CHAPTER 2

LITERATURE REVIEW

MODES OF ACTION INVOLVED IN BIOCONTROL OF PLANT PATHOGENS, WITH SPECIAL REFERENCE TO FUNGAL PATHOGENS

"No man is an island, entire of it self; every man is a piece of the continent, a part of the main." – John Donne

No single organism can ever exist on its own. It will always be linked within a matrix of complex interactions with other organisms occurring in the same environment. These interactions can be beneficial, have no effect or can be harmful to one or more of the organisms involved (Gooday, 1988). Managing the interactions between microorganisms is the basis of biocontrol (Bellows, 1999). Understanding the mechanisms through which this can be achieved, is critical to the eventual improvement and wider use of biocontrol (Fravel, 1988). To keep plants healthy, the impact of microorganisms detrimental to the plant must be reduced and the effect of beneficial ones exploited (Milner *et al.*, 1997). Few studies focus on mode of action of biocontrol agents of fungal plant pathogens alone, despite the many articles on biocontrol. This review attempts to alleviate some confusion regarding modes of action.

1. HISTORY OF BIOCONTROL

Fungal pathogens, especially those causing postharvest diseases, are most commonly controlled by means of chemical fungicides (Wilson & Wisniewski, 1989; Roberts, 1994). According to van Driesche & Bellows (1996), the worldwide use of pesticides has increased twelve fold since the 1950's. Essentially, fungicides are toxins and some are carcinogens. To persist in the environment and function optimally, fungicides are formulated not to be easily biodegradable (Campbell, 1989). However, serious environmental problems develop due to the accumulation of pesticide residues in natural resources, affecting non-target organisms (Campbell, 1989). Pesticide residues in food also affect human health. In the USA, up to 3000 hospitalisations and 200 fatalities are due to pesticide misuse annually (Campbell, 1989). Pathogens may build up resistance against fungicides when used incorrectly, leading to less effective control of target plant pathogens (Kotzé *et al.*, 1982; Wilson *et al.*, 1994). New or alternative fungicides must subsequently be developed, which is costly. In some instances, chemical sprays leave residues on fruit surfaces that must be removed manually (Denner & Kotzé, 1986), thereby increasing production cost. It is therefore important to

develop alternative disease control measures to ensure global food security (Skidmore, 1976; Payne & Lynch, 1988; Van Driesche & Bellows, 1996; El-Ghaouth, 1997).

The plant pathologist's definition of biological control is the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists (Baker & Cook, 1974). In the 1920's, the first biological control measures were developed to control insect pests by introducing their natural predators to the environment (Campbell, 1989). At approximately the same time investigations were conducted in the use of non-pathogenic microorganisms to control plant disease (Campbell, 1989). Since then many studies focused on the potential of biological control, which resulted in an increase in knowledge of interactions between host, pathogen and antagonist.

Most reported biocontrol programs focus on control of root pathogens within the rhizosphere environment (Payne & Lynch, 1988). Although many biocontrol agents have been tested *in vitro*, very few agents have been commercialised (Payne & Lynch, 1988; Milner *et al.*, 1997). Biocontrol agents do not provide consistent levels of control and variable results are frequently generated (Wilson *et al.*, 1994; Milner *et al.*, 1997). Environmental parameters influence disease progress. Few biocontrol studies have extended their focus to include the effect of environmental parameters on biocontrol efficacy over time. In addition, the interaction of the antagonist with other microbial inhabitants on the plant surface has not been studied extensively. Biocontrol is often not as effective as chemical control in disease prevention (Dubos, 1984; Wilson *et al.*, 1994). An understanding of the interactions between the environment, host, and pathogen and antagonist populations, may therefore help explain the variation found in effectiveness of biocontrol agents under field conditions.

2. MODES OF ACTION INVOLVED IN BIOCONTROL

Keeping the mode or modes of action of an antagonist in mind is crucial for developing successful biocontrol programs (Milner *et al.*, 1997). Even in successful biocontrol strategies, a more in depth understanding of the mechanism of action may improve the reliability of the agent as well as broadening of its application to other host-pathogen combinations (Milner *et al.*, 1997). It may also help to increase the biocontrol product's activity, optimising the method and timing of its application, and developing more appropriate formulations to enhance efficacy and consistency of its performance. Should important modes of action of biocontrol agents

be identified, it could be used in the isolation of more effective antagonists and ensure improved efficacy (Wilson & Wisniewski, 1989; Milner *et al.*, 1997).

It is relatively easy to study pathogen-antagonist interactions *in vitro*. However, it is much more difficult to prove mechanism of interactions in nature (Mari & Guizzardi, 1998; Calvente *et al.*, 1999). Figure 2.1 gives a brief overview of modes of action involved in biocontrol. The mode of action of the antagonist is not always easy to determine. Thus far, most conclusions regarding modes of action have been based on indirect evidence (Droby & Chalutz, 1994). In the case of *Pseudomonas cepacia*, the antagonist produces an antibiotic, pyrrolnitrin. It is also able to control *Penicillium digitatum* (Pers.: Fr.) Sacc. which is known to be resistant to this antibiotic. Antibiosis can therefore be excluded as the mode of action in this interaction (Mari & Guizzardi, 1998).

Baker's possession principle (Baker, 1987) describes the activity of any antagonistic microbe: "A microorganisms already in a substrate may retain possession against vigorous competitors by: rapidly converting nutrients into its own propagules ("personalized packages") making it unavailable to others; polluting the infection site with antibiotics, phytoalexins, or toxic chemicals; preventing accumulation in the infection site of excess nutrients that attract competitors; or modifying the water potential, rate of oxygen diffusion, pH, or other conditions of the host tissue to limit growth of competitors."

Generally, the modes of action of antagonists are poorly understood. Today it is commonly accepted that a range of different modes of action is involved. These modes of action can be grouped into two categories: those acting indirectly and those that act directly upon the pathogen (Figure 2.1). The type of interaction can be density dependent and can be "regulated" or "switched on" by the specific growth phases in the life cycle of the pathogen or antagonist (Dunny & Leonard, 1997). Microbial interactions should, therefore, be seen over time and space. Modes of action frequently involved in biocontrol systems include antibiosis, induced resistance, competition for space and nutrients, parasitism, and even plant growth promotion (Payne & Lynch, 1988; Milner *et al.*, 1997; Bellows, 1999).

2.1. Indirect interactions

Indirect interactions include cross-protection, hypo virulence and growth stimulation where the antagonist does not act directly on the target pathogen. These interactions act on the host, stimulating its resistance

mechanisms or changing the ecology of the surrounding area to discourage pathogens from germinating and infecting the plant (Payne & Lynch, 1988; Fravel, 1988).

