagonistic activity was observed between *B. subtilis* and the avocado pathogens. The antagonist was found to be unable to effectively inhibit radial growth of the various fungal pathogens tested at low temperatures. However, at higher temperatures, inhibition increased. In a previous study, higher culturing temperatures (30 and 37 ºC) increased the growth and subsequent yield of cell mass of *B. subtilis* (Korsten & Cook, 1996).

The previously reported variability in efficiency of *B. subtilis* in postharvest studies may be attributed to temperature under commercial conditions. In this study, the antagonist worked most effectively at 30 to 37 ºC *in vitro*. Picked avocado fruit take around two hours to reach the packinghouse (Personal communication, Derek Donkin, South African Avocado Growers' Association, South Africa, 2004). After fruit pass through the packinghouse, they are stored at between 5.5 to 7 ºC for up to 48 h (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). Fruit are containerized or placed in a cool truck for transport to the ports for export, or local markets, and are kept at between 5.5 and 7.5 ºC, depending on the moisture content of the fruit (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). From packing to arrival at the European market can take on average 27 d (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). The temperatures at which fruit are maintained after packing is not optimal for the *in vitro* activity of *B. subtilis* and can contribute to its variable effectiveness. Upadhyay *et al.* (1991) found that by increasing the temperature from 18 to 37 ºC antagonism of *Pseudomonas cepacia* against *Trichoderma viride* Pers could be improved. Postharvest heat treatment of oranges was found to enhance the efficacy of *Pseudomonas glathei* against *P. digitatum* (Pers.) Sacc. (Huang *et al.*, 1995). After treatment with the antagonist, the fruit were stored at 30 ºC for 24 hours, stimulating the multiplication of the antagonist and delaying pathogen spore germination. Incubation of fruit at 20 to 30 ºC might also increase the efficacy of *B. subtilis* against postharvest diseases on avocado. However, in this study the pathogens were found to grow well at these temperatures and this might neutralize the possible advantage in increased antagonism. The effect of temperature on the shelf life of the

fruit must also be taken into consideration. By choosing antagonists that are active at the prevailing conditions when infection occurs, can enhance biocontrol efficacy (Wilson & Pusey, 1985; Mari & Guizzardi, 1998). Postharvest diseases of apple, caused by *Penicillium expansum* Link., were effectively controlled at 5, 10 and 20 ºC (Sholberg *et al.*, 1995). These *B. subtilis* isolates originated from apples stored for six to seven months at 1 ºC. Leibinger *et al.* (1997) found that *B. subtilis* applied preharvestly survived well in the field, but population size decreased after storage at 2 ºC. Temperature was also found to affect the modes of action involved in biocontrol (Gupta & Utkhede, 1986; Fiddaman & Rossall, 1993; Ohno *et al.*, 1995; Graumann *et al.*, 1997).

In the current study, various nutrients were found to enhance the *in vitro* antagonistic efficacy against avocado stem-end rot and anthracnose pathogens. In *in vitro* studies, medium with citric acid, D-(+)-galactose, pyruvate and benzoate incorporated into it was found to provide a basis for sustained inhibition by *B. subtilis* against *C. gloeosporioides* (Havenga *et al.*, 1999). The formation, size and sustainability of inhibition zones were used as criteria to study the impact of nutrient sources on biocontrol, and not percentage inhibition of the pathogen radial growth as was used in other studies (Skidmore & Dickinson, 1976; Whipps, 1987; Upadhyay & Rai, 1987). In this study, the enhancing effect of pyruvate and the amino acids L-(+)-asparagine and L-aspartic acid on *B. subtilis* antagonism against *C. gloeosporioides*, as well as the low effectiveness of starch to increase antagonism was confirmed (Havenga *et al.*, 1999). Various studies showed the effective enhancement of biocontrol by adding nutrients (Dik, 1991; Upadhyay *et al.*, 1991; Janisiewicz *et al.*, 1992; Janisiewicz & Bors, 1995; Manjula & Podile, 2001). The mode of action of a biocontrol agent can also be affected by nutrients (Wisniewski *et al.*, 1991; Slininger & Jackson, 1992; Krebs *et al.*, 1993; Fiddaman & Rossall, 1994; Milner *et al.*, 1995; Duffy & Défago, 1999).

Nutrients incorporated into a commercial product should enhance the antagonist activity without supporting pathogen growth (Janisiewicz *et al.*, 1992; Havenga *et al.*, 1999). From the current study it was found that the best amino acids to incorporate into a commercial formulation against the tested pathogens would be L-glutamic acid, L-glutamine and L-(+)-asparagine. Ammonium chloride, L-cysteine and L-arginine also supported antagonism without increasing the growth of most of the tested fungal pathogens. It was further found that carbon sources most effective for enhancing antagonistic potential of the biocontrol product are D-arabinose and D-(+)-mannitol. Citrate is also a good alternative, but it did not sustain antagonism against *C. gloeosporioides*. However, in the previous study (Havenga *et al.*, 1999) it did support the production of an inhibition zone.

In the initial Avogreen® formulation, a rich growth medium was used in the fermentation process to obtain high total counts. However, it was modified after Korsten *et al.* (1998) found that the incidence of postharvest decay increased when Avogreen® was used in postharvest dips compared with the control treatment. Currently, the liquid and powder Avogreen® formulations consists of a minimal medium containing tri-ammonium citrate and L-aspartic acid as carbon and nitrogen sources respectively (Personal communication, Prof P.L. Steyn, Stimuplant CC, Pretoria, South Africa). The use of specific nutrients in the formulation of a commercial product is supported by the results obtained in this study. Fuchs *et al.* (2000) found that the medium in which an antagonist is grown affect its subsequent activity. A less-rich growth media helped *Pseudomonas* sp. Pf153 to protect cucumber roots to a greater extent than when grown on a rich media. A formulation of *P. fluorescens* F113 controlled damping-off of sugarbeet, but was less-effective when nutrients were added (Moënne-Loccoz *et al.*, 1999). These findings support the use of a minimal medium. Both the powder and liquid formulations have a shelf life of at least one year (Studies done by W. Havenga at Stimuplant CC, Pretoria, South Africa). The consistent efficacy of the product under various preharvest commercial conditions is currently still under investigation but preliminary results indicate the product is effective (Madel van Eeden, personal communication, University of Pretoria).

