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Mini review

Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases

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Abstract

Mechanisms involved in the biological suppression of infection and inoculum potential of *Botrytis cinerea* are numerous and variable and the involvement of two or more mechanisms has been demonstrated in several systems. Reported combinations include antibiosis with enzyme degradation of *B. cinerea* cell walls; competition for nutrients followed by interference with pathogenicity enzymes of the pathogen or with induced resistance; and alteration of plant surface wettability combined with antibiosis. Since germinating *B. cinerea* conidia are dependent on the presence of nutrients, competition for nutrients is regarded as important in systems where biocontrol is involved. Conidial viability and germination capacity are also potentially affected by the presence of antibiotics produced by biocontrol agents and present in the phyllosphere. Slower in action are mechanisms involving induced resistance in the host plant and production of hydrolytic enzymes that degrade *B. cinerea* cell walls. The latter has been demonstrated much more convincingly *in vitro* than in the phyllosphere. Biocontrol in established lesions and reduction of sporulation on necrotic plant tissues is a means to minimize the pathogen inoculum.

Abbreviations: BCA – bio-control agent; *Bc* – *Botrytis cinerea*; PG – polygalacturonase; PL – Pectin Iyase; PME – Pectin methyl esterase; PR – pathogenesis related; VPD – vapour pressure deficit.

The arena for biocontrol – the phyllosphere

There are numerous reports of attempts to control foliar pathogens, including *Bc*, by means of antagonists (See for instance Tronsmo, 1992; Andrews, 1992), and onIy a few will be reviewed here. Species of leaf bacteria (Blakeman and Brodie, 1976), yeast (Fokkema et al., 1979) and filamentous fungi (Dubos and Bulit, 1981; Fokkema, 1973) can inhibit pathogens by competing for nutrients. Fungi and a few bacteria are capable of direct parasitism (Kranz, 1981; Scherff, 1973). Antibiosis has been attributed to many bacterial strains (Leben and Daft, 1965), but also to fungi (Andrews, 1985; Ghisalberti and Sivasithamparam, 1991), and yeasts (Baigent and Ogawa, 1960).

The organisms involved in bio-control of foliar diseases include the pathogen, the host, the BCA and phyllosphere microorganisms. These are all affected by one another, by cultural practices and by pesticides. Necrotrophic pathogens, such as species of *Botrytis*, use exogenous nutrients in many circumstances, in order to germinate and to grow on the plant surface before penetration. Reduction of nutrient concentrations generally results in a reduced rate of pathogen conidial germination and slower germtube growth, thereby reducing the number of infection courts and the extent of subsequent necrosis incited by the pathogen (Blakeman, 1975, 1985; Blakeman and Fokkema, 1982). Mechanisms and attributes other than those which can be evaluated in Petri dishes are of importance under field conditions. Among these are the ability to live and grow on the plant surface under varying nutritional and microclimatic conditions, and to colonize the plant in such a way as to prevent the

establishment of the pathogen. This makes it very difficult to select antagonists for field applications, and the problem is further complicated by the fact that an isolate, although effective against a certain pathogen on one crop, may be ineffective against the same pathogen on another crop.

The chemical exudates on the plant surface contain macro- and micro-elements, sugars, sugar alcohols, pectic substances, amino acids, and organic acids (Tukey, 1970). The quality and quantity of leachates from plants are affected by plant age (Blakeman, 1972) and factors such as temperature, VPD and surface moisture, light, fertilization and pollen (Sol, 1967; Tukey, 1970; Weissman, 1964) which change constantly (Burrage, 1971). These changes may affect phyllosphere microflora directly (Dik et al., 1992) or have an indirect effect by modifying leaf characteristics, e.g., metabolic state, morphology (Cutter, 1978) and surface chemistry (Hallan and Juniper, 1971). As nutrients fluctuate there are community changes in colonization by bacteria, yeasts and filamentous fungi (Blakeman, 1982, 1985; Fokkema, 1981). Plant surfaces are covered with hydrophobic wax layer on which water is distributed as discrete droplets. The wettability of the plant surfaces can be modified by weathering, plant status and surfactants. Furthermore, microorganisms are known to produce surfactants (Cooper, 1986); thus, they can induce changes in the wettability of plant surfaces.

The pathogen and diseases caused by it

Bc infects the leaves, stems, flowers and fruits of various crops. On fruits, it causes a typical rot that is frequently covered with a grey mould and which may serve as a source of inoculum within the crop. On some fruits (tomato, pepper), flowers (gerbera, rose, cyclamen) or phylloclades (ruscus) the pathogen also induces small, necrotic lesions, usually surrounded by a bright halo (named 'ghost spot' in the case of tomato infection) (Elad, 1988, 1989; Elad et al., 1992; Salinas et al., 1989; Verhoeff, 1970). In greenhouse vegetables, stems of plants can be infected either by invasion by the fungus, through the petiole or directly through wounds, after pruning and harvesting. The infection may ultimately girdle the stem and kill the entire plant (Jarvis, 1989; Yunis et al., 1990). Infections may remain quiescent in the developing fruit or infected flowers of several crops, e.g., grape in vineyards, strawberry and other berry crops in the open field, and tomato, eggplant or pepper grown in greenhouses.