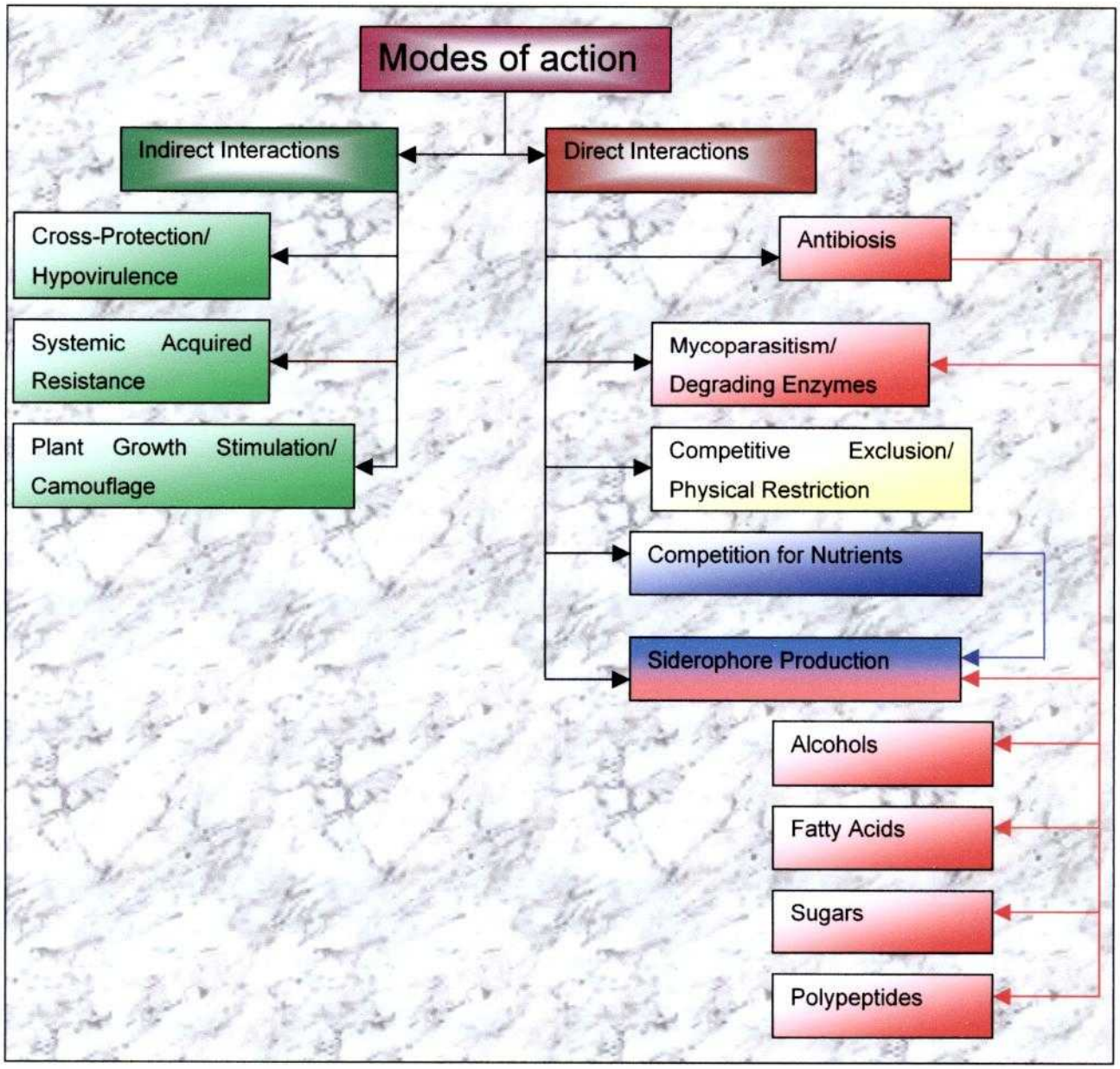

Figure 2.1: Modes of action involved in biocontrol and their relation to each other.

2.1.1. Cross-protection or hypo virulence

Cross-protection occurs when an established virus prevents a related virus from fully expressing its disease-causing ability (Milner *et al.*, 1997; Dodds, 1999; Bellows, 1999). The challenging virus usually fails to accumulate in the host. Since this mode of action occurs only with viruses, it will not be discussed further.

2.1.2. Systemic acquired resistance

Systemic acquired resistance is the development of resistance throughout the whole plant. Plants are challenged by biotic and abiotic elicitors (agents inducing resistance), including non-pathogenic organisms. These induce the plant defence mechanisms and limit or even prevent subsequent infections by pathogens (Van Driesche & Bellows, 1996; Bellows, 1999). Resistance development is seen in areas distant from the original inoculation site and can be very unspecific in its target pathogen (Bellows, 1999).

Elicitors can be grouped into three categories: organic molecules, chemicals and antagonists. Organic elicitors commonly involved in induced resistance include ethylene, chitin, chitosan oligosaccharides and salicylic acid (Wilson *et al.*, 1994; Droby *et al.*, 1996). Chemicals able to induce resistance in plant tissue include benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester, β-aminobutyric acid and 2,6-dichloroisonicotinic acid (Benhamou *et al.*, 2001). Antagonistic organisms are also able to induce resistance, usually by producing a wide range of organic molecules. These molecules include pathogenesis related proteins and jasmonates (Kogel *et al.*, 1995), as well as some of the organic molecules mentioned earlier (Wilson *et al.*, 1994; Droby *et al.*, 1996; Dodds, 1999; Benhamou *et al.*, 2001). Several studies on different fruit crops implicate host exposure to antagonists resulting in induced resistance (Table 2.1). In harvested crops, elicitation can also occur through physical means. Physical treatments that can induce resistance include heat treatment, wounding, gamma radiation and UV-C light (Wilson *et al.*, 1994; Mari & Guizzardi, 1998).

Induction signalling molecules are produced by the plant upon treatment with the elicitor and are used to prime the activation of resistance in the rest of the host (Benhamou *et al.*, 2001). The host act upon the signalling molecules by triggering its gene expression (Benhamou *et al.*, 2001). Defence systems include the lignifications of cell walls through the addition of chemical cross-linkages in cell wall peptides, making it difficult for the pathogen to establish infection through lysis; suberification of tissues, where fatty substance suberin infiltrates cell walls, making them corklike; production of phytoalexins (Paul *et al.*, 1998), chitinases and β-1,3-glucanases (Bellows, 1999). When challenged, a necrotic hypersensitive reaction is elicited in some cases (Benhamou *et al.*, 2001).

Benhamou *et al.* (2001) tested several elicitors against *Fusarium oxysporum* (Schltdl.: Fr.), the casual agent of tomato crown and root rot. The tomato root cells underwent several modifications when treated with the

elicitors. The nature and spectrum of the observed modifications differed depending on the elicitor. This suggests that there may be more than one general pathway followed after elicitation.

Induced resistance is recognized as an important form of resistance in vegetative plant tissues and similar mechanisms may function in harvested fruit. Some reports indicate that certain postharvest biocontrol agents may interact with host tissues, particularly during wounding and thereby enhancing wound healing (Droby & Chalutz, 1994). In studies done by El-Ghaouth (1998), *Candida saitoana* Montrocher also stimulated the production of papillae and other protuberances in the tissue underlying the wounded area. These protuberances might contain phenolic-like substances and could help the tissue to restrict the spread of the invading pathogens.

In many cases a combination of induced resistance and other modes of action may be involved in biocontrol. Weller (1988) used a strain of *Pseudomonas fluorescens* CHAo, to suppress *Thielaviopsis basicola* (Berk. & Broome) Ferraris, causing black root rot of tobacco. In the case of *P. fluorescens*, both the production of antibiotics and siderophores are used in its action. However, it was found that hydrogen cyanide production is also important. Mutant strains of *P. fluorescens* deficient in producing HCN were less suppressive than the normal strain, proving the involvement of induced resistance in its mode of action (Weller, 1988).

2.1.3. Plant growth stimulation and camouflage

A more subtle or indirect mechanism may play a role in keeping plants healthy. Gilbert *et al.* (1994) observed a change in the microbial community surrounding the roots when treating soybeans with *Bacillus cereus* strain UW85. The community changed from one resembling the rhizosphere microbiology to that resembling the soil microbiology. This happened without *B. cereus* UW85 becoming the dominant organism in the community. Coinciding with the population change came a reduction in root disease. These results lead to the development of Gilbert's "camouflage hypothesis". According to this hypothesis, the microbial community changes the root ecology so that it resembles that of the soil, disguising the root (meaning the rhizosphere niche) so that the pathogen will not detect it, or making it less attractive to pathogens thereby protecting it from disease. However, this hypothesis has not been tested or challenged (Gilbert *et al.*, 1994; Milner *et al.*, 1997). According to Milner *et al.* (1997), this may be one mechanism in which antibiotics produced by antagonists work. It may not act directly on the pathogen, but indirectly influencing the microbial community to adapt.

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Table 2.1: Cases where induced resistance was found to be involved in antagonism of pathogenic fungi

Host	Control agent	Disease	Pathogen	Molecules involved	Reference
Apple *Malus sylvestris* Mill.	*Candida saitoana* Montrocher	Postharvest rot	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link	Chitinase induction	El-Ghaouth *et al.*, 1998
Apple *Malus sylvestris* Mill.	*Pichia guilliermondii* (US-7) Wickerham	Postharvest rot	*Penicillium digitatum* (Pers.: Fr.) Sacc.	Phytoalexin scoparone, phenylalanine ammonia lyase, peroxidase induction	Wilson *et al.*, 1994
Peach *Prunus persica* Sieb. & Zucc.			*Botrytis cinerea* (Pers.: Fr.)		
Citrus					
Grapevine *Vitis vinifera* L.	*Bacillus* spp.	Grey mould	*Botrytis cinerea* (Pers.: Fr.)	Phytoalexin induction	Paul *et al.*, 1998
Groundnut *Arachis hypogaea* L.	*Bacillus subtilis* AF1	Crown rot	*Aspergillus niger* Tiegh.	Lipoxygenase	Sailaja *et al.*, 1997
Tobacco *Nicotiana tabacum* L.	*Pseudomonas fluorescens* CHAo	Black root rot	*Thielaviopsis basicola* (Berk. & Broome) Ferraris	Hydrogen cyanide production	Ahl *et al.*, 1986; Stutz *et al.*, 1986
Tomato *Lycopersicon esculentum* Mill.	*Pythium oligandrum* Drechsler	*Fusarium* crown and root rot disease	*Fusarium oxysporum* f.sp. *radicis-lycopersici* Jarvis & Shoemaker	Elicitin: Oligandrin production	Benhamou *et al.*, 2001

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It is not only root-related pathogens that may be controlled using this approach. Biocontrol in the postharvest arena have also been challenged by indirect manipulation of total populations that already occur on the plant surface. According to Wilson & Wisniewski (1989), there are two basic approaches available in postharvest biocontrol: promoting and managing natural antagonists that already exist on the surface, or by artificial introduction of target antagonists. It has been suggested that certain hosts may control the microbial populations on its surfaces through expression of its own genes (Wilson & Wisniewski, 1989). The host manipulates the microbial community to be more suppressive to diseases. It may become possible in future to modify the host genetics, ensuring that a disease-suppressive microbial population will be sustained.