In conclusion, this study highlighted the importance of temperatures and nutrients on the efficacy of *B. subtilis*. Antagonists must be able to not only survive, but also flourish in the environment where they are applied. The antagonist, *B. subtilis* is most effective at temperatures of 15 °C or higher. In order for antagonists to be successful, the formulation in which it is applied must be optimised. The biocontrol agent formulation must be active against all tested pathogens, as well as being affordable and practical. Since there is no single carbon and nitrogen source that will ensure optimal antagonist efficacy, while maintaining minimal growth of all the pathogens, a compromise must be made in the choice of ingredients. A minimal medium with specific carbon and nitrogen sources that will not support the growth of the target pathogens, but provide the required nutrition for optimal antagonist activity would be the preferred choice. Future studies should focus on the efficacy of the formulated product Avogreen® under commercial conditions both in pre- and postharvest applications.

6. REFERENCES

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Table 5.1: Nitrogen sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*

Phomopsis perseae	*Colletotrichum gloeosporioides*	*Dothiorella aromatica*	*Lasiodiplodia theobromae*
L-arginine	NH_4Cl	NH_4Cl	NH_4Cl
L-cysteine^{D}	L-valine^{PC}	L-glutamic acid^{o}	L-aspartic acid^{D}
L-(+)-asparagine^{o}	L-tyrosine^{C}	L-alanine	L-cysteine^{D}
L-glutamic acid^{o}	L-histidine^{C}	L-(+)-asparagine^{o}	L-glutamic acid^{o}
L-glutamine^{o}	L-arginine	L-arginine	L-lysine^{DL}
L-serine^{C}	L-glutamic acid^{o}	L-proline^{PC}	L-valine^{PC}
	L-(+)-asparagine^{o}	L-cysteine^{D}	L-glutamine^{o}
	L-glutamine^{o}	L-aspartic acid^{D}	L-(+)-asparagine^{o}
	L-alanine	L-lysine^{DL}	L-proline^{PC}
	L-proline^{PC}	Glycine^{CD}	L-histidine^{C}
	L-serine^{C}	L-glutamine^{o}	L-isoleucine
	L-aspartic acid^{D}		
	Glycine^{CD}		
	L-phenylalanine^{PC}		

^{P} - Stimulated *P. perseae* growth
^{C} - Stimulated *C. gloeosporioides* growth
^{D} - Decreased *D. aromatica* growth
^{L} - Decreased *L. theobromae* growth
^{o} - Source does not support pathogen growth and supports antagonism against all four pathogens tested

Table 5.2: Carbon sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*

Phomopsis perseae	*Colletotrichum gloeosporioides*	*Dothiorella aromatica*	*Lasiodiplodia theobromae*
L-(-)-arabinose^{P}	D-(+)-glucose^{CDL}	D-(+)-trehalose^{PDL}	D-(+)-mannitol^{o}
Acetate^{DL}	D-(+)-trehalose^{PDL}	D-(+)-glucose^{CDL}	D-gluconic acid^{L}
Glycerol^{PC}	Glycerol^{PC}	D-(-)-sorbitol^{PDL}	D-(+)-trehalose^{PDL}
D-(+)-trehalose^{PDL}	L-(-)-arabinose^{P}	D-(+)-mannitol^{o}	L-(-)-arabinose^{P}
Peptone^{PCL}	Pectin^{CDL}	Pyruvate^{L}	Peptone^{PCL}
Pectin^{CDL}	Pyruvate^{L}	Starch^{DL}	Citrate^{o}
D-(-)-arabinose^{o}	D-(+)-mannitol^{o}	Peptone^{PCL}	D-(-)-sorbitol^{PDL}
Citrate^{o}	Peptone^{PCL}	Pectin^{CDL}	Pectin^{CDL}
D-(+)-glucose^{CDL}	D-(-)-sorbitol^{PDL}	D-gluconic acid^{L}	Glycerol^{PC}
D-(-)-sorbitol^{PDL}	Starch^{DL}	L-(-)-arabinose^{P}	Starch^{DL}
			D-(+)-glucose^{CDL}
			Pyruvate^{L}
			Acetate^{DL}
			D-(-)-arabinose^{o}

^{P} - Stimulated *P. perseae* growth
^{C} - Stimulated *C. gloeosporioides* growth
^{D} - Stimulated *D. aromatica* growth
^{L} - Stimulated *L. theobromae* growth
^{o} - Source does not support pathogen growth, supporting high levels of antagonism against some pathogens