Low VPD, free moisture on plant surfaces and cool weather conditions are considered the most important environmental factors which promote infection by *Bc* (Blakeman, 1980; Elad and Shtienberg, 1995). Optimum temperatures for infection are between 10 and 20 °C, but infection can occur and grey mould may develop even at 2 °C and above 25 °C Elad, 1989; Elad et al., 1989; Jarvis, 1980; Marois et al., 1988; Salinas et al., 1989). Conidia of *Bc* require nutrients for germination and for subsequent germ tube growth.

Pathogen hydrolytic enzymes are crucial for infection by *Bc* (Elad and Evensen, 1995; Verhoeff, 1980). These include: cutinase, which hydrolyses secondary ester linkages of the cutin polymer (Salinas et al., 1992); cell-wall-degrading enzymes, i.e., pectolytic enzymes (exo- and endo-PG, PL and PME), during the first phase of host-pathogen interactions (Collmer and Keen, 1986; Johnston and Williamson, 1992; Leone, 1992); cellulase and a trans-eliminase (Verhoeff and Warren, 1972); xylanases, arabinase, β-glucosidase, β-galactosidase, β-mannosidase, and α-galactosidase (Urbanek and Zalewska-Sobczak, 1984).

A BCA should be capable of inhibiting the pathogen during one or more of the key stages of its disease cycle. Infection with *Bc* can be reduced by pre-inoculation of the phylloplane with epiphytic filamentous fungi, bacteria or yeasts (Reviewed by Blakeman, 1993; Blakeman and Fokkema, 1982) or by treating wounds at pre- or post-harvest stages. BCAs may reduce conidial germination and tissue penetration and may also interfere with lesion development (Elad et al., 1994a, b) and sporulation (Fokkema, 1993; Köhl and Fokkema, 1993; Köhl et al., 1995a, b). Many investigations of the bio-control of *Bc* have been reported (reviewed by Dubos, 1992; Elad and Shtienberg, 1995; Elad et al., 1995; Gullino, 1992), they will be referred to here only with respect to the mode of action through which control is imposed.

Interference with infection

The process of *Bc* conidial germination and subsequent host penetration are affected by microorganisms introduced into the phyllosphere. Microorganisms may change the physical properties of the host surface or may attach to the conidia of the pathogen, so affecting their behaviour through physical or biochemical association. Further, microorganisms compete with germi-

nating conidia of *Bc* for nutrients or space and may even secrete compounds inhibitory to the germinating conidia. Further contributions to the biocontrol of the pathogen are made by interference with the pathogenicity enzymes of *Bc* and even induced resistance. As will be shown below, most of the studies demonstrating biocontrol of *Bc* emphasize competition and inhibitory compounds as important mechanisms, whereas, the roles of other modes of action have so far received less attention in research.

Surface activity

Microorganisms can change the wettability of plant surfaces, as has been shown for introduced surface-active *Pseudomonas* spp. and for natural populations stimulated by added nutrients (Bunster et al., 1986). The surface effect induced by microorganisms may interfere with the attachment of pathogens to their hosts (Bunster et al., 1986). *Bacillus brevis* has been found effective against grey mould of Chinese cabbage. The antagonist, by causing drops to spread and dry, decreased the periods of wetness observed on leaves, thus restricting the occurrence of conditions favourable for *Bc* (Edwards and Seddon, 1992). This phenomenon is probably widespread but has been neglected in our search for potent mechanisms of biocontrol. Moreover, apart from the active antagonist, the few biocontrol preparations available contain additives which are active in changing the behaviour of water on plant surfaces, which by itself could affect the ability of *Bc* to germinate. Such a phenomenon of drop spreading and drying is observed when suspensions of formulated agents containing spreader compounds are applied on leaves (Elad, unpublished) and should be regarded as important on plant surfaces affected by *Bc*.

Attachment of biocontrol agents to the fungal pathogen

An example of the interaction between *Bc* conidia and potential BCAs is demonstrated in Figure 1. Cells of BCAs may attach to their hosts, and attachment mechanisms are recognized to play a role in cell-cell interactions in fungi and other microorganisms, including yeasts (Douglas, 1987). Lectins have been described as crucial for such interactions, including those involved in biocontrol systems (Barak et al., 1985, 1986; Elad, 1995a; Manocha, 1990) Attachment of cells of the antagonistic yeast, *Pichia guilliermondi* to *Bc* hyphae was observed by Wisniewski et al. (1991). The attachment could be blocked by agents that alter protein integrity (salts, proteases, etc.) and certain sugars (Wisniewski et al., 1991). The attachment of various antagonistic yeasts (*Rhodotorula glutinis* and *Cryptococcus albidus*) to conidia of *Bc*, and their self-aggregation was tested in order further to assess the nature of these phenomena (Elad, unpublished). The attachment of the yeast cells to conidia of *Bc* is typically associated with the presence of a fibrillar material, as seen in Figure 1. Attachment of yeast cells to conidia was evident for four of the five BCAs and aggregation in the presence of the conidia was also pronounced in four cases out of five. These phenomena were prevented in two of the yeasts by boiling the host conidia. Sugars either promoted or inhibited the attachment or self-aggregation, indicating a specific lectin involvement in the systems. Moreover, in some cases, interaction was decreased by protease or trypsin, HCl and NaOH which affected a protein moiety, or by β-glucanase which degraded