Some microbial antagonists may play a role in stimulating growth of the host. In the case of *B. cereus* UW85, nodulation of soybeans were increased when treated with the antagonist. This may contribute to plant health (Milner *et al.*, 1997). The possibility of having antagonists that act more indirectly by promoting plant health or altering microbial populations and keeping plants healthy may prove useful in the future.

2.2. Direct interactions

A more direct interaction between the antagonist and pathogen are more commonly found. To date, direct interactions have been more extensively studied compared to indirect interactions. Direct interactions include antibiosis, parasitism, competition for space and nutrients, as well as the production of siderophores and volatiles. However, there is no clear distinction between the direct interactions since siderophore production (molecules produced to sequester iron ions, making them more readily available to the producer organism) can also be seen as competition for nutrients and iron. Also, the production of enzymes, volatiles, and toxic substances by the antagonist can be seen as antibiosis (Fravel, 1988). However, interaction definitions are dependent on the view of the researcher.

2.2.1. Antibiosis

Baker & Cook (1974) defined antibiosis as the inhibition of one organism by a metabolite of another. Fravel (1988) described antibiotics as low molecular weight organic compounds produced by antagonists that are deleterious to the growth of other microbes. Milner *et al.* (1997) saw antibiosis as the production of toxic metabolites like antibiotics, lytic enzymes, and volatile substances. However, antibiosis could result from production of an alcohol or change in pH of the environment or by the production of simple substances not commonly considered to be antibiotics. Thus, metabolites of all types could qualify as products involved in

antibiosis. The use of enzymes and siderophores as biocontrol modes of action are discussed in section 2.2.2 and 2.2.5 respectively.

Of the various forms of antagonism, antibiosis has numerous advantages. Direct physical contact between the antagonistic organism and the target pathogen is not necessary (McKeen *et al.*, 1986). Antibiotic substances may diffuse in water films and water filled pores, or in the case of volatiles, through air filled pores (Fiddaman & Rosall, 1993). The area in which the antibiotic is active will be greater, and its impact will be more rapid and even more effective than other modes of action such as competition or hyper parasitism. Antibiosis may even continue to play a role even after growth ceased from the antagonist. Such reports of antibiotic release from senescent cells within the colony have previously been documented (Baker & Cook, 1974).

In nature, antibiotic production is a common phenomenon and several bacterial antagonists are reported to produce antibiotics that may play a role in protecting crops from pathogen attack. Antibiotics that are produced *in vitro* will not necessarily be produced at the site of action or on the host surface or *in vivo* (Fravel, 1988; Droby & Chalutz, 1994). Some antibiotics are produced only in culture where conditions are optimal (Baker & Cook, 1976; Fravel, 1988). Antibiotics may therefore not always be produced in the phyllosphere. This can be seen where culture filtrates of antagonists are inhibitory to pathogens, while washed cells are not. According to Pfender (1996), this may be due to poor growth of the applied antagonist, or to a lack of antibiotic production under nutritional and physical conditions existing on the phyllosphere. Neighbouring organisms can easily break down antibiotics or it can be inactivated by adsorption to the surface of the host (Blakeman & Brodie, 1976). According to Baker & Cook (1974), antibiosis works best where nutrients are abundant or in excess, such as at specific micro sites i.e. leaking plant parts or organic debris.

Less attention has been given to antibiosis mediated through the production of volatile substances compared to non-volatile antibiotics (Fravel, 1988). Volatile substances produced by antagonists include molecules like ammonia, alkyl pyrones, ethanol, isobutanol, isoamyl alcohol, and isobutyric acid (Fravel, 1988). These molecules can directly inhibit fungi *in vitro*. Fiddaman & Rossall (1993) found that *Bacillus subtilis* produces a volatile substance that is able to inhibit the growth of *Rhizoctonia solani* (J. G. Kühn) and *Pythium ultimum* (Trow).

Milner *et al.* (1997) defined four approaches of determining the role of antibiosis in antagonism. Firstly, mutants unable to produce the antibiotic are tested for activity. If the non-producing strain is unable to give control over the pathogen, antibiosis is involved (Fravel, 1988; Gutterson, 1990; Weller, 1988; O'Sullivan & O'Gara, 1992; Leifert *et al.*, 1995). Secondly, the antibiotic can be purified and tested. If it is effective in the field situation, it is involved in antagonism. However, antibiotics can adsorb onto host tissue and soil particles, inactivating it (Blakeman & Brodie, 1976). The third approach involves the use of a pathogen that is insensitive to the antibiotic. If the antagonist inhibits the pathogen, the antibiotic is not involved and another mechanism may play a role. Finally, genes coding for the antibiotic can be cloned into non-expressing microorganisms. Clones can be evaluated for activity against the pathogen. If it becomes inhibitory, the antibiotic is involved in antagonism.

These strategies were employed to show the involvement of the antibiotic phenazine-1-carboxylic acid in the activity of *P. fluorescens* strain 2-79, against *Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx, casual agent of take-all of wheat (Weller & Cook, 1983; Bull *et al.*, 1991). The antagonist significantly decreased not only the size, but also the number of lesions, indicating that the antibiotic is a factor in suppression of primary infection by the pathogen. The antibiotic was also isolated from the rhizosphere of healthy roots. A mutant strain, unable to produce phenazine antibiotic was unable to suppress lesion formation, indicating that the antibiotic plays a role in antagonism (Bull *et al.*, 1991). A pyoverdine siderophore and an additional antifungal factor, playing a minor role in disease suppression, were also produced (Hamdan *et al.*, 1991; Slininger & Jackson, 1992; Milner *et al.*, 1997).

Different approaches exist in practice to exploit antibiosis. In the case of Tsuge *et al.* (1996), the antibiotic itself was produced, partially purified and used as a biocontrol agent. Iturin A, produced by *B. subtilis* NB22, was obtained using solid-state fermentation of okara (soy bean curd residue). This approach resulted in a high-efficiency and low production cost of the antibiotic. However, one can reason that following this approach is similar to more traditional chemical control.

An antagonist can produce an array of different antibiotics. Pseudomonads are known to produce a variety of inhibitory compounds that all contribute to disease suppression, though not all work against all pathogens on all hosts (O'Sullivan & O'Gara, 1992; Milner *et al.*, 1997). *P. fluorescens* strain CHAO produces several antifungal compounds, including hydrogen cyanide; antibiotics, including pyoluteorin and 2,4-diacetylphloroglucinol; as well as a siderophore, pyoverdine. Pyrrolnitrin contributes to the suppression of *R.*

solani, while pyoluteorin suppresses *P. ultimum* on cotton seedlings. Tobacco root rot caused by *T. basicola* is suppressed by the production of hydrogen cyanide and 2,4-diacetylphloroglucinol. The antibiotic, 2,4-diacetylphloroglucinol, suppresses take-all of wheat, caused by *G. graminis*. The causal agent of damping-off of cress, *P. ultimum*, is suppressed by pyoluteorin (Milner *et al.*, 1997). *B. subtilis* cell-free filtrates protect fruit from *Monilinia fructicola* (Wint.) Honey (McKeen *et al.*, 1986). The active material were isolated and several iturin peptides were identified, having a low toxicity and lacking allergenic properties, which is active against few bacteria but a wide variety of fungi (Gueldner *et al.*, 1988).

Antibiotics work in different ways. Thus far, the direct effect of a few antibiotics has been determined. By-products of the metabolic activity of *B. cereus* UW85 are antagonistic to oomycete and accumulate in culture supernatants (Milner *et al.*, 1996). Some exhibit their antagonistic activity through the sequestering of calcium and the production of large amounts of ammonium (Milner *et al.*, 1997). This increases the pH of the medium and causes the lysis of oomycete zoospores. However, the increase of the ammonium to calcium ratio does not account for the main ability of *B. cereus* UW85 to suppress disease. Antibiotics, including zwittermicin A and antibiotic B, are also produced. Zwittermicin A is a linear aminopolyol with a broad host range that includes both fungi and bacteria. Its activity includes the reversible inhibition of germ tube elongation of *Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell. Antibiotic B is an aminoglycoside. It has a narrower target range than zwittermicin A, but also shows activity against both bacteria and fungi. These two antibiotics do not account for all antifungal activity of *B. cereus* UW85, suggesting that it produces additional metabolites with antifungal activity (Milner *et al.*, 1997).