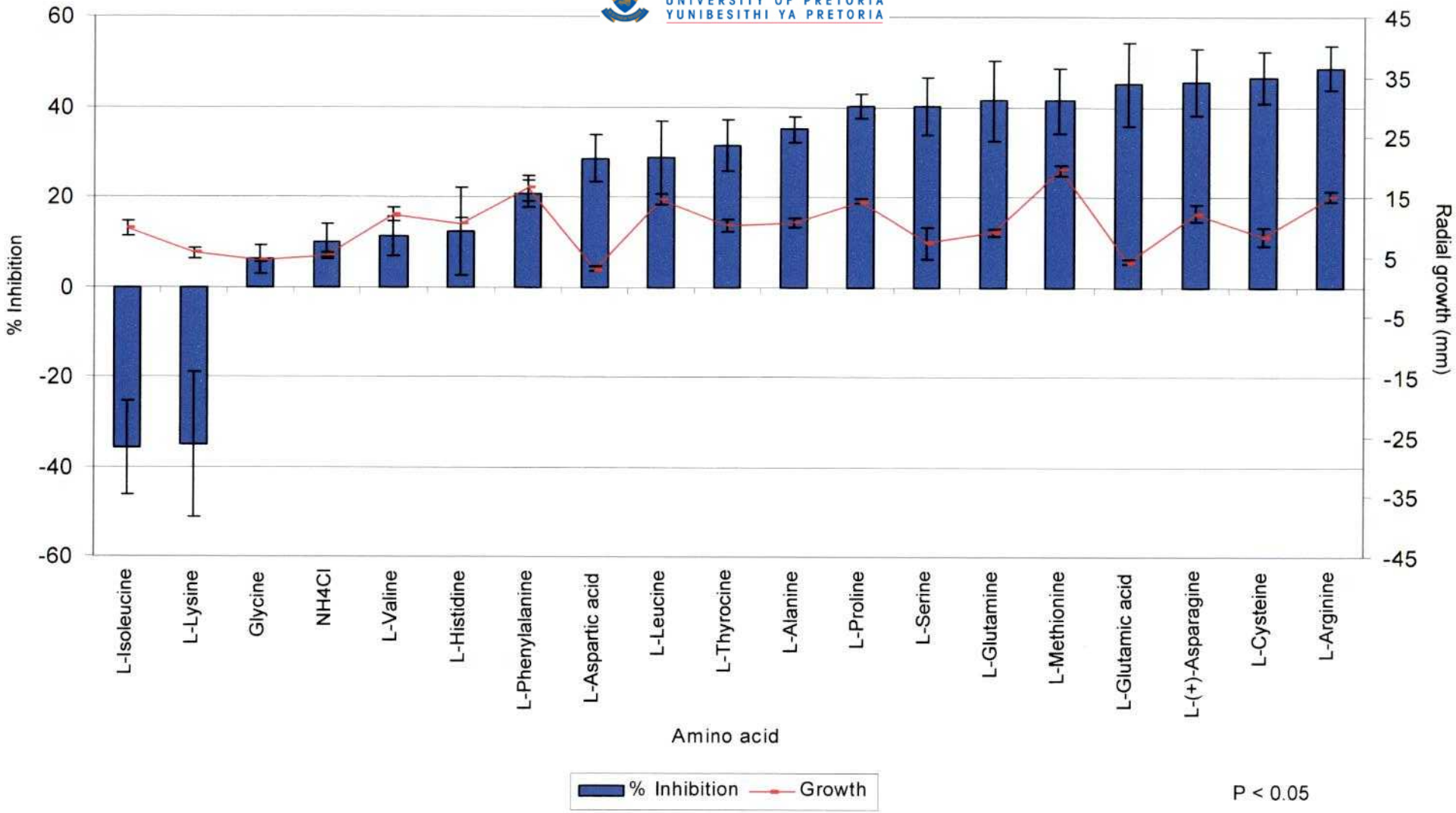

Figure 5.5: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Phomopsis perseae*.

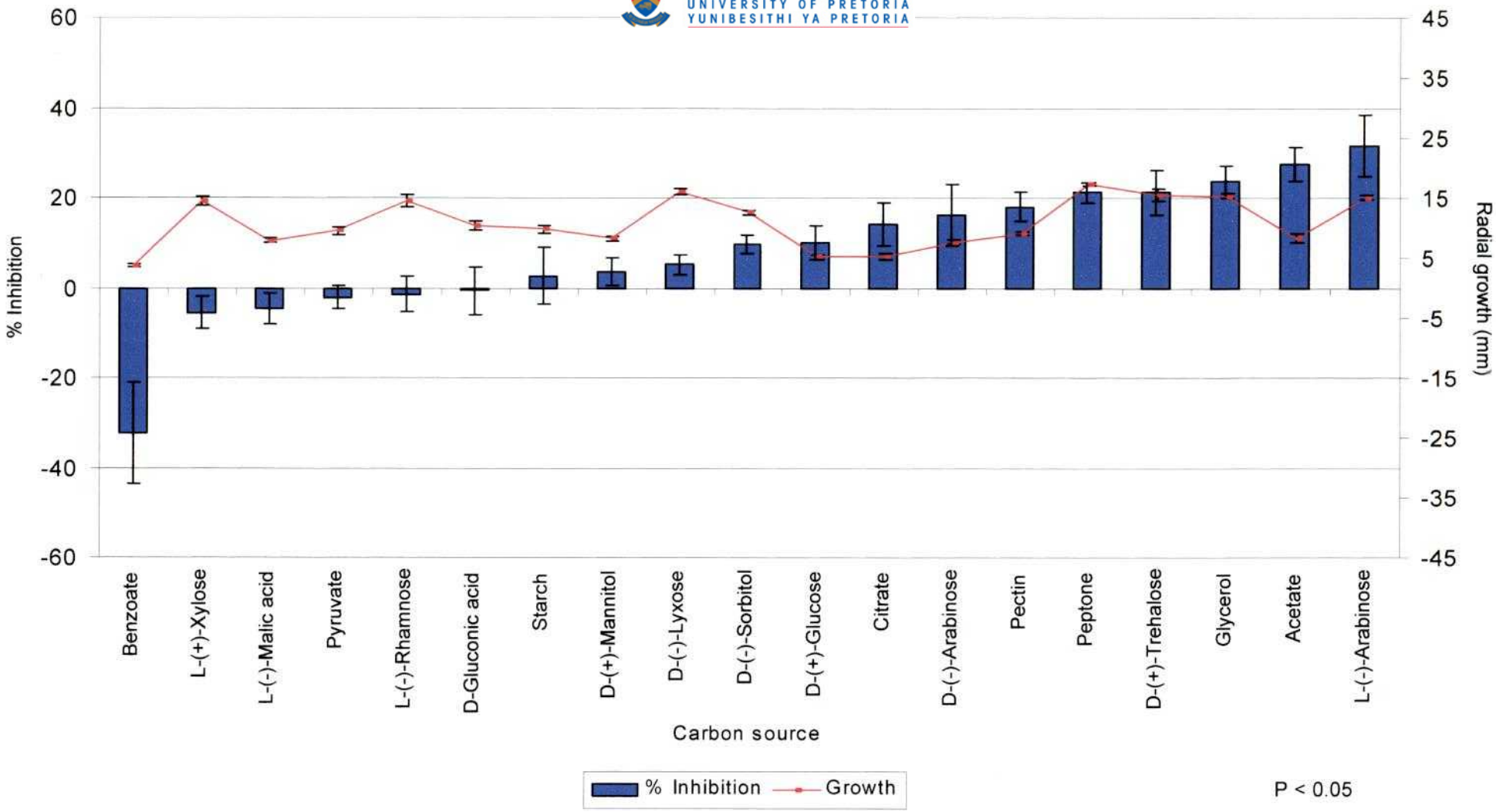

Figure 5.6: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Phomopsis perseae*.