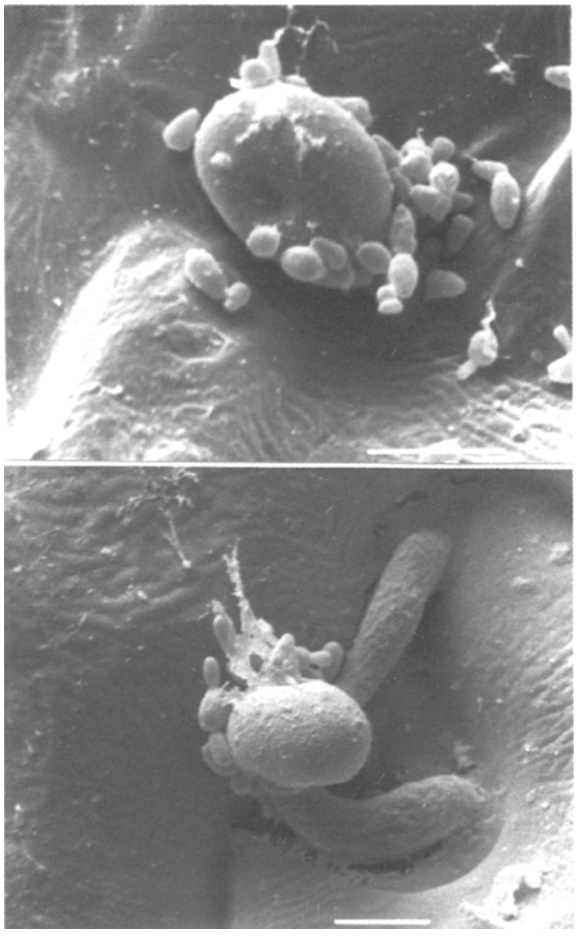

Figure 1. Scanning electron micrographs of the interaction between yeast cells and conidia of *Bc* (Bar = 5 μm).

a glucan moiety, both associated with lectins. Salts, SDS and β-mercaptoethanol, which affected the interactions, may have denatured the proteins or affected their activity and dispersal. The data indicate a variable involvement of lectin-like compounds whose role and origin are different in each yeast pathogen system (Elad, unpublished). However, their direct role in biocontrol of *Bc* has not yet been elucidated and should be studied further. However, Cook and Long (1995) successfully used the attachment phenomenon to screen potential biocontrol agents among phyllosphere bacteria and yeasts which adhere to hyphae of *Bc*.

Competition with germinating conidia of B. cinerea

Nutrients present in water on plant surfaces may be utilized by both *Bc* and saprophytic microorganisms. *Pseudomonas* sp. from the leaf surface removed amino acids much more rapidly than did conidia of the pathogen (Brodie and Blakeman, 1975). On the other hand, streptomycin applied to beet root leaves increased germination of the pathogen, because of reduced development of the bacterial population (Blakeman and Sztejnberg, 1974). Competition for nutrients or space was demonstrated when post-harvest biocontrol was tested with various yeasts on apple fruits, e.g., the yeast strain 87 of *Candida* sp. (McLaughlin et al., 1990), the basidiomycetous yeast *Cryptococcus laurentii* (there was no evidence of inhibitory substances or induction of wound healing) (Roberts, 1990), *Sporobolomyces roseus* (Janisiewicz et al., 1994) and *Candida oleophila* (Mercier and Wilson, 1994).

T. harzianum T39, which is the core of a commercial preparation that controls grey mould (Elad et al., 1994c), is a competitor against germinating *Bc* conidia at early stages of interaction (Zimand et al., 1995). Isolates of the yeasts *Rhodotorula glutinis* and lit Cryptococcus albidus, of the bacteria *Xanthomonas maltophilia, Bacillus pumilus, Lactobacillus* sp. and *Pseudomonas* sp., and of *Gliocladium catenulatum* were able to reduce germination of conidia of *Bc* and the severity of rot symptoms on detached leaves, and to control the disease on whole plants. Since the nutrient concentration on leaves had an effect on control efficacy, activity was partially attributed to competition for nutrients.

Bc needs external nutrients for germination and is very susceptible to the absence of nutrients in the phyllosphere. This is the reason for the widespread idea that microorganisms capable of efficient nutrient utilization should be good BCAs of this pathogen. However, the role of competition for nutrients or for space is not easily proved. Indeed, in the above mentioned cases, the fact that dead cells of the BCAs were also effective raised the possibility of the involvement of induced resistance (Elad et al., 1994a, b). In the presence of propagules of *Bc* on the symptomless leaf, the yeast population was reduced as compared with that on noninfected leaves, probably due to deprivation of nutrients by the pathogen. However, on established lesions, the yeast population proliferated. The introduced yeasts probably consumed the leaking nutrients from the damaged host tissue (Dik et al., 1991), thus protecting the leaves (Köhl et al., 1991) and affecting the developing grey mould.

