Biological Control of White Mold of Bean (*Phaseolus vulgaris* L.) by *Epicoccum purpurascens* Ehrenb. ex Schlecht

by

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Biocontrol of White Mold of Bean by *Epicoccum purpurascens*

Abstract

Ph. D. Zhou Ting Plant Pathology

Biological Control of White Mold of Bean (*Phaseolus vulgaris* L.) by *Epicoccum purpurascens* Ehrenb. ex Schlecht.

After a wild-type isolate of *Epicoccum purpurascens* was exposed to shortwave ultraviolet light, several new strains were recovered which were improved in sporulation, fungicide tolerance, and performance in suppression of white mold caused by *Sclerotinia sclerotiorum*. The efficacy of *E. purpurascens* in controlling white mold of snap bean (*Phaseolus vulgaris*) was assessed in greenhouse and field trials. White mold was significantly reduced in both greenhouse and field trials when 2-4 sprays of *E. purpurascens* conidial suspensions (in 1% malt extract) were sprayed onto the plant surface during the flowering period. Germination of *E. purpurascens* conidia on senescent petals was greater than on younger flowers. Addition of malt extract to conidial suspensions improved germination on flowers and increased colonization of emerging flowers. Application of *E. purpurascens* did not accelerate senescence of bean leaves or affect pod yield of bean in greenhouse trials. Mycoparasitism of *S. sclerotiorum* by *E. purpurascens* was found only rarely in *in vitro* tests and was not observed on flower disks. Production of inhibitory compounds by *E. purpurascens* was the most important mechanism in suppression of white mold but competition for nutrients also appeared to play a role in biocontrol. The influence of nutrients on conidial germination, growth, sporulation and production of antifungal compounds by *E. purpurascens* were also investigated.

RESUME

Ph.D. Zhou Ting Pathologie végétale

Contrôle biologique de la pourriture blanche du haricot (*Phaseolus vulgaris* L.) par *Epicoccum purpurascens* Ehrenb. ex Schlecht.

L'exposition d'une souche sauvage de *Epicoccum purpurascens* à une faible longueur d'onde de lumière ultraviolet, a permis d'obtenir plusieurs nouvelles souches, améliorées quant à leur sporulation, leur niveau de tolérance aux fongicides et à leur aptitude à contrôler la pourriture blanche causée par *Sclerotinia sclerotiorum*. Des essais en milieu contrôlé (serre) et en milieu naturel ont permis d'évaluer l'efficacité de *Epicoccum* à contrôler la maladie. Des applications répétées (2 à 4) d'une suspension sporale (conidies) de *E. purpurascens* (dans 1% d'extrait de malt) pendant la floraison a permis de réduire de façon significative le développement de la pourriture blanche tant en serre qu'en milieu naturel. La germination des conidies de *E. purpurascens* était supérieure sur les corolles sénescentes comparativement à celle observée sur les jeunes fleurs. Cependant, l'addition d'extrait de malt aux suspensions sporales a permis d'augmenter le taux de germination sur les fleurs et de favoriser la colonisation des nouvelles fleurs. Des essais en serre ont permis de démontrer que l'application de *E. purpurascens* n'accélère pas la sénescence des feuilles et n'a aucun effet sur la production de gousses. Seuls quelques rares cas de mycoparasitisme ont pu être observés de la part de *E. purpurascens* envers *S. sclerotiorum* lors d'essais in vitro mais aucun sur des disques de pétales de fleurs. La production de substances inhibitrices par *E. purpurascens* était le mécanisme le plus important pour le contrôle de la pourriture blanche cependant la compétition pour les éléments nutritifs semblait également un moyen efficace de biocontrôle de *S. sclerotiorum*.

D'autres facteurs ont fait l'objet d'étude plus particulièrement l'influence d'additifs nutritionnels sur la germination des conidies, la croissance et la production de substances antifongiques.