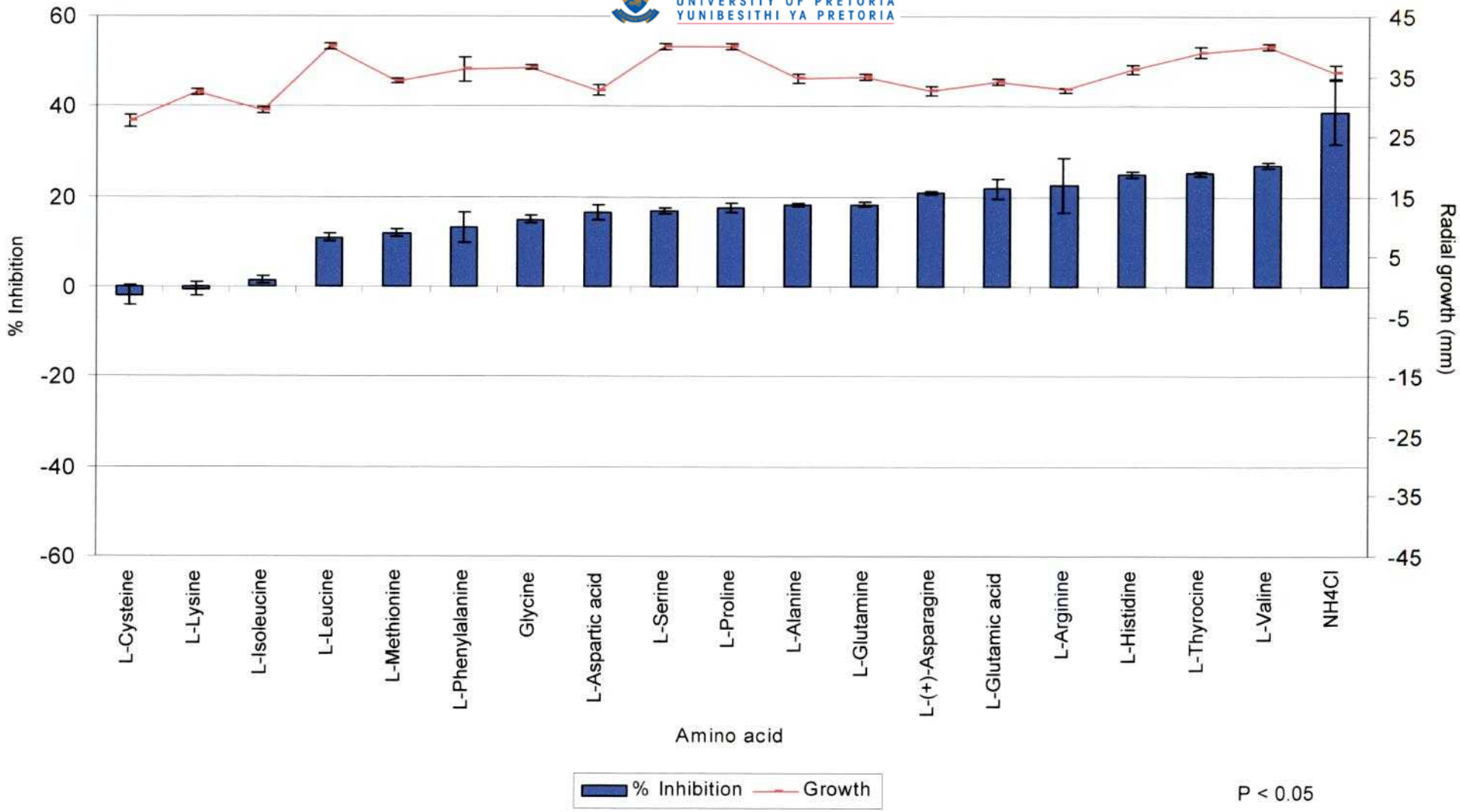

Figure 5.7: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*.