Inhibitory and toxic substances

Germinating conidia of *Bc* are also susceptible to the presence of inhibitory compounds in their vicinity. Production of compounds inhibitory to fungi, including *Bc*, is common among microorganisms. Tronsmo and Raa (1977) and Tronsmo and Dennis (1977) found that *T. pseudokoningii* and *T. viride* were able to produce substances which inhibited the pathogen on fruits of strawberries and apples. Grey mould of snap bean pods and blossom was reduced by 77–97% by *T. hamatum* (Nelson and Powelson, 1988), which produced inhibitory volatiles. On the other hand, the successful BCA, *T. harzianum* T39 does not act by means of volatile or nonvolatile inhibitory products (Zimand et al., 1994). An isolate of *Penicillium chrysogenum* was found to produce inhibiting products which reduced conidial germination of *B. fabae* and lesion development on leaves of faba bean (Jackson et al., 1994). Janisiewicz and Roitman (1988) found an isolate of *Pseudomonas cepacia* to be effective against *Botrytis, Penicillium* and *Mucor* rots of apples and pears, by production of the powerful antibiotic pyrrolnitrin. *Bacillus brevis* (Edwards and Seddon, 1992) is effective against grey mould of Chinese cabbage by secret on of gramicidin S (and alteration of leaf wettability). The antibiotic itself was very effective against *Bc*. Leifert et al. (1995) related the control of *Bc* by *B. subtilis* and *B. pumilus* to antibiotics.

Although the ecological relevance of tests in Petri dishes is open to doubt (Elad and Chet, 1995), antagonism in culture has been routinely studied. For instance, *Trichoderma viride* showed two successive steps in antagonism, *in vitro*: the first one fungistatic, possibly resulting from diffusible antibiotics; and the

second a fungicidic effect which was contact dependent (Lamy et al., 1981). Growth of *Bc, Monilia laxa, M. fructigena* and *Phomopsis viticola* was inhibited when they were inoculated on agar containing more than 10^8 heat killed conidia of *T. viride* per ml (Ale-Agha et al., 1974). *T. viride*, which controlled chocolate spot of bean, inhibited three *Botrytis* spp. to the same extent as its culture filtrate (Morris and Lane, 1990). Recently, reports have been published that the peptaibol antibiotics trichozianins A1 and B1, produced by *T. harzianum*, and gliotoxin, produced by *Gliocladium virens* in culture, inhibited spore germination and hyphal elongation in *Bc* (Di Pietro et al., 1993; Schirmböck et al., 1994). Bélanger et al. (1995) found that in dual cultures of *T. harzianum* and *Bc*, antagonism was initially by antibiosis leading to cell death, which was followed by degradation of the cell by means of chitinolytic enzymes.

The role of antibiotics in biocontrol can be convincingly established by testing mutants which lack antibiotic production. Although multiple mutations or pleiotropic for other relevant activities may have occurred during the production of mutants, the mutation serves as one of the tools for testing the role of antibiotic production. Antibiotic production was responsible for the biocontrol of *Bc* by *B. subtilis* on *Astilbe* micro-plants. A UV-induced antibiotic negative mutant strain of this culture showed no activity on seedling bioassay *in vivo* (Leifert et al., 1995). Furthermore, Li and Leifert (1994) found that an antibiotic-producing isolate of *B. subtilis* was capable of protecting *Astilbe* microplants as long as resistance did not develop in the *Bc* population. However, this points to the potential risk of resistance development when reliance is placed on antibiosis-based biocontrol agents.

Restraining pathogen hydrolytic enzymes

Interference with pathogenicity processes has not been thoroughly studied yet, except for one system: the effect of *T. harzianum* T39 on germination of and subsequent lesion production by *Bc* was tested on bean leaves. Initially, the biomass of germ tubes of the pathogen was reduced by the BCA, 20 h after application (nutrient competition), but no difference in the amount of pathogen mycelium could be observed 48 h after inoculation. Nevertheless disease was significantly reduced by *T. harzianum* T39 (Zimand et al., 1995). In this case, the activity of pectolytic enzymes PG, PME and PL, produced by Bc on bean leaves was reduced in the presence of the antagonist. These reductions in enzyme activities were detected even 4 days after inoculation (Zimand et al., 1995). The activities of chitinase, β-1,3-glucanase and cutinase, but not that of cellulase, were also reduced in the presence of the BCA (Kapat et al., unpublished). These new findings indicate the possible discovery of a new mechanism by which biocontrol affects the pathogen: interference with the pathogenicity process. The precise steps leading to this effect are not clear yet. The effect may be the result of direct action of T39 on *Bc* hydrolytic enzymes; it could also be an indirect effect, via plant factors which induce enzymatic activity in *Bc*. It is speculated that in the future there will be more reports on the importance of this mechanism in suppressing infection by *Bc* and other pathogens.

Enzymes degrading fungal cell wall and mycoparasitism

Cell-wall-degrading enzymes of microbial origin. Fungal-cell-wall degrading enzymes are associated with degradation of hyphae of many pathogens (Elad, 1995a) but there have been only a few reports on this phenomenon in relation it to *Bc. Trichoderma* and *Gliocladium* spp. are known as mycoparasites (Elad, 1995a), and Tronsmo and Raa (1977) and Tronsmo and Dennis (1977) claimed that *Trichoderma* controls *Botrytis* rot of strawberry and apple, presumably by direct parasitism and antibiotic production (mentioned above). Labudova and Gogorova (1988) found isolates of *T. reesei* and *T. harzianum* capable of producing proteinase, mananase, laminarinase and chitinase and claimed that this implied that the nature of the antagonism of the two isolates was based on mycoparasitism. When fructifications of the antagonist were observed on the margins between necrotic and healthy areas on the rotting berries, microscopic observations revealed coiling and penetration of the mycelium of the latter by the antagonist (Dubos, 1987).