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1. General Introduction

Common beans (*Phaseolus vulgaris* L.), including dry beans and snap beans, are grown widely in the world (Adams *et al.* 1985). In North America, snap beans are one of the most important vegetables but production often is affected by various diseases, which are responsible for serious losses in yield and quality of bean products (Tu 1984). One of the most destructive and economically important diseases of beans is white mold, incited by *Sclerotinia sclerotiorum* (Lib.) de Bary (= *Whetzelinia sclerotiorum* (Lib.) Korf and Dumont) (Steadman 1983; Tu and Beversdorf 1982). Severe epidemics of the disease occur frequently, and disease incidence and yield reductions reaching 100% have been reported (Purdy 1979; Tu 1984). Dramatic losses of the products also can arise from the spread of the pathogen from diseased to healthy tissues in storage. Shipments of snap beans in New York (U.S.A) are rejected at processing plants when more than 2% of the pods are diseased (Hunter *et al.* 1978).

At present, the most effective general control for white mold on snap bean is the application of a fungicide spray during the flowering period. Although applications can be effective in controlling disease, application may result in fungicide residue in the products, pollution of the environment and, possibly, the development of resistance of the pathogen to the fungicide. Some cultural control methods have been recommended to control this disease, however, they usually are ineffective. Also, some cultivars and breeding lines of white bean have shown useful field tolerance. Resistance, however, generally is not available (Steadman 1983; Tu 1984; Tu and Beversdorf 1982). Therefore, development of an alternative measure to control white mold effectively becomes essential. Biological control by means of application of antagonists has been chosen as the subject of this study.

The objectives of the research reported here were to produce and select strains of *Epicoccum purpurascens* Ehrenb. ex. Schlecht. tolerant to

several commonly used fungicides and with improved control of white mold; to determine the efficacy of *E. purpurascens* in controlling white mold of snap bean under greenhouse and field conditions; to investigate the behavior of *E. purpurascens* on beans and evaluate its effects on flower abscission, pod abortion, pod yield and senescence of leaves; to study the interactions between *S. sclerotiorum* and *E. purpurascens* and their effect on control of white mold; to assess effects of nutrients on *E. purpurascens* regarding conidial germination, mycelial growth, sporulation, and production of antifungal compounds.

* * * *

This thesis is submitted in the form of original papers suitable for journal publications. The thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines Concerning Thesis Preparation, Section 7, Manuscripts and Authorship. The relevant section of the regulation follows:

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The text of the above shall be cited in full in the introductory sections of any theses to which it applies."

The results presented in this thesis have been published as:

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Zhou, T., Reeleder, R.D. and Sparace, S.A. xxxx. Influence of nutrients on

Epicoccum purpurascens. Can. J. Bot. xx: xxx-xxx. (Section 7).

Although all studies presented in this thesis are the responsibility of the candidate, the project was supervised by Dr. R. D. Reeleder, Adjunct Professor, Department of Plant Science, Macdonald College of McGill University. That part of the study concerning antifungal compounds was under the supervision of Dr. S. A. Sparace, Department of Plant Science, Macdonald College of McGill University. The experiment "Generation of initial fungicide-tolerant strains of *E. purpurascens*" in section 3 was carried out by Thierry Legros under the direction of Dr. Reeleder.