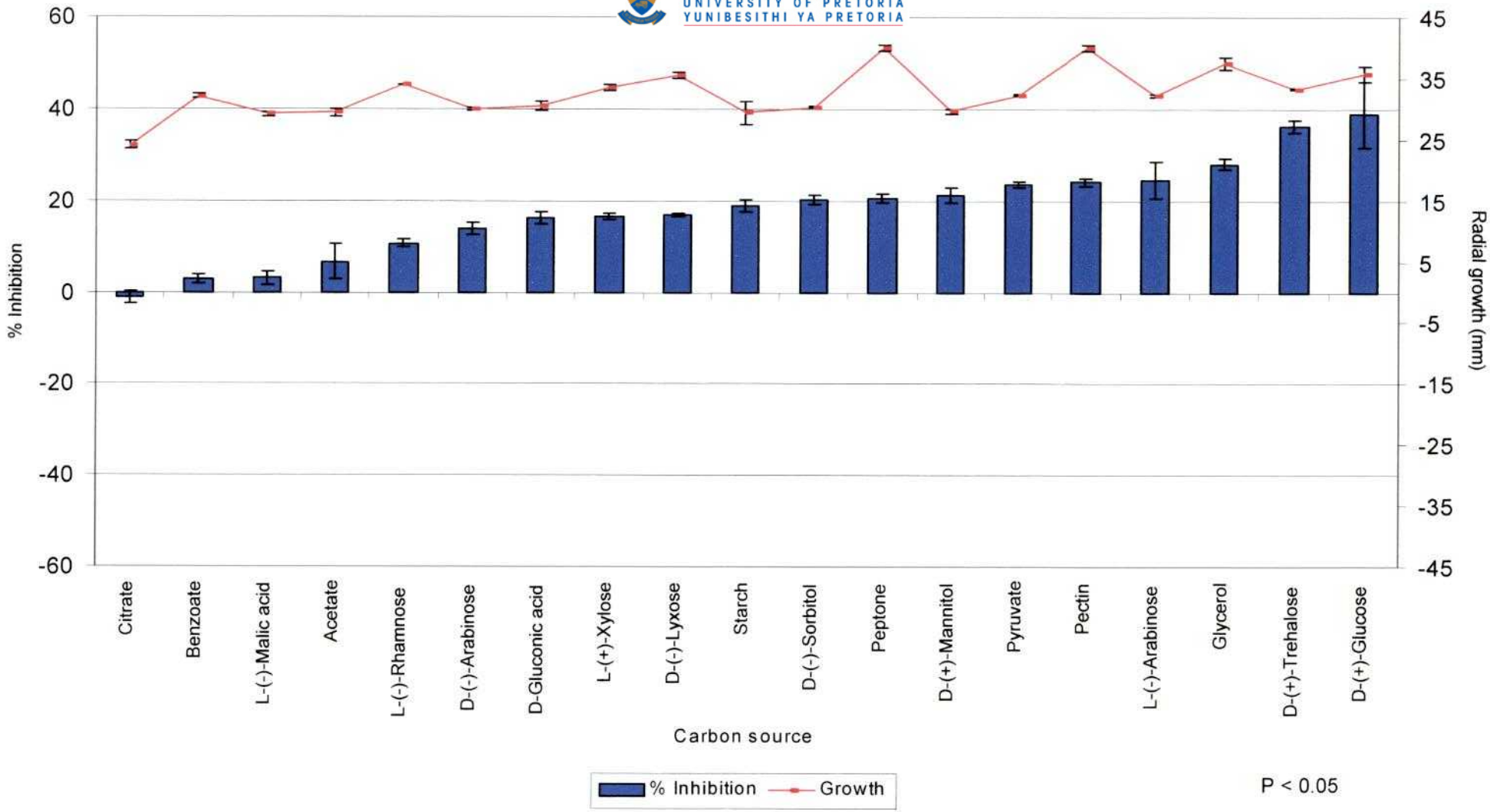

Figure 5.8: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*.

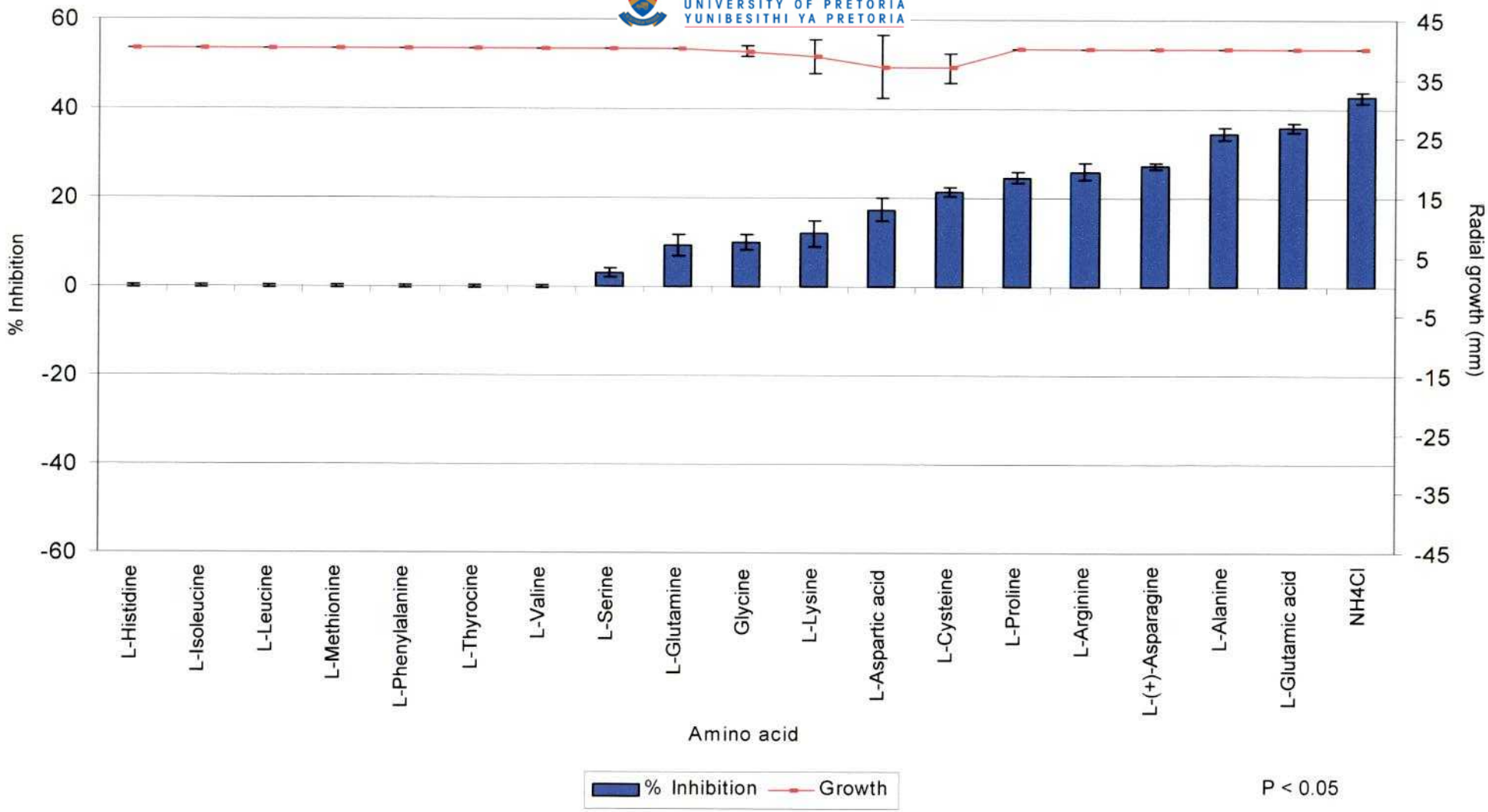

Figure 5.9: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Dothiorella aromatica*.