In vitro conidial germination of *Bc* served as a bioassay for testing hydrolytic enzymes and antibiotics of *T. harzianum* and enzymes of *Enterobacter cloacae* (Lorito et al., 1993a, b; Schirmböck et al., 1994). A synergistic activity of chitinolytic enzymes of the two microorganisms and bacterial cells was found (Lorito et al., 1993a); combination of endochitinase and chitobiosidase of *T. harzianum* resulted in a synergistic increase in antifungal activity, which by itself was more pronounced than the activity of the bacterial chitinases (Lorito et al., 1993b). An antifungal syner-

gistic effect was observed when the chitinases, β-1,3-glucanase and the above-mentioned peptaibols were present in the conidial suspension of *Bc* (Schirmböck et al., 1994). Similarly, endochitinase from *G. virens* had synergistic antifungal activity in combination with gliotoxin (Di Pietro et al., 1993). It was suggested recently (Wisniewski et al., 1991) that the mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*, combines tenacious attachment with secretion of cell-wall degrading enzymes. The role of chitinases and β-1,3-glucanase in the control achieved by *T. harzianum* T39 was assessed by comparing enzymatic activities of five isolates of the same species and correlating them with control achievements (Zimand et al., 1991); there was no correlation between ability to degrade *Bc* cell-wall polymers and biocontrol activity.

The involvement of cell wall degrading enzymes produced by antagonists, in *Bc* control under *in vitro* conditions is open to doubt. These enzymes have not been convincingly shown to take part in disease suppression. Furthermore, the role of mycoparasitism in control of *Bc in vivo* is doubtful since it is too slow to be effective against such a pathogen, which is capable of fast germination and subsequent host penetration.

Host-plant pathogenicity-related proteins. The presence of chitinase, a PR, plant-defense protein has been recorded in infected kiwi fruit (McLeod and Poole, 1994) and in transgenic tobacco plants (Roby et al., 1990) infected by *Bc*. In the latter, the chitinase promoter was activated during attack. Transgenic tobacco plants carrying genes of PR1 a (acidic PR1), PR2 (basic β-1,3-glucanase), PRQ' (acidic glucanase), PR3 (basic chitinase) or PR3b (acidic chitinase) were less susceptible to *Bc* than wild-type tobacco plants (Shaul, Elad, Kapulnik and Chet, unpublished). The induction of PR1, PR2 and PR3 in leaves of tobacco plants infected by *Bc* was tested (Elad, 1995b; Shaul et al., 1995). Tobacco plants were either wild-type or transgenic, containing PR1 and PR2 genes with β-glucuronidase (GUS) promoter. Northern analysis of RNA extracts from infected leaves of regular plants revealed the presence of PR1, PR2 and PR3. In the transgenic plants, PR1 and PR3 were found. However, immunoblotting revealed the presence only of acidic and basic PR3 proteins and not others. The identity of the actual inducing fragment and the chain of events leading to induction of PR proteins is not clear. Furthermore, the role of the PR proteins in combating the infection caused by *Bc* is also not clear. In the tobacco system it was induced and detected after the appearance of the initial symptoms, but transgenic tobacco plants carrying genes of PR proteins were less susceptible to *Bc* than wild-type plants.

Induced resistance

Induced resistance is also recognized as an important mode of action of biocontrol in vegetative tissues (Kuc, 1987; Sequeira, 1983). Wilson (1989) suggested that part of the mode of action of yeasts against certain citrus fruit rots may involve induced resistance in the fruit. Antagonists applied to harvested fruits may induce wound healing processes: in grapefruits, production of ethylene, phenylalanine ammonia Iyase and peroxidase was induced (Droby et al., 1991). Dead cells of antagonistic yeasts and bacteria were found capable of reducing grey mould similarly to live cultures in some cases (Elad et al., 1994a, b). The application of dead yeast cells was associated with an increase in the indigenous populations of bacteria and yeasts, but the indigenous populations were not high enough to reduce germination of *Bc* conidia and their penetration of the host tissue (Elad et al., 1994a). The BCAs did not induce resistance when applied at a short distance from the pathogen, and did not produce detectable inhibitory compounds. It was concluded that the activity of the BCAs was associated with their cells or cell walls, and at least part of the activity was not associated with live cells. Similarly, dead cells of *T. harzianum* T39 were also capable of partial control of *Bc* infection (Kapat and Elad, unpublished). Thus, locally induced resistance, along with competition for nutrients, resulted in the inhibition of grey mould on bean and tomato.

Cellulase from *T. viride* applied to a suspension cell culture of grapevine induced a hypersensitivity-like response in it. The elicitor treatment induced phenolic metabolism and formation of H_2O_2 and of the phytoalexin resveratrol (Calderon et al., 1993). The formation of extracellular peroxidases and of a new form of basic peroxidase, which were correlated with the formation of resveratrol oxidation products and tissue browning were found (Calderon et al., 1994). The authors suggest this elicitation process as a potential mechanism of induced resistance incited by *T. viride*. It seems that induced resistance is a credible mechanism by which suppression of *Bc* may be achieved, and it is tentatively proposed that natural biocontrol of *Bc* may be imposed by this mechanism. This is a relatively new direction for research and more information is needed in order to evaluate the role of induced resistance in *Bc* control.