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2. Review of Literature

Biological control on the phylloplane

The phylloplane

The phylloplane is generally considered to consist of the leaf surface (Last and Deighton 1965). In a larger sense, however, the phylloplane often includes stem, bud, and floral surfaces or surfaces of other aerial plant parts (Spurr 1990). The air space surrounding the surface may also be included; however, most investigators use the term "phyllosphere" when air space under the influence of the phylloplane and the phylloplane itself are being discussed (Last 1955). The phylloplane provides a habitat for a wide range of microorganisms including bacteria, yeast-like fungi, filamentous fungi, and microfauna (Blakeman 1985; Last 1972). The activities of these biological components, however, are dependent on the physical and chemical components of the phylloplane (Dickinson 1976; Dickinson and O'Donnell 1977), which differ from those of the rhizoplane and rhizosphere and vary among the different tissues found on the aerial parts of plants (Juniper 1983; Campbell 1989). The atmosphere around the aerial surfaces of plants is subject to fluctuations in temperature, gas concentration, radiation level, wind velocity, moisture, and air pollution (Burrage 1971, 1976; Smith 1976; Lepp and Fairfax 1976). In addition, the characteristics of aerial surfaces of plants are affected by their physical structure; cell walls, cuticles and waxes of the epidermis, stomata, trichomes etc. all contribute to the physical nature of the surface. Variations in these components result in differences in roughness, wettability and nutrient status of the aerial surfaces (Blakeman 1985; Juniper 1983). Nutrients for the microorganisms on the phylloplane are primarily derived from plant tissues as a result of leakage and exudation by trichomes and other cells and may be supplemented

by deposits of pollen and honeydew later during the growing season (Morgan and Tukey 1964; Dickinson 1976; Warren 1972). Also, leaves of certain plants may secrete to the surface phenolic or terpenoid substances inhibiting microbial growth (Blakeman and Atkinson 1981). Considering the conditions on the phylloplane, it is likely that the growth of microorganisms is normally severely restricted by environmental factors and limitation of nutrients. Thus any benefit that can be obtained from shelter is advantageous (Campbell 1989; Cullen and Andrews 1984a).

Biological control

Biological control is defined as "the use of one organism to eliminate or reduce disease caused by another" (Holliday 1989). Biological control of pathogens on the phylloplane has progressed more slowly than that of pathogens affecting roots. This is mainly due to successful control of pathogens on the phylloplane by chemical methods, poor knowledge of the ecology of microorganisms present on aerial surfaces (Cullen and Andrews 1984a; Rishbeth 1988), and complicated environmental conditions compared to those in soil (Leben 1985). Attention to biological control of pathogens on the phylloplane increased greatly as pathogens developed resistance to fungicides (Dennis and Davis 1979) and as environmental pollution was identified as an important problem. The increase also was encouraged by progress in microbial ecology and development of biological control techniques. This progress has been reviewed or summarized by many authors (Preece and Dickinson 1971; Dickinson and Preece 1976; Blakeman 1981; Blakeman and Fokkema 1982; Cullen and Andrews 1984a; Windels and Lindow 1985; Fokkema and Van den Heuvel 1986; Dubos 1987; Blakeman 1988; Jayapal Gowdu and Balasubramanian 1988; Rishbeth 1988; Campbell 1989).

Two general approaches are employed to encourage biological control of pathogens on the phylloplane. One is manipulation of the ecosystem to favor increases in the populations of indigenous antagonists. The manipu-

lation may be made to the physical and/or chemical environment of crops. Populations of *Sporobolomyces roseus* on wheat leaves varied with different relative humidities. The population decreased at 65% RH but resumed growth when exposed to 95% RH. In addition, application of nutrients to wheat leaves stimulated the population of *S. roseus* resulting in reduction of infection of wheat leaves by *Cochliobolus sativus* (Bashi and Fokkema 1977). Another approach is the introduction of antagonists in large numbers at sites where the pathogen is, or may become, established. This antagonist may be chosen either from the naturally occurring phylloplane population or from other habitats (foreign antagonists), and may be either a saprophyte or a non-pathogenic variant of the pathogen. *Chaetomium globosum* isolated from apple leaf reduced severity of scab caused by *Venturia inaequalis* when it was sprayed onto the leaves of apple seedlings (Andrews *et al.* 1983). *Trichoderma* species from soil or plant debris are the best known examples of foreign antagonists applied to the phylloplane (Dubos 1987; Tronsmo 1986). *Cladosporium*, a phylloplane-dominating saprophytic fungus, was used to control blossom blight and green fruit rot of strawberry in the field (Bhatt and Vaughan 1962) and *C. cladosporioides* reduced the number of white mold lesions on bean leaves up to 51% compared to the control in a greenhouse test (Boland and Hunter 1988). Application of nonpathogenic isolates of *Alternaria* sp. to leaf surfaces of tobacco before inoculation with the pathogen, *A. alternata*, protected tobacco leaf from infection by the pathogen (Spurr 1977). Indeed, attempts at biological control of pathogens on the phylloplane have most frequently involved applying antagonists to the plant surface (Andrews *et al.* 1983; Fokkema *et al.* 1979; Nelson and Powelson 1988; Rai and Singh 1980).