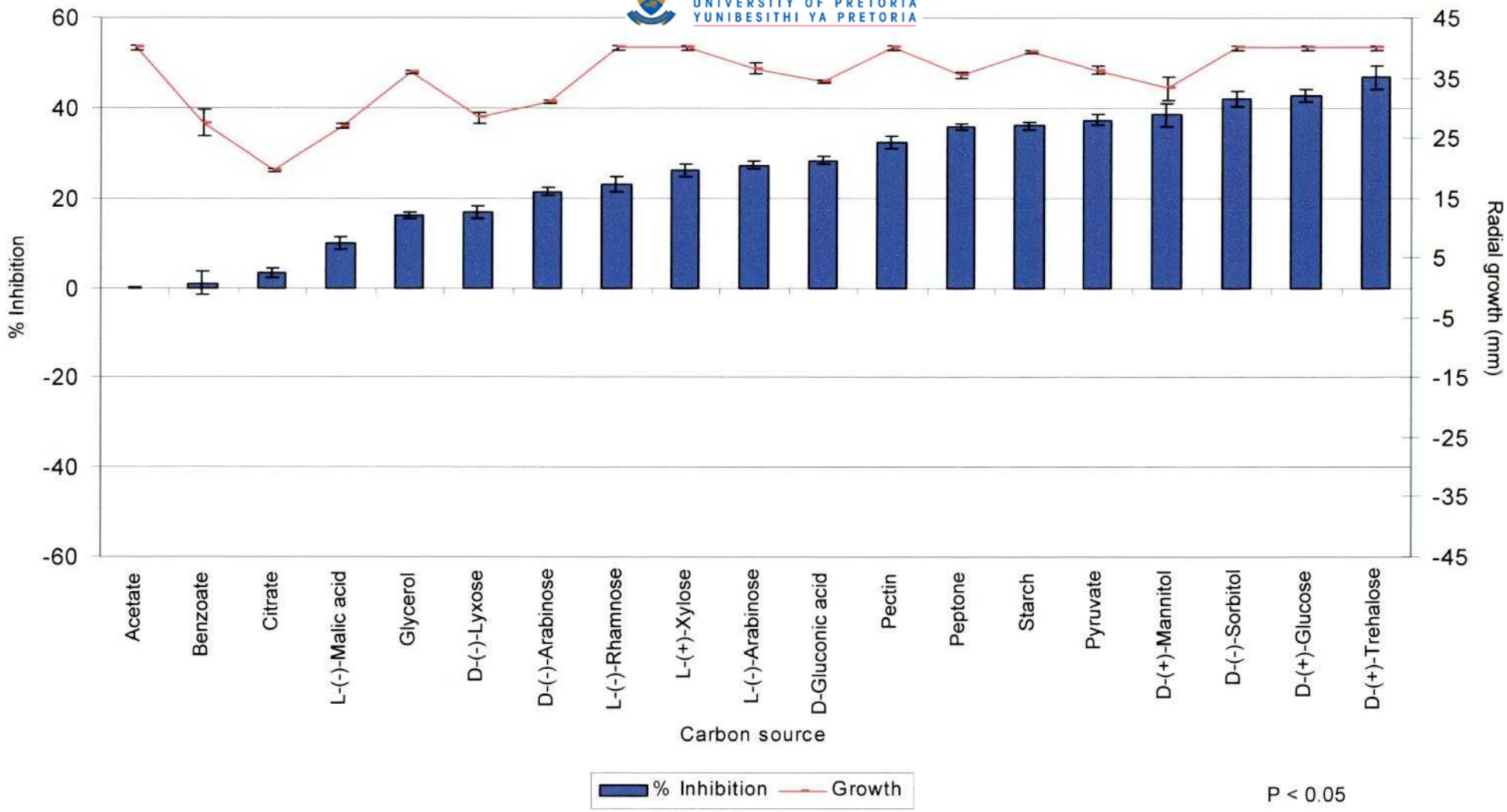

Figure 5.10: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Dothiorella aromatica*.