Control of pathogen inoculum

Inoculum of the pathogen on necrotic tissue or on plant debris threatens both the crop in the field and outside it, in the season of production and also in later seasons. That is why research into restraining inoculum production and survival is considered important and has been carried out for several crops. The possibility of disrupting the life cycle of *Bc* at these late stages was found fruitful when control of lesion development and conidial production, by means of various microorganisms were attempted.

Colonization of necrotic plant material by microorganisms

The invasion of dead tissue by *Bc* was regarded by Newhook (1951) and Wood (1951) as a suitable process for inhibition of the pathogen by BCAs, since the substrate supports active saprophytic growth. They inoculated senescent lettuce leaves with antagonists such as *Fusarium* sp. and *Penicillium claviforma*, isolated from the same crop, in order to prevent primary establishment of *Bc*. Saprophytic micro-organisms established naturally on dead lettuce tissue in the field gave a considerable degree of control of *Bc* (Newhook, 1951). Establishment of *Cladosporium herbarum* and *Penicillium* sp. on dead petals adhering to tomato fruit reduced infection from 46–80% in trusses of non-treated control to 1–3% (Newhook, 1957). Later, *C. herbarum* effectively controlled grey mould on strawberries by protecting the flowers, even under field conditions (Bahtt and Vaughan, 1962). *Trichoderma* effectively protected floral caps of grapes from invasion by *Bc*. Isolations from floral caps showed that 61% of the caps in control plots carried *Bc* whereas only 12% of the caps in *Trichoderma*-sprayed plots carried it (Dubos et al., 1982). Thus, application of the antagonist at the flowering stage can prevent establishment of *Bc* on dead flower parts and can delay the development of the first foci of disease in vineyards. Similarly, the suppressive effect of *Gliocladium roseum* (Peng and Sutton, 1990; Sutton, 1990) described below is due to better colonization than by the pathogen of dead strawberry leaves. Substrate competition rather than antibiosis or hyperparasitism is the key biocontrol mechanism in this system (Sutton and Peng, 1993).

Suppression of sporulation of B. cinerea

Botrytis spp. sporulate abundantly on necrotic tissue and crop remains, and the conidia from successive cycles of infection contribute to the development of an epidemic within the crop (Köhl et al., 1995a; Sutton, 1990; Sosa-Alvarez et al., 1995; Yunis and Elad, 1993). Köhl, Fokkema and coworkers (Fokkema et al., 1991; Köhl and Fokkema, 1993; Köhl et al., 1992, 1995bc), working with *Botrytis* spp. on dead leaves of onion and lily, demonstrated that reduction of pathogen sporulation by means of several isolates of fungi may minimize the conidial load in the crop. Peng and Sutton (1990) tested various antagonists for their ability to control sporulation of *Bc* on strawberry leaflets and found that *Trichoderma* and *Gliocladium* isolates were most effective. *G. roseum* and *Myrothecium verrcaria* have been reported effectively to suppress sporulation of the pathogen on black spruce seedlings (Zhang et al., 1994), and several microorganisms, including *Penicillium* sp., *Arthrinium montagnei, Ar. phaeospermum, Sesquicillium candelabrum, Chaetomium globosum, Alternaria alternata, Ulocladium atrum* and *T. viride*, reduced sporulation of the pathogen in previously established lesions. These sporulation-inhibiting fungi did not reduce the infection of leaves by *Bc*. Most of these selected fungi and some bacteria were also found to be capable of reducing lesion expansion (Elad et al., 1994a). Köhl et al. (1995c) selected BCAs according to their capability to suppress sporulation after exposure to interrupted periods of leaf wetness. In this way Köhl et al. (1995c) were able to obtain an isolate of *U. atrum* which is capable of coping with field conditions. Unlike Peng and Sutton (1990), Köhl et al. (1995c) found that *Gliocladium* did not perform well under field conditions whereas *U. atrum* suppressed sporulation by more than 90% (Köhl et al., 1995c).

The interaction of saprophytes with germinating propagules of pathogens is different from their interaction with the sporulating phase of the pathogens, with respect to the length of the interaction time (Fokkema, 1993). In addition, the interaction with germination takes place on the undamaged plant surface, whereas the interaction with sporulation occurs in necrotic leaf tissue. The epidemiological implementation of pre- and post-infection biological control in the field may result in reduced disease spread. Reductions in production of inoculum followed by a suppression of its ability to infect would create an accumulative effect over several disease cycles (Jarvis, 1980; Köhl et al., 1992, 1995a) in greenhouses where the population of *Botrytis* is developing independently of exogenously contributing inoculum (Jarvis, 1980; Yunis et al., 1990) and also in field crops where inoculum produced within the field makes the main contribution to the develop-

ment of epidemics (Köhl et al., 1995a; Jarvis, 1962; Jordan and Pappas, 1977).