Mechanisms of biological control using antagonistic fungi

A wide range of fungi possess antagonistic activity to plant pathogens *in vitro* and/or *in vivo* but few are successfully used for biological control, especially under field conditions. This is partially

due to a lack of knowledge about the mechanisms involved in biological control (Cullen and Andrews 1984a; Howell 1990). Mechanisms of biological control are dependent upon antagonists used (species or strains) and the target pathogen. Various antagonist:pathogen relationships may differ with respect to mechanism and most often more than one mechanism is involved (Campbell 1956; Dubos 1987). Generally, however, antagonistic fungi have three mechanisms to achieve their success in the biological control. These are mycoparasitism, production of antibiotics, and competition for nutrients and space (Blakeman and Fokkema 1982; Cullen and Andrews 1984a; Jayapal Gowdu and Balasubramanian 1988; Fravel 1988; Weller and Thomashow 1990).

Mycoparasitism

This refers to a direct attack of one fungus (mycoparasite) on another. Mycoparasites in relation to biological control have been widely studied, especially those which operate on soil-borne pathogens (Adams 1990; Baker 1987; Whipps *et al.* 1988). About 210 species of mycoparasites have been documented (Campbell 1989). Mycoparasites may be obligate or facultative. Most of the mycoparasites which are obligate do little harm to their host since they depend on the host for their continued development. In contrast, facultative mycoparasites destroy host tissue by means of enzymes and toxins, and continue to absorb nutrients from the dead host cells. The latter has great potential in the control of fungal plant pathogens (Burge 1988) since it can reduce the inoculum level of pathogens.

Many cases of phylloplane mycoparasitism studied to date have involved attacks on rust and powdery mildews by rather more specialised fungi (Jarvis and Slingsby 1977; Swendsrud and Calpouzos 1972; Sharma and Heather 1983; Sundheim 1982). *Darluca filum*, one of the most intensively studied mycoparasites has been reported to attack 362 species of rust (Kranz 1981; Kuhlman *et al.* 1978; Swendsrud and Calpouzos 1972). Under greenhouse conditions, inoculation of *D. filum* on wheat with *Puccinia*

graminis and *P. triticina* showed 100 % infection at high humidity (Prasada 1948). However, Swendsrud and Calpouzos (1972) found that *D. filum* applications onto wheat were only effective if inoculated after the appearance of the rust *Puccinia recondita*. Other species such as *Scytalidium uredinicola* and *Verticillium lecanii* also are likely to be effective in rust control (Kuhlman 1981; Spencer 1980; Spencer and Atkey 1981). *Ampelomyces quisqalis* has been used to control powdery mildew (*Erysiphe cichoracearum* and *Sphaerotheca fuliginea*) on cucumber (Jarvis and Slingsby 1977; Sundheim 1982) and parasitized these pathogens extensively.

Dicyma (*Hansfordia*) *pulvinata* is a mycoparasite of several leaf-spot fungi such as *Cercospora personatum* (Hughes 1951) and *Cerosporidium personatum* on peanut (Taber *et al.* 1981) and *Cladosporium fulvum* on tomato (Peresse and Le Picard 1980). Although *D. pulvinata* does not prevent initial infection by the pathogen, it quickly colonizes the leaf-spot lesions and destroys the conidiophores and conidia of the pathogen, thus, suppresses secondary inoculum formation and dispersal of the pathogen (Mitchell *et al.* 1987). Similarly, *Nectria inventa* suppressed *Alternaria brassicae* (black spot) on oilseed rape only on fallen leaves, thereby reducing carryover of