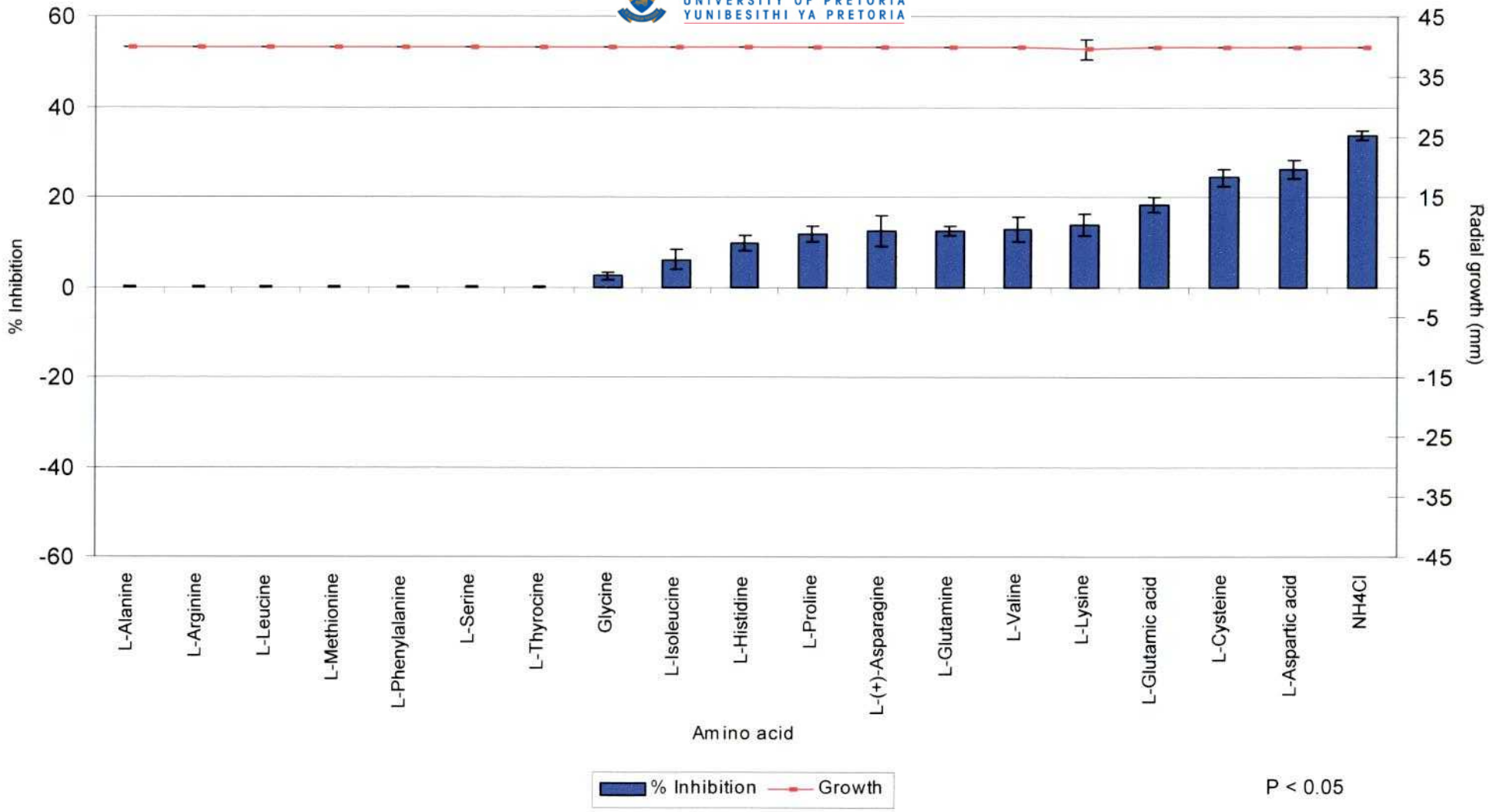

Figure 5.11: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae*.

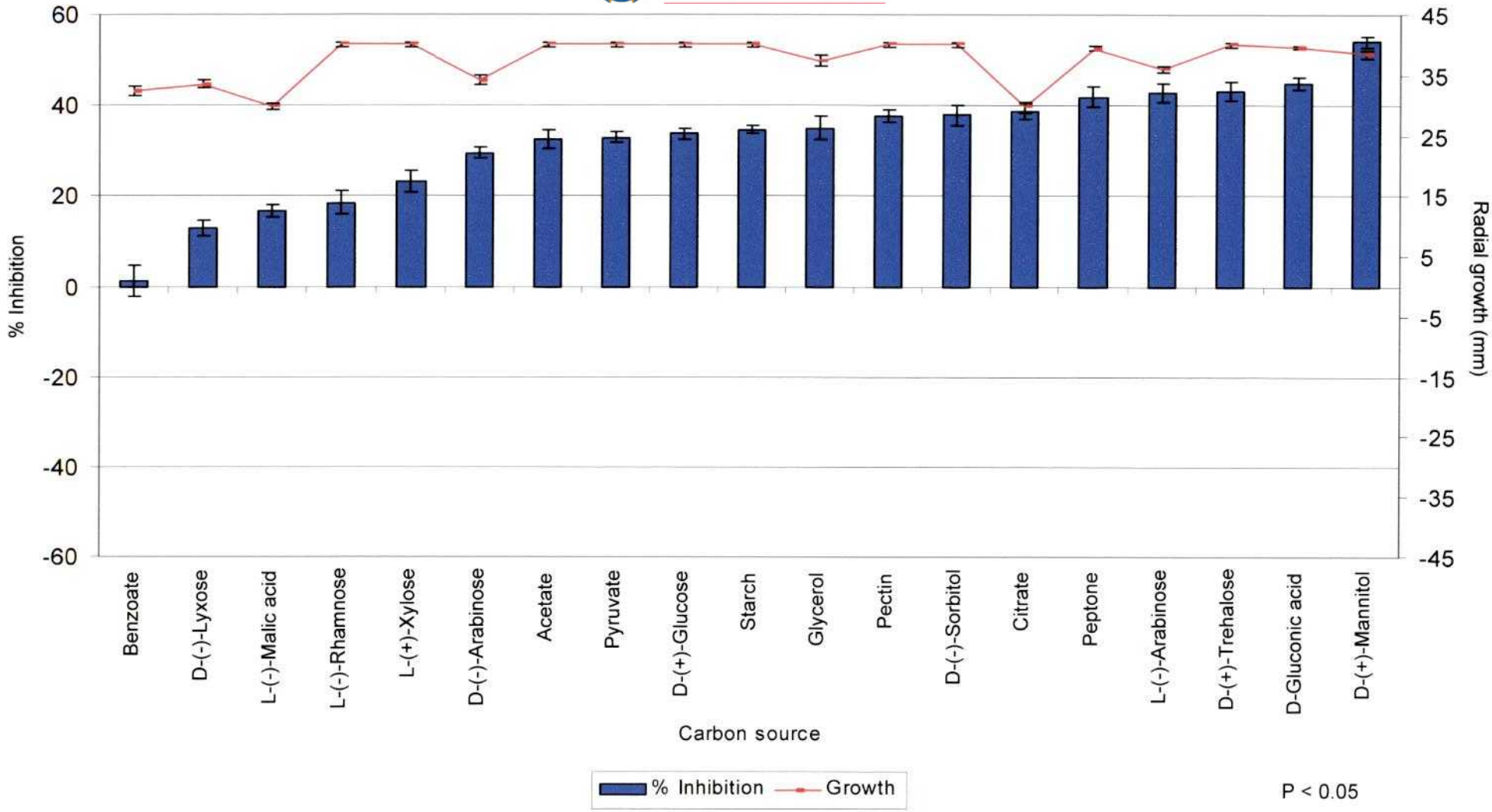

Figure 5.12: Effect of different carbon sources on *Bacillus subtilis* antagonism of against *Lasiodiplodia theobromae*.

Blakeman, J.P. & Brodie, I.D.S. 1977. Competition for nutrients between the epiphytic micro organisms and germination of spores of plant pathogens on beetroot leaves. Physiological Plant Pathology 10: 29 – 42.