Colonization of sclerotia

Sclerotia of *Bc* are claimed to be an important source of primary inoculum in certain agrosystems (Nair and Nadtotchei, 1987). Therefore, the limitation of the viability and infectivity of sclerotia surviving on the canes of grapes and in soil may essentially influence the epidemics in the following year. Mycoparasites such as *Gliocladium roseum, Trichoderma viride, Acrostalagmus roseus, T. pseudokoningii, T. lignorum, Coniothyrium minitans* and *Teratosperma oligocladum* have been isolated from sclerotia of various *Botrytis* spp. or found capable of parasitizing the sclerotia (Summarized by Ghaffer, 1988). Among the mycoparasites, the ability of *Trichoderma* spp. to rot sclerotia of *Botrytis* spp. is well substantiated (Coley-Smith and Cooke, 1971; Coley-Smith, 1980). As sclerotia are mainly produced in temperate-zone vineyards, cold tolerant antagonists are essential for their decay. Indeed, Köhl and Schlösser (1989) found 19 isolates which macerated sclerotia of *Bc in vivo* at 5 °C. On grape vines, the production of sclerotia as resting structures was partly hindered by *Trichoderma* spp. which had been applied in late summer (Dubos et al., 1982). However, the treatment of sclerotia left in the field with the purpose of control of *Bc* was not thoroughly implemented so its potential for grey mould suppression is not yet clear.

Improved biological control

It is obvious that a biocontrol microorganism will not persist and effectively control disease unless it becomes established, survives, and is adapted to the plant environment. Nonetheless, insufficient research efforts have been directed towards the development of treatments or cultural practices capable of enhancing the establishment, survival and activity of the BCA (Elad, 1990; Windels and Lindow, 1985). Ecophysiological research on biocontrol agents by Magan (1995) points to the possibility of improving the tolerance of BCAs to low water availability in the phyllosphere. Mycelium and conidia of *Gliocladium roseum* has been tested for improved germination at lowered water availability (Magan, 1995), by manipulation of carbon concentrations and water availability to affect the accumulation of specific polyols in the thallus of the fungus.

The addition of various sugars, amino acids or other nutrient sources was attempted by researchers who were aiming at improved biocontrol of *Bc*. However, such nutritional fortification failed, probably because the pathogen was encouraged to germinate and infect by the added nutrients. It is probably impossible to find nutrients which would encourage the antagonist while not encouraging the pathogen.

Biological control of Botrytis rot of apple fruits by strains 87 and 101 of *Candida* sp. was enhanced when wounds were treated with $CaCl_2$, KCl or $CaCO_3$, (McLaughlin et al., 1990). In another study, Sugar et al. (1994) found enhanced activity of microorganisms in combination with several salts; these authors suggest that the salt ions affect the growth and survival of the microorganisms, or that they stimulate physiological processes in the microorganisms that result in excretion of new or additional metabolites. Wisniewski et al. (1995) found enhanced activity of two isolates of *Candida oleophila* in the presence of $CaCl_2$, and suggested that this was due to the direct inhibitory effect of calcium ions on pathogen germination and metabolism, and indirectly due to the ability of the yeast to maintain normal metabolism in the presence of toxic levels of calcium. Similarly, some salts ($CaCO_3$, $MgCl_2$ and KCl but not $CaSO_4$, enhanced the survival of *T. harzianum* T39 on bean and tomato leaves (Elad, unpublished). Some of the salts (KCl, $CaSO_4$, $CaCO_3$ and $MgCl_2$ enhanced biocontrol activity of this BCA. Enhancement of biocontrol of *Bc* by salts has been observed also on rose and gerbera cut flowers (Elad, unpublished; Kerssies, 1993).

Field examination of *T. harzianum* T39 activity (Elad et al., 1993) revealed better performance of the BCA at a low VPD (but not in the presence of wetness) and at high temperatures (25 °C). Under controlled conditions, the effect of VPD on the *T. harzianum* population was marked: higher populations were obtained under lower VPD. On the other hand, the effect of temperature could not be generalized; although there was a tendency towards higher populations at lower temperatures, it could be attributed to the relatively lower VPD conditions (within the limits of 0.35–0.55 kPa) that may occur at lower temperatures.

Formulation of a biocontrol agent can improve the activity, widen the range of conditions under which it is effective, increase its ability to withstand drastic changes in the phyllosphere and to survive well under unfavourable microclimatic conditions. This was shown by O'Neill et al. (1996) on wounded tomato stems. Temperature had a greater effect than vapour pressure deficit (VPD) on the efficacy of biocontrol. Suppression of *B. cinerea* incidence by *T. harzianum*

on stem pieces was significant at 10 °C and higher temperatures up to 26 °C. Control of infection was significantly lower at a VPD of 1.3 kPa (60% reduction), than at VPD < 1.06 kPa (90–100% control). Reductions in the severity of stem rotting and the sporulation intensity of grey mould were generally not affected by VPD in the range 0.59–1.06 kPa. Survival of *T. harzianum* on stems was affected by both temperature and VPD and was greatest at 10 °C at a low VPD and at 26 °C at a high VPD (O'Neill et al., 1996).

Use of information on the effects of microclimatic conditions on biocontrol and survival of *T. harzianum* T39 led to the development of a weather-oriented integrated control system (named Botman) for optimal use of the BCA with minimized use of chemical control means (Elad and Shtienberg, 1995). Enhancement of the *T. harzianum* population on plants fertilized with 3, 5, 8% NPK emphasizes another effect of cropping practice which is important for the establishment of the BCA in the phylloplane (Elad and Kirshner, 1992, 1993). Studies such as this one are important for gaining a better understanding of the introduction of the BCA into its target space; lack of attention to this point may lie behind cases of unexplained success or failure of biocontrol experiments which involve such a BCA.