With the use of recombinant DNA technology, antibiosis can be readily manipulated and exploited to enhance disease suppression (Spadaro & Gullino, 2004). Antibiotic production regulation and biosynthesis has received a lot of attention recently. New approaches may enhance the amount of antibiotic synthesized or to improve the pattern of antibiotic synthesis. The synthesis of many antibiotics is influenced by specific nutrients and may be applied to enhance the activity of the biocontrol agent (Martin & Demain, 1980; Fravel, 1988; Gutterson, 1990; Thomashow *et al.*, 1990; Clarke *et al.*, 1992; Slininger & Jackson, 1992). Once the genes involved in the production of antibiotics are cloned, they can be placed under the control of a promoter that can be regulated giving control over the amount of antibiotic produced as well as timing of antibiotic production. Multiple copies can be cloned into the biocontrol organism that will also increase the amount of antibiotics produced. However, increasing antibiotic production does not necessarily imply that the biocontrol activity will be enhanced as well. In the case of *P. fluorescent* CHAO, increasing the production of

pyoluteorin, active against *P. ultimum*, resulted in the antagonist becoming phytotoxic to cress and sweet corn (Maurhofer *et al.*, 1992).

There remains some public concern over the use of antibiotic producing antagonists as biocontrol agents of postharvest diseases (Spadaro & Gullino, 2004). Introducing antibiotic producing antagonists into food supplies may have adverse effects on human resistance to antibiotics (Spadaro & Gullino, 2004). Pathogens are also more likely to develop resistance to antibiotics, possibly by only a single mutation, and the antagonist's efficacy is lost. Natamycin is an example of an antibiotic that has been widely used for food preservation to which very little resistance has been found (Droby & Chalutz, 1994). Some reports of members of the genus *Bacillus* producing antibiotics targeting prokaryotic and eucaryotic organisms are summarized in Table 2.2.

2.2.2. Mycoparasitism and Cell-wall degrading enzymes

Parasitism of soil borne and foliar fungal diseases by antagonists is well known (Wilson & Wisniewski, 1994). The term parasitism covers various interactions, including morphological disturbances, overgrowth of one organism by another (especially in the case of fungi), penetration and direct parasitism by production of haustoria, or the lysis of one organism by another (Wilson & Wisniewski, 1994). Necrotrophic interactions occur when the parasite derives nutrients from dead host cells, killed usually by the parasite itself, even though it may not invade the host (Skidmore, 1976).

Several enzymes produced by antagonists are involved in biocontrol, including glucose oxidase, lipase, protease, laminarinase, β-glucosidases, mannanase, xylanase, cellulases, chitinase and chitosanase (Fravel, 1988; Droby *et al.*, 1996; Nielsen & Sørensen, 1997; Frändberg & Schnürer, 1998; Picard *et al.*, 2000). Enzymes involved in biocontrol distort the distinction between parasitism and antibiosis. According to Fravel (1988), antagonists producing cell wall degrading enzymes that may simultaneously parasitise the pathogen and inhibit it through antibiosis. Other enzymes may cause only antibiosis where the antibiotic is an enzymatic end product. In the case of *Talaromyces flavus* (Klöcker) Stolk & Samson TF1, glucose oxidase is produced that catalyses the production of hydrogen peroxide from glucose (Kim *et al.*, 1980). In the case of *Bacillus* spp. X-b, a complex of different enzymes (chitinase, chitosanase, laminarinase, lipase and protease) are produced (Helistö *et al.*, 2001). Several examples of enzymes involved in biocontrol are summarised in Table 2.3.

Table 2.2: Antibiotics produced by members of the genus *Bacillus*

Antagonist	Type	Structure	Group / antibiotic	Host	Pathogen	Disease	Reference
Bacillus brevis	Peptide antibiotics	Cyclic peptide	Gramicidin S	-	-	-	Katz & Demain, 1977
Bacillus brevis	Peptide antibiotics	Cyclic peptide	Tyrocidine	-	-	-	Katz & Demain, 1977 Mootz & Marahiel, 1997
Bacillus brevis	Peptide antibiotic	Peptide lactone	Brevistin	-	-	--	Shoji, 1978
Bacillus brevis	Peptide antibiotics	Linear peptide	Edeine	-	-	-	Katz & Demain, 1977
Bacillus cereus	Peptide antibiotic	Linear peptide	Cerexins A to D	-	-	-	Shoji, 1978
Bacillus cereus UW85	3-amino-3-deoxy-D-glucose	-	Kanosamine	Alfalfa *Medicago sativa* L. Tobacco *Nicotiana tabacum* L.	*Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell *Pythium torulosum* Coker & P. Patt	Damping-off	Milner *et al.*, 1996 Silo-Suh *et al.*, 1998 Shang *et al.*, 1999
Bacillus cereus UW85	Aminopolyol	-	Zwittermicin A	Alfalfa *Medicago sativa* L. Tobacco *Nicotiana tabacum* L.	*Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell *Pythium torulosum* Coker & P. Patt	Damping-off	Milner *et al.*, 1995 Raffel *et al.*, 1996 Silo-Suh *et al.*, 1998 Shang *et al.*, 1999
Bacillus circulans ATCC 21656	Peptide antibiotic	Cyclic peptide	EM-49	-	-	-	Loeffler *et al.*, 1986
Bacillus circulans ATCC 21656	Peptide antibiotic	Cyclic peptide	Octapepin C_1	-	-	-	Shoji, 1978
Bacillus colistinus	Peptide antibiotics		Colistin	-	-	-	Katz & Demain, 1977
Bacillus licheniformis	Peptide antibiotics	Cyclic peptide	Bacitracin A to F	-	-	-	Katz & Demain, 1977 Podlesek *et al.*, 2000
Bacillus mesentericus	Peptide antibiotic	Peptide lactone	Esperin	-	-	-	Shoji, 1978
Bacillus polymyxa	Peptide antibiotics		Gatavalin	Cauliflower *Brassica oleracea* L. var. *botrytis* L.	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	Cyclic peptides	Polymyxin	-	-	-	Katz & Demain, 1977 Pichard *et al.*, 1995 Shoji, 1978
Bacillus polymyxa	Peptide antibiotics	-	Jolipeptin	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	BN 109	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	LI-F	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	Gavaserin	-	*Xanthomonas campestris* pv. *campestris*	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	Saltavalin	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	Linear peptides	Tridecaptin A, B, C	-	-	-	Shoji, 1978
Bacillus subtilis	Peptide antibiotics	Cyclic peptide	Mycobacillin	-	-	-	Katz & Demain, 1977 Shoji, 1978
Bacillus subtilis	Lipopeptide	-	Iturin: Bacillomycin L_c	-	*Ophiostoma ulmi* (Buisman) Nannf.	Dutch Elm Disease	Eshita *et al.*, 1995

Table 2.2 (cont.)

Antagonist	Type	Structure	Group / antibiotic	Host	Pathogen	Disease	Reference
Bacillus subtilis	Lipopeptide	-	Surfactin	-	-	-	Jacques *et al.*, 1994 Tsuge *et al.*, 1996
Bacillus subtilis	Lipopeptide	Cyclic peptides	Iturin A, C	Peach *Prunus persica* Sieb. & Zucc.	*Monilinia fructicola* (Wint.) Honey	Brown rot	Gueldner *et al.*, 1988 Jacques *et al.*, 1994
Bacillus subtilis	Lipopeptide	Cyclic peptides	Bacillomycin D, L, F	-	-	-	Jacques *et al.*, 1994 Peypoux *et al.*, 1984
Bacillus subtilis 370	Lipopeptide	-	Mycosubtilin	-	-	-	Peypoux *et al., 1986* Jacques *et al.*, 1994
Bacillus subtilis S499	Lipopeptide	-	Lipobacillin	-	Fungi	-	Jacques *et al.*, 1994
Bacillus subtilis FR-2	Lipopeptide	-	Bacillopeptins A, B, C	Garlic *Allium sativum L.*	*Fusarium oxysporum* (Schltdl.: Fr.)	Basal rot	Kajimura *et al.*, 1995
Bacillus subtilis F-29-3	Peptide antibiotic	Linear peptide	Bacilysin	Mungbean *Phaseolus aureus* Roxb.	*Rhizoctonia solani* (J. G. Kühn)	-	Loeffler *et al.*, 1986
Bacillus subtilis A14	Dipeptide	-	Bacilysin, Anticapsin	-	Bacteria: *Staphylococcus aureus*	-	Sakajoh *et al.*, 1987
Bacillus subtilis 168	Peptide antibiotic	Linear peptide	Bacilysin	-	-	-	Hilton *et al.*, 1988
Bacillus subtilis ATCC 6633		-	Bacilysin	-	Bacteria, fungi	-	Kugler *et al.*, 1990
Bacillus subtilis F-29-3	Lipopeptide	-	Fengymycin / Fengycin	-	-	-	Loeffler *et al.*, 1986 Chen *et al.*, 1995 Lin *et al.*, 1998
Bacillus subtilis YB8	Lipopeptide	-	Plipastatin B1	-	-	-	Tsuge *et al.*, 1996
Bacillus subtilis	Lantibiotic	Peptide lactone	Subtilin	-	Bacteria	-	Shoji, 1978; Klein *et al.*, 1996
Bacillus subtilis ATCC 6633	Phosphono-oligopeptide	-	Rhizocticin A to D	-	Fungi	-	Kugler *et al.*, 1990
Bacillus subtilis ATCC 6633		-	Subsporin A to C	-	Fungi	-	Kugler *et al.*, 1990
Bacillus isolates	Phenolic derivatives	-	Coumarin and isocoumarin: Amicoumacin	-	Bacteria	-	Pinchuk *et al.*, 2002
Bacillus sp. HIL Y-85, 54728	Lantibiotic	-	Mersacidin	-	*Staphylococcus aureus*	-	Bierbaum *et al.*, 1995