Brodie, I.D.S. & Blakeman, J.P. 1976. Competition for exogenous substrates *in vitro* by leaf surface micro-organisms and germination of conidia of *Botrytis cinerea*. Physiological Plant Pathology 9: 227 – 239.

Dik, A.J. 1991. Interactions among fungicides, pathogens, yeasts, and nutrients in the phyllosphere. Pages 412 – 429 In: Microbial Ecology of Leaves. Andrews, J.H. & Hirano, S.S. (Eds). Springer-Verlag, New York.

Duffy, B.K. & Défago, G. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Applied and Environmental Microbiology 65: 2429 – 2438.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Fiddaman, P.J. & Rossall, S. 1994. Effect of substrate on the production of antifungal volatiles form *Bacillus subtilis*. Journal of Applied Bacteriology 76: 395 – 405.

Fuchs, J.-G., Moënne-Loccoz, Y. & Défago, G. 2000. The laboratory medium used to grow biocontrol *Pseudomonas* sp. Pf153 influences its subsequent ability to protect cucumber from black root rot. Soil Biology and Biochemistry 32: 421 – 424.

GenStat for Windows. 2000. Release 4.2. Fifth Edition. Oxford: VSN International.

Graumann, P., Wendrich, T.M., Webber, M.H.W., Schröder, K. & Marahiel, M.A. 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Molecular Microbiology 25: 741 – 756.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. & Cutler, H.G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 36: 366 – 370.

Gupta, V.K. & Utkhede, R.S. 1986. Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. Journal of Phytopathology 117: 9 – 16.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Hase, C., Mascher, F., Moënne-Loccoz, Y. & Défago, G. 1999. Nutrient deprivation and the subsequent survival of biocontrol *Pseudomonas fluorescens* CHA0 in soil. Soil Biology and Biochemistry 31: 1181 – 1188.

Huang, Y., Deverall, B.J. & Morris, S.C. 1995. Postharvest control of green mould on oranges by a strain of *Pseudomonas glathei* and enhancement of its biocontrol by heat treatment. Postharvest Biology and Technology 5: 129 – 137.

Ippolito, A. & Nigro, F. 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruit and vegetables. Crop Protection 19: 715 – 723.

Janisiewicz, W.J. & Bors, B. 1995. Development of a microbial community of bacterial and yeast antagonists to control wound-invading postharvest pathogens of fruits. Applied and Environmental Microbiology 61: 3261 – 3267.

Janisiewicz, W.J., Usall, J. & Bors, B. 1992. Nutritional enhancement of biocontrol of blue mold on apples. Phytopathology 82: 1364 – 1370.

Knox, O.G.G., Killham, K. & Leifert, C. 2000. Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. Applied Soil Ecology 15: 227 – 231.

Korsten, L. & Cook, N. 1996. Optimizing culturing conditions for *Bacillus subtilis*. South African Avocado Growers' Association Yearbook 19: 54 – 58.

Korsten, L., Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Krebs, B., Junge, H., Ockhardt, A., Höding, B., Heubner, D. & Erben, U. 1993. *Bacillus subtilis* – an effective biocontrol agent. Pesticide Science 37: 427 – 433.

Leibinger, W., Breuker, B., Hahn, M. & Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 87: 1103 – 1110.

Manjula, K. & Podile, A.R. 2001. Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. Canadian Journal of Microbiology 47: 618 – 625.

Mari, M. & Guizzardi, M. 1998. The postharvest phase: emerging technologies for the control of fungal diseases. Phytoparasitica 26: 59 – 66.

Milner, J.L., Raffel, S.J., Lethbridge, B.J. & Handelsman, J. 1995. Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Applied Microbiology and Biotechnology: 43: 685 – 691.

Moënne-Loccoz, Y., Naughton, M., Higgins, P., Powell, J., O'Connor, B. & O'Gara, F. 1999. Effect of inoculum preparation and formulation on survival and biocontrol efficacy of *Pseudomonas fluorescens* F113. Journal of Applied Microbiology 86: 108 – 116.

Morris, C.E. & Rouse, D.I. 1985. Role of nutrients in regulating epiphytic bacterial populations. Pages 63 – 82 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Ohno, A., Ano, T. & Shoda, M. 1995. Effect of temperature on production of lipopeptide antibiotics, Iturin A and Surfactin by a dual producer, *Bacillus subtilis* RB14, in solid-state fermentation. Journal of Fermentation and Bioengineering 80: 517 – 519.

Sholberg, P.L.., Marchi, A. & Bechard, J. 1995. Biocontrol of postharvest diseases of apple using *Bacillus* spp. isolated from stored apples. Canadian Journal of Microbiology 41: 247 – 252.

Skidmore, A.M. & Dickinson, C.H. 1976. Colony interaction and hyphal interference between *Septoria nodorum* and phylloplane fungi. Transactions of the British Mycological Society 66: 57 – 64.

Slininger, P.J. & Jackson, M.A. 1992. Nutritional factors regulating growth and accumulation of phenazine 1-carboxylic acid by *Pseudomonas fluorescens* 2-79. Applied Microbiology and Biotechnology 37: 388 – 92.

Snedecor, G.W. & Cochran, W.G. 1980. Statistical Methods. 7th Edition. Iowa State University Press. Ames, Iowa.

Spadaro, D. & Gullino, M.L. 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology 91: 185 – 194.

Upadhyay, R.S. & Rai, B. 1987. Studies on antagonism between *Fusarium udum* Butler and root region microflora of pigeon pea. Plant and Soil 101: 79 – 93.

Upadhyay, R.S., Visintin, L. & Jayaswal, R.K. 1991. Environmental factors affecting the antagonism of *Pseudomonas cepacia* against *Trichoderma viride*. Canadian Journal of Microbiology 37: 880 – 884.

Van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.

Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. The New Phytologist 107: 127 – 142.

Wilson, C.L. & Pusey, P.L. 1985. Potential for biological control of postharvest plant diseases. Plant Disease 69: 375 – 378.

Wisniewski, M.E., Biles, C., Droby, S., McLaughlin, R., Wilson, C.L. & Chlautz, E. 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*: I. Characterization of attachment to *Botrytis cinerea*. Physiological and Molecular Plant Pathology 39: 245 – 258.