Interaction of BCAs with other phyllosphere microorganisms

In the phyllosphere, the introduced BCA faces not only the pathogen but also a variety of other microorganisms with which it constantly interacts. The colonization of apple wounds by natural microorganisms and an introduced yeast, *Candida oleophila*, was monitored by Mercier and Wilson (1994) who found that the presence of naturally occurring microflora (the yeasts, *Aurobasidium pullulans* and *Sporobolomyces roseus*, and isolates of the bacteria *Erwinia, Pseudomonas* and *Gluconobacter* did not interfere with the biocontrol of storage rot; in some cases it was even beneficial.

The effect of the introduced *T. harzianum* T39 on other phylloplane microorganisms has also been studied recently (Elad and Kirshner, 1992) and it was found to be either promotive or inhibitory to bacteria, yeasts or filamentous fungi, depending on plant nutrition and the conditions under which the plants were incubated. The influence of *T. harzianum* may be attributed to an increased population of the introduced BCAs, related to the experimental conditions, or to the effect of the BCA on the interactive balance among the microbial populations. The results (Elad and Kirshner, 1992, 1993) do not provide a simple picture of phyllosphere BCA and *Bc* populations, because of complicating feedbacks within the phyllosphere, which is a dynamic system involving many interdependent variables. Moreover, the results also reflect the condition of viable propagules at the leaf surface; these may themselves have complex relationships with the activity of mycelial phases (where these exist) inside the plant tissues.

Conclusions

The mechanisms involved in the biological suppression of pathogenicity and inoculum potential of *Bc* are numerous and varied. Most of the possible modes of action studied for biocontrol of plant pathogens have also been demonstrated in systems where *Bc* was affected by BCAs. However, in many cases these mechanisms were not tested under *in vivo* conditions, but in cultures with artificial systems that mimic the phyllosphere arena poorly. Our ultimate goal is to elucidate the contributions of mechanisms which act at the sites of interaction on plant tissues. Knowledge related to the *in vivo* processes which take place in systems of successful biocontrol of *Bc* is still lacking.

It is apparent that, usually, in many systems, more than one mode of action is responsible for biocontrol. Indeed, as described in the course of this review, the integration of two or more mechanisms has been demonstrated in several cases. These include combinations such as: antibiosis with enzymes which degrade *Bc* cell walls; competition for nutrients followed by interference with pathogenicity enzymes of the pathogen or with induced resistance; and alteration of plant surface wettability with antibiosis. It is likely that further study into documented biocontrol systems will reveal more cases of multiple modes of action.

The relative importance of any of the above-mentioned mechanisms is open to speculation. Since germinating *Bc* conidia are dependent on the presence of nutrients, competition for nutrients should be regarded as important in systems where biocontrol is involved. Conidial viability and germination capacity are also potentially affected by the presence of antibiotics produced by BCAs and present in the phyllosphere. However, it should be noted that production of antibiotics on defined media in cultures does not necessarily mean that the same compounds will be

produced in high enough quantity under the chemical environment of the phyllosphere nor that they will be stable there. The activity of antibiotics as well as their production by BCAs in the phyllosphere is affected by the physiological status of the host and by its leachates. The effect of an introduced BCA on the hydrolytic enzymes of *Bc*, which are capable of dissolving the plant cuticle and cell walls, is a novel mode of action discovered as yet in only one system; it explains the prevention of infection even when *Bc* produces significant hyphal biomass on plant surfaces. In this situation, in the absence of the BCA, infection would have occurred and developed earlier and much more intensively. Mechanisms which are slower in action include those involving induced resistance in the host plant and production of hydrolytic enzymes that degrade *Bc* cell walls. The demonstration of the latter has been much more convincing in cultures than in the phyllosphere.

The contribution to effective biocontrol of the specific attachment of yeast BCA cells to *Bc* hyphae or conidia has not yet been elucidated; it has been found to be associated with degradation of hyphae of *Bc* but not of conidia. However, it may be speculated that the tenacious attachment of yeast cells to *Bc* improves the ability of the former to inhibit the activity of the latter by competition or by any other potential mode of action. The enhancement of activity by various salts is also not fully understood; it may be that the salts enhance the effects of certain components of biocontrol.

Treatment of established *Bc* with effective BCA results in competition between saprophytically growing mycelium of the pathogen and of the antagonist. The effects of these are: i. reduction of infection via necrotic plant parts adhering to healthy parts; ii. reduction of initial inoculum; and iii. reduction of secondary inoculum. Thus reduction of the threat to the crop in the same season or the next season, by conidia and subsequent epidemic development is achieved.

The effect of environmental and host factors on the functioning of the biocontrol mechanisms responsible for suppression of *Bc* infection and sporulation are of utmost importance for the success of biocontrol. However, these are the least understood basic features of the systems presented throughout this review. We are far from understanding the roles of microclimate, host species, host nutrition and leachates and host physiological status, and the contributions of these factors to the efficacy of *Bc* biocontrol and to the strength of the mechanisms by which it is achieved.

In the past, many studies were devoted to description of cases in which microorganisms successfully suppressed infection by or sporulation of the pathogen; attention was not always paid to the mechanisms responsible for successful suppression. The recovery and the thorough understanding of *Bc* biocontrol systems and the study of factors which affect them or enhance their potential are crucial for the development of reliable and effective systems for suppression of *Bc*.

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