Table 2.3: Enzymes involved in biocontrol of fungal pathogens

Antagonist	Pathogen	Enzymes	Reference
Aureobasidium pullulans (de Bary) G. Arnaud	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. *Aspergillus niger* Tiegh.	Exochitinase β-1,3-glucanase	Castoria *et al.*, 2001
Pichia guilliermondii Wickerham	*Botrytis cinerea* (Pers.: Fr.)	β-1,3-glucanase	Wisniewski *et al.*, 1991
Bacillus subtilis AF1	*Aspergillus niger* Tiegh.	-	Podile & Prakash, 1996
Bacillus sp. X-b	Wood decay fungi	Chitinase Chitosanase Laminarinase Lipase Protease	Helistö *et al.*, 2001
Pythium oligandrum Drechsler	*Phytophthora parasitica* (Breda de Haan) Tucker	Cellulase	Picard *et al.*, 2000
Bacillus pumilus *Bacillus polymyxa*	*Aphanomyces cochleoides* Drechsler *Pythium ultimum* Trow *Rhizoctonia solani* (J. G. Kühn)	Glucanolytic and proteolytic enzymes	Nielsen & Sørensen, 1997
Bacillus pabuli K1	Various fungi	Chitinases	Frändberg & Schnürer, 1994
Species of: Pseudomonads, *Corynebacterium-Arthrobacter* group, *Streptomyces, Bacillus*	*Penicillium roqueforti* Dierckx (J5)		
Pichia anomala Strain K (E.C. Hansen) Kurtzman	*Botrytis cinerea* (Pers.: Fr.)	Exo-β-1,3-glucanase	Jijakli & Leoivre, 1998
Candida saitoana Montrocher	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link	Chitinase β-1,3 glucanase	El-Ghaouth *et al.*, 1998
Bacillus cereus UW85	-	-	Gilbert *et al.*, 1994

In the postharvest arena, little is known about biocontrol agents that directly parasitise pathogens (Wilson & Wisniewski, 1994). The yeast, *Pichia guilliermondii* Wickerham, attaches very effectively to the fungal pathogen *Botrytis cinerea* (Pers.: Fr.) hyphae. There is evidence that the antagonist produces hydrolases that may be responsible for the degradation of the fungal cell wall (Droby & Chalutz, 1994; Droby *et al.*, 1996). Cells of *C. saitoana* attach to *B. cinerea* spores and hyphae, preventing the proliferation of the pathogen. In this interaction El-Ghaouth *et al.* (1998) postulated that the yeast might have affected the ability of the fungus to degrade the host tissue. Fungal hyphae were atypical where it was in contact with the yeast, showing alterations ranging from cell wall swelling to degradation of the cytoplasm. These alterations showed similarity to symptoms observed in aged and starved fungal hyphae. Nutritional starvation of the fungi may be caused by the rapid colonization of the yeast. The yeast is also able to produce fungal cell wall degrading enzymes, including chitinase and β-1,3 glucanase. The production of these enzymes may explain some of the observed alterations where the yeast is in contact with the fungal cell wall (El-Ghaouth *et al.*, 1998).

2.2.3. Competitive exclusion and physical restriction

Competition is the interaction between two or more microbial populations that has a simultaneous demand for the same resource, whether it is living space or nutrients (Droby & Chalutz, 1994). If a microbial population already colonized an area, it naturally excludes newcomers. To establish an antagonist before the pathogen arrives is important, especially when the antagonist is not an aggressive colonizer (Morris & Rouse, 1985; Wilson & Wisniewski, 1989; Andrews, 1992). There is no clear distinction between competitive exclusion and competition for nutrients (see section 2.2.4), since the ability to colonize a specific surface goes hand in hand with its ability to take up nutrients thereby depleting the nutrient supply to the surrounding microbes. According to Kinkel *et al.* (1996), competitive ability consists of two distinct parts. Firstly, it describes the ability of a microbe to reduce the relative fitness of co-existing organisms. This can be achieved through predation, antibiosis, parasitism or competition. Secondly, it refers to the ability of the microbe to resist the reduction in its own fitness. It is difficult to directly measure the process of competition. By measuring the reduction in reproductive output, biomass, or vigour of competing microbes, the intensity of competition can be quantified.

Several studies show that exclusion of the pathogen occurs on the host and is an important mode of action in biocontrol. Some *Pseudomonas* species needs to be aggressive colonizers of roots to be successful as biocontrol agents (Bull *et al.*, 1991). In the case of *Cladosporium herbarum* (Pers.: Fr. Link), a saprophytic

fungus, extensive growth on pollen rich rye leaves could prevent the pathogen spores from actually reaching the leaf surface (Skidmore, 1976). When leaf surfaces were inoculated with ice nucleation-deficient strains of *P. syringae* before producing strains were inoculated, the producing strains were excluded (Andrews, 1992). Exclusion may play a role in the suppression of crown gall caused by *Agrobacterium tumefaciens* by *A. radiobacter,* but it has not been proven (Milner *et al.*, 1997).

This mode of action is not usually taken into consideration in the initial selection of antagonists, since screening for it involves more labour-intensive methods. Also, when other more aggressive modes of action, like antibiosis, are involved, the impact of competitive exclusion is seldom directly observed.

2.2.4. Competition for nutrients

Environmental factors severely restrict microbial growth on leaves. The growth of some of the microbes on the plant surface may be limited by the low quantity of nutrients present at any one time (Morris & Rouse, 1985). On newly expanded leaves, the supply of nutrients may be limited. However, as the leaf starts to age, the amount of nutrients on the leaf surface increase. Sometimes water availability will be variable and affect biocontrol (Campbell, 1989; Mercier & Wilson, 1995). The availability of nutrients is not only restricted on leaves, but also on fruit surfaces. Competition for nutrients is described on the phylloplane (Campbell, 1989; Droby & Chalutz, 1994; Bellows, 1999) and various researchers discuss its involvement in biocontrol (see table 2.4). However, indisputable evidence supporting its role in biocontrol is lacking (van Dijk & Nelson, 2000).

The different members of the microbial population that makes up the ecosystem on the plant surface, differs in their ability to grow and take-up nutrients (Droby *et al.*, 1996). According to Droby & Chalutz (1994), bacteria and yeasts are able to take-up nutrients from a dilute solution more rapidly and in greater quantities than the germ tubes of filamentous fungi, due to their large surface-to-volume ratio. Competition can be important at two stages in the pathogen's life cycle (Bellows, 1999). There may be competition during the initial establishment of fresh resource that was not previously colonized by microorganisms. Then, after initial establishment, there can be further competition to secure enough of the limited resources present to permit survival and eventual reproduction (Bellows, 1999).

Most postharvest infections occur through wounds inflicted during harvest and handling (Roberts, 1994). In order to control wound pathogens, the antagonist must be present at the wound site where the antagonism is

to take place (Wilson & Wisniewski, 1989; Roberts, 1994). Wound competence is the ability of the antagonist to grow rapidly at the wound site, be an effective utiliser of nutrients at low concentrations, and survive and develop better than the pathogen on the surface of the fruit and at the infection site under extreme temperature, pH and osmotic conditions. Most postharvest antagonists are able to survive and increase their numbers rapidly in wounds (Droby & Chalutz, 1994).

Competition for nutrients was demonstrated in the interaction of the yeast, *P. guilliermondii*. The antagonist and pathogen were co-cultured on a minimal synthetic medium or in wound leachate solutions (Wisniewski *et al.,* 1991). Spore germination and growth of the pathogen was inhibited when co-cultured with the antagonist. The antagonist concentration determined the inhibition and adding exogenous nutrients reversed the inhibition. This indirect evidence points to the role of nutrient competition in the interaction. There exists a delicate balance at the wound site between the numbers of antagonist and pathogen propagules, affecting the outcome of the interaction and determining whether the wound will become infected or not (Wilson & Wisniewski, 1989). However, Droby & Chalutz (1994) showed that the number of antagonist cells at the wound site does not always determine its efficacy.

To demonstrate nutrient competition as a mechanism of action, it is assumed that the pathogen needs external sources of nutrients for germination and penetration into the host tissue and this assumption is difficult to prove (Droby & Chalutz, 1994). Indirect evidence of the role of nutrient competition can be obtained if the following occurred: 1) spore germination and/or pathogen growth inhibition occurred during co-culturing with the antagonist; 2) pathogen inhibition was dependent on the concentration of the antagonist propagules; and 3) the inhibition could partially or completely be reversed by adding exogenous nutrients (Droby & Chalutz, 1994). Examples of antagonistic interactions where competition for a limited resource is summarised in table 2.4. Siderophore-mediated competition for iron is implicated mostly in rhizosphere systems (see section 2.2.5). Antagonists grow in a microsite, depleting it of certain nutrients. In some cases, fungal spores need exogenous nutrients to germinate (Skidmore, 1976; Andrews, 1992).

Plant-derived long-chain fatty acids are the limiting nutrient in the competition between the antagonist, *Enterobacter cloacae*, and the seed-rotting damping-off fungus *P. ultimum* (Van Dijk & Nelson, 2000). Fatty acids (linolenic acid) are required by the fungus to elicit its germination. Mutants of the antagonist were made with mutations in the fatty acid metabolism (beta-oxidation and fatty acid uptake). These mutants

could not metabolise linoleic acid and they were unable to suppress *Pythium* seed rot. However, when mutants were introduced to complement the mutants, suppression was obtained.

2.2.5. Siderophore production

Iron (Fe^{3+} ions) is limited in soil and on plant surfaces. Siderophores are extracellular, low molecular weight compounds produced by microorganisms and plants to sequester these ions, making them more readily available and giving the produce organism a competitive advantage (Fravel, 1988; Van Driesche & Bellows, 1996). Several antagonistic microbes producing siderophores involved in biocontrol have been described (Table 2.4). Siderophores produced by microbes surrounding the plant surface may enhance plant growth by increasing the availability of iron (Alexander & Zuberer, 1991).

Siderophores are implicated in the inhibition or limitation of growth of some organisms (Bellows, 1999). This is seen in the interaction between some antagonists and pathogens. Some microorganisms produce highly efficient siderophores that out compete those produced by pathogens, limiting their growth (Van Driesche & Bellows, 1996; Bellows, 1999; Calvente *et al.*, 1999). In some cases, siderophores are even implicated in the induction of systemic resistance (Lindow & Wilson, 1999).

Not only Pseudomonads are known to produce siderophores involved in biocontrol. Calvente *et al.* (1999) was able to control blue mould on apple using a combination of a yeast antagonist, *Rhodotorula glutinis* (Fresen.) F.C. Harrison, and its siderophore. Greater control was achieved using the combination rather than the antagonist alone.

The type of fungal pathogen that is to be controlled must be taken into consideration. Some pathogen fungal spores need exogenous sources of nutrients in order to germinate (Blakeman, 1985). Conidia are dormant cells that require a large input of iron to germinate and siderophores may reduce germination through chelating iron, subsequently reduce mycelial growth (Charlang *et al.*, 1981).

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Table 2.4: Antagonists producing siderophores involved in biocontrol of fungal pathogens

Host	Pathogen	Disease	Control agent	Reference
Tobacco *Nicotiana tabacum* L.	*Thielaviopsis basicola* (Berk. & Broome) Ferraris	Black root rot	*Pseudomonas fluorescens*	Ahl *et al.*, 1986
Wheat *Triticum aestivum* L.	*Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx	Take-all	*Pseudomonas fluorescens*	Weller & Cook, 1983 Pierson & Weller, 1994 Hamdan *et al.*, 1991 Slininger & Jackson, 1992 Milner *et al.*, 1997
Wheat *Triticum aestivum* L.	*Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx	Take-all	*Pseudomonas putida* B10	Buyer *et al.*, 1989
Cotton *Gossypium hirsutum* L.	*Rhizoctonia solani* (J. G. Kühn)	Damping-off	*Pseudomonas* sp. EM85	Pal *et al.*, 2000
Barley *Hordeum vulgare* L.	-	-	*Pseudomonas* spp.	Buyer *et al.*, 1993

3. FACTORS AFFECTING MODES OF ACTION

Disease suppression by antagonists in the laboratory does not always work as effectively in the field (Milner *et al.*, 1997). One possible explanation is the variable conditions in the field. Physical, chemical and biological environments are continuously changing. The host itself is continuously fluctuating in its surface properties, physically, chemically and biologically. Characteristics of the pathogen will also determine the antagonist's efficacy.

The antagonist population as a biological system fluctuates according to the existing environmental conditions, and in accordance to its population size as well as the presence of other microbial colonizers (Milner *et al.*, 1997). The influence of the host, pathogen and antagonist on biocontrol as well as their interaction with one another over time within an environment will be described in this section (Figure 2.2). The influence of these factors on the efficacy of the biocontrol agent will also be discussed.

3.1. Plant host affecting biocontrol modes of action

The plant host has a dual role in biocontrol. Through its own resistance mechanisms it reduces the pathogen's activities (Clarke, 1996; Droby *et al.*, 1996). It also provides the meeting ground for the pathogen and antagonists in which they will interact. The host affects the environment in which the interaction between the pathogen and the antagonist will occur by its excretion of exudates, ion and water uptake, gaseous exchange, as well as the UV light and temperature on its surface (Baker & Cook, 1974; Campbell, 1989; Leibinger *et al.*, 1997; Bellows, 1999). Environmental factors also drive the dynamic nature of microbial communities on plant surfaces and influence plant growth and development as well as all plant-microbe and even microbe-microbe interactions (Spurr, 1994). Microbial ecosystems are extremely complex and the interactions on the plant surface are affected by both physical and chemical (or nutritional) variables (Morris & Rouse, 1985).

In the preharvest scenario, the strategy of disease control is to ultimately keep the plant as healthy as possible and keep the inoculum levels of the pathogen as low as possible (Arul, 1994; Ippolito & Nigro, 2000). The major factors causing decay of fresh produce in the postharvest scenario are fungal infection, and/or physiological processes such as senescence (Arul, 1994; Roberts, 1994). Factors that accelerate senescence and favour microbial growth can promote postharvest decay. These include physiological and mechanical injuries and exposure to undesirable storage conditions (Arul, 1994). Any treatment that slows

the rate of senescence and inhibits microbial growth is a key factor in controlling disease in the postharvest arena (Roberts, 1994).

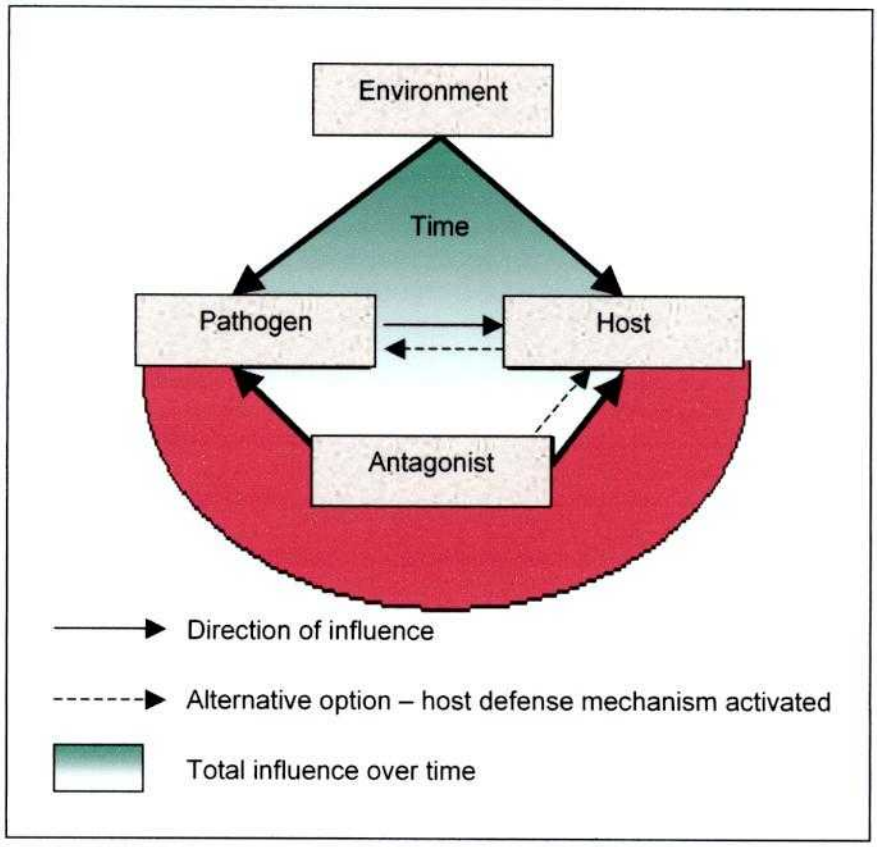

Figure 2.2: A schematic representation of the factors that influence biocontrol efficacy.

Aerial plant surfaces are hostile environments to the colonizing microorganisms (Campbell, 1989; Andrews, 1992). Microbial growth is restricted on plant surfaces due to the impact of environmental factors. Nutrient levels are low (Derridji, 1996), and microclimate variables exist, including surface moisture, temperature and irradiation, which may impact on the microbial community growth (Van Driesche & Bellows, 1996). Colonization, as well as growth and effectiveness of antagonism are affected (Romantschuk *et al.*, 1996).

Only a thin layer of air, a few millimetres thick, separates the leaf surface from the macro environment. The leaf itself influences the temperature, moisture and air currents within this layer (Andrews, 1996). This is a dynamic environment due to fluctuating environmental factors, including temperature, relative humidity, dew, rain, wind and UV-radiation, which may be cyclic or noncyclic in nature. Factors such as the position in the plant canopy, shape, size and surface topography of the leaf and fruit also influences the environment (Andrews, 1992). In the phyllosphere, leaf age and position influences the size of colonizing microbial populations (Jacques, 1996). On senescent and necrotic leaves, microbes are exposed to greater

environmental extremes, including humidity and temperature fluctuations, than exists on similarly exposed living leaves (Pfender, 1996). In the postharvest scenario, the environment is more easily manipulated. Temperature, humidity and gas composition can easily be controlled under storage conditions (Pusey, 1994).

Physical characteristics of plant surfaces vary among plant species, cultivars of the same species, different growth stages as well as distribution within the same plant (Andrews, 1996). The physical surface of a plant is made up of epidermal, guard and special functional cells such as trichomes, or leaf hairs, which varies considerably in size and shape, structure and density (Spurr, 1994). The phylloplane is a rough environment, giving the impression of hills, peaks, valleys and craters. Leaf veins, epidermal cells and cuticles may vary in shape and size, while trichomes, stomata and hydatodes may be present or absent and also varies in shape and size (Andrews 1992). Leaf surfaces may contain wax, embedded within and upon the cuticle, which is made up of long chain hydroxycarbons, alkyl esters, free primary alcohols and fatty acids. These waxes vary in thickness, composition, distribution and resistance to abrasion. Young leaves can regenerate wax (Andrews, 1992). However, this ability declines with age resulting in retention of water films and leaching of nutrients (Andrews, 1992).

The chemical composition of plant surfaces varies greatly (Derridji, 1996). The phylloplane is not an energy-rich environment, although there may be temporary patches of excess carbon compounds. Nutrients on the phylloplane originate endogenously or exogenously and include diverse carbohydrates, amino acids, organic acids, sugar alcohols, mineral trace elements, vitamins, hormones as well as antimicrobial compounds such as phenols and terpenoids (Andrews, 1992; Derridji, 1996). In the phylloplane, nutrients are important since they directly provide a growth substrate for microbes and indirectly induce the synthesis of secondary metabolites such as antibiotics and siderophores. Exogenous sources of nutrients include soil particles, dust, ions and solutes in rainwater, aphid honeydew, pollen, dead microbes, as well as bird and insect excrements (Spurr, 1994). Endogenous nutrients are removed from leaves by wounding, the leaching action of rain, dew and fog, or active exudation through guttation by hydatodes or even cuticles (Derridji, 1996; Jacques, 1996; Schönherr & Baur, 1996). Exudate concentrations vary both quantitatively and qualitatively with host, leaf position, leaf surface, age of plant, light temperature, fertility, pH, leaching medium and leaf injury. Nutrients are frequently limiting on the phylloplane. One nutrient affects the utilization of another, while some nutrients may be toxic to some microbial species (Andrews, 1992). In senescent and necrotic tissue, the distinction between the surface and the interior of the plant becomes difficult (Pfender, 1996),

making more nutrients available as the leaf dies. However, some nutrients are exported to other parts of the plant.

The biological composition on the plant surface is consists of bacteria, yeasts and fungi. These microbes succeed one another over time, so that the composition of the interacting community on the plant surface is not constant (Andrews, 1996). Microbial population size and composition vary under the influence of biotic and abiotic factors that affect population density and influence the host (Jacques, 1996). There are two different types of surface inhabitants: endophytes (parasitising the internal plant tissues and using nutrients from the plant to grow) and epiphytes (growing on the plant surface and utilizing available nutrients) (Spurr, 1994). Bacteria are usually the first colonists of the phylloplane, followed by yeasts and then filamentous fungi (Andrews, 1996). Sources of inoculum include soil, seed, air, and buds as well as over wintering shoots. Air carried spores are probably the primary source of filamentous fungi.

The available inoculum, the environment and host phenology directs the sequential microbial colonization of the phylloplane (Blakeman, 1985). Local effects like the degree of insect infestation, existing weather conditions and cropping practices alter this pattern. Factors that may influence colonization are births, immigration, emigration and growth rate of colonists, as well as the position of the leaf in the tree canopy (Andrews, 1996; Lindow, 1996). The preferred colonization sites on the leaf are along the veins and in grooves above the anticlinal wall of epidermal cells. This may be because of the localized concentrations of nutrients, protection from erosion, trapping and deposition of the colonists, as well as the retention of water films (Andrews, 1992).

The plant host has genes that, when expressed, affect the microbial community on its surface and the area surrounding it (Milner *et al.*, 1997). Gilbert *et al.* (1994) found that in certain cases the rhizosphere communities surrounding disease-resistant cultivars showed more similarities to the microbial communities in the surrounding soil than those of susceptible cultivars (see section 2.1.3). This may play a role in protecting the host from pathogen attack.

3.2. Pathogen affecting biocontrol modes of action

The pathogen remains one of the most important considerations in the selection of biocontrol agents, since each pathogen differ in their interaction with the host. This is due to genetic diversity and variable levels of ecological fitness (Blakeman, 1985). Successful plant surface colonizing fungi has to locate nutrients and

convert it into a viable reproductive or migratory form without yielding to competition with neighbours, unfavourable conditions or unfavourable host responses (Rayner, 1996). Pathogens also react differently to antagonists, since pathogens can vary in terms of virulence, they may also differ in their susceptibility to antagonist action. Blakeman (1985) describes three different types of pathogens: unspecialised necrotrophes, specialized necrotrophes and biotrophic pathogens. For biocontrol to be successful, the weak link in the pathogen's life cycle should be identified as the "the window of opportunity". The target biocontrol agent's mode of action should fit into the window of opportunity, disrupting the disease cycle.

Unspecialised necrotrophic pathogens include *B. cinerea*, *Cladosporium*, *Alternaria*, *Cochliobolus* and *Septoria* species (Blakeman, 1985; Andrews, 1992). They grow saprophytically on the plant surface before formation of an infection structure (Blakeman, 1985; Andrews, 1992). Nutrients from the spore itself, nutrients leaked from the plant surface and other nutrient sources like pollen and aphid honeydew, act to sustain the growth of this group of pathogens. The window of opportunity that is applicable in this circumstance is to obstruct the uptake of nutrients required for growth (Andrews, 1992). The saprophytic phase will be controlled when the antagonist competes for nutrients, preventing its establishment. When antagonistic organisms are present in high enough numbers in the area surrounding the pathogen spores, loss of endogenous nutrients from the spore may occur, reducing or preventing germination (Blakeman, 1985). The production of enzymes or antibiotics by the antagonist may also be effective against these pathogens (Blakeman, 1985).

The saprophytic phase of the specialized necrotrophic pathogens on the plant surface prior to penetration may be totally absent or very limited. They require less exogenous nutrients than unspecialised necrotrophic pathogens (Blakeman, 1985). Pathogens belonging to this group include some species of *Colletotrichum*, causing anthracnose. They form well-developed appressoria directly from the spore, from either no or a very short germ tube. Excess nutrients encourage the pathogen to grow saprophytically, suppressing the normal pathogenic behaviour (Blakeman, 1985). The weak link in the disease cycle of specialized necrotrophic pathogens is therefore the formation of appressoria (Blakeman, 1985). By adding competing bacteria to the spores, the extension of the germ tube is reduced and the number of appressoria formed increases since nutrient depravation enhances appressoria development (Koomen & Jeffries, 1993). The possibility exists that siderophores may directly assist in controlling fungi (Fravel, 1988). Iron may be fixed at a site within the conidium of the pathogen, inducing germination (Blakeman, 1985). Should germination take place in unsuitable conditions, pathogenesis may be reduced.

No saprophytic phase occur before biotrophic spores germinate and penetrate the host, even though long germ tubes can be produced (Blakeman, 1985; Andrews, 1992). Endogenous nutrients inside the spore entirely support growth of biotrophic pathogens. This means that competition for nutrients from epiphytes and biocontrol agents will not be effective against biotrophic pathogens. In the case of biotrophic pathogens, it was found that germ tubes and appressoria are sites of nutrient leakage. Yeasts and yeast-like fungi were found to stimulate germination of uredospores and growth of germ tubes of some rust fungi, benefiting the pathogen (Blakeman, 1985). These pathogens are difficult to control using biocontrol. Direct acting modes of action, like antibiosis and direct parasitism, are the best modes of action to combat biotrophic pathogens (Blakeman, 1985; Roberts, 1994).

The way a pathogen reacts to competition can be important at two stages in the pathogen's life cycle. Competition may occur during the initial establishment of new niche's that was not previously colonized by microorganisms. Then, after initial establishment, there can be further competition to secure enough of the limited resources present to permit survival and eventual reproduction (Bellows, 1999).

Microorganisms show traits that classify them as either adept at the initial colonizing phase, or as able to withstand and prevent subsequent phases of competition. A feature of r-strategists (ruderal species) is a highly reproductive capacity (Atlas & Bartha, 1987). They produce large amounts of reproductive bodies, like spores, increasing the chance that some may find newly available resources. They are efficient at the dispersal of these reproductive bodies, which is mostly resistant to harmful environmental conditions, and establish themselves in disturbed habitats or available resources. In contrast to the r-strategists, K-strategists flourish in more stable environments (Atlas & Bartha, 1987). They are able to cope with competition for space and limited resources. Plant pathogens are spread across this r – K range of characteristics. These traits will determine which mode of action will be the most effective against the pathogen (Bellows, 1999).

3.3. Antagonist affecting biocontrol modes of action

As with pathogens, biocontrol agents are also spread across the r – K range of evolutionary adaptive strategies (Andrews, 1992). An antagonist will be most effective when it is set in its optimal relationship (Janisiewicz & Korsten, 2002). Some pathogens infect hosts when some kind of disturbance occurs, such as wounding or when there is a change in the microbial ecology on the host surface (Blakeman, 1985). Biocontrol agents showing r-strategist characteristics will be able to colonize available resources, reducing

the available amount of nutrients, and have some survival mechanism, such as spores, which will persist on the host (Bellows, 1999). These agents will be in place before the pathogen infection cycle can begin. In cases where the pathogen has already infected the host, K-strategist biocontrol agents will be the best option, as a more competitive species will be required to be effective (Bellows, 1999).

The antagonists timing of application is also of importance (Andrews, 1992). Most antagonistic organisms currently used for biological control are most effective when they are applied prior to infection by the pathogen and do not appear to control previously established infections (Ikediugwu *et al.,* 1994; El-Ghaouth, 1997; El-Ghaouth *et al.*, 1998). Smilanick *et al.* (1993) tested two yeasts and two *Pseudomonas* species against *M. fructicola*, causal agent of brown rot of stone fruit. When the two yeasts were applied prior to pathogen inoculation, they were able to protect fruit from infection. However, when applied after inoculation by the pathogen, they were unable to control decay. On the other hand, the two bacteria were able to protect wounds up to 12 hours after inoculation with the pathogen (Smilanick *et al.*, 1993). It seems that it depends on the mode of action employed by the antagonist will be able to control established infections or not.

Since the action of the antagonist is under genetic control, its ability to take-up nutrients, produce antibiotics, siderophores and enzymes can be self-regulated (growth phase, nutrient status). It can also be triggered by reigning environmental conditions (exogenous nutrients, temperature, humidity). In the case of spore-forming bacteria, like *Bacillus* species, antibiotics are produced when sporulation is initiated, or when the stationary phase of growth is reached (Nakano & Zuber, 1990). Genes involved in Fengycin synthesis, an antibiotic produced by *B. subtilis*, is initiated during two different stages of cell growth. The promoter for the genes, are active during the log phase, and again during the early stationary phase (Lin *et al.*, 1999). Cell density, or quorum sensing, may also affect the production of antibiotics. Phenazine, an antibiotic produced by *Pseudomonas aureofaciens* is regulated by cell density (Pierson *et al.*, 1994). Nutrients may also affect the production of antibiotics. Milner *et al.* (1995) found temperature and the nutrient base for growth affected the accumulation of the antibiotic zwittermicin A, produced by *B. cereus* UW85.

4. NEW AND EMERGING TECHNOLOGIES IN THE USE OF BIOCONTROL AGENTS

Changing the environment surrounding the biocontrol agent, host and pathogen has received recent attention in an attempt to enhance biocontrol efficacy (Gilbert *et al.*, 1994). Manipulation of the biocontrol agent or the plant host may lead to improved disease control and it is possible not only to add resistance

genes to plants but also to induce changes in the plant environment to promote colonization by antagonists (Andrews, 1996). According to Morris & Rouse (1985), an alternative to inoculate the plant surface with large quantities of biocontrol microbes is to alter the ecosystem to favour the increase of the indigenous antagonists. This can be accomplished by adding certain nutrients to the plant surface. This strategy, however, requires a comprehensive understanding of the ecosystem.

By combining biocontrol agents a mixture of different biocontrol systems, the genetic diversity of the biocontrol systems pool increases (Pierson & Weller, 1994; Milner *et al.*, 1997). The resulting treatments may utilize a wide range of modes of action under a broader range of environmental conditions, and may even persist longer in the environment. The combination may also suppress a wider range of pathogens (Pierson & Weller, 1994). Two *Pseudomonas* isolates were tested against take-all of wheat, both in combination and on their own. The mixture was up to 30 % more effective in the field than using either of the isolates alone. They postulated that the combined treatment might have enhanced root colonization or increased the complexity of the protective barrier to the take-all fungus (Weller & Cook, 1983; Pierson & Weller, 1994).

Biocontrol agents may never give us the same level of control as that of chemicals. However, it may help reduce the amount of chemicals used on a crop, thereby decreasing the cost and improving the impact on the environment (Dubos, 1984; El-Ghaouth, 1997). By combining hot water spray and fruit brushing, the incidence of postharvest disease caused by *Alternaria alternata* (Fr.: Fr. Keissl.) improved on mango (Prusky *et al.*, 1999). Using alternative control measures such as these together with biocontrol agents might improve the total control achieved (Pusey *et al.*, 1986). Janisiewicz *et al.* (1998) evaluated calcium treatment and biocontrol agents, either alone or in combination, to control postharvest decay of apples. Each of the treatments gave some control, but not as high as combining the calcium treatment with the biocontrol. Similar results have been found on citrus (Obagwu & Korsten, 2003).

5. CONCLUSION

Biocontrol is currently one of the few alternatives for using chemicals to control fungal diseases. However, the success of biocontrol agents has been inconsistent (Andrews, 1996). This fact has lead to the study of the interactions between the host, pathogen, and biocontrol agent, as well as factors that influence them. We now know more about how biocontrol systems work compared to a few years ago. With a better understanding of biocontrol systems, future biocontrol products can be selected and screened more

successfully. By deciding on a biocontrol agent with specific modes of action, it may be possible to select an agent that is suitable for a specific pathogen-host-environment combination.

To ensure the future success of a biocontrol agent, it is crucial to keep the interaction between the biocontrol agent, host, pathogen and environment in mind when doing laboratory studies. According to Upper & Hirano (1996), it is important to make sure that the behaviour of a system in the greenhouse or growth chamber mimics the in the field scenario before starting extensive and exhaustive laboratory studies, particularly in the case of plant–microbe interactions.

Focusing on altering the application of biocontrol agents, combined treatments as well as genetic enhancement is no longer futuristic, and has received much attention recently. It will be possible that in time, and with a more comprehensive understanding of biocontrol systems and factors, biocontrol may still provide a practical, holistic and economically viable solution for consistent disease control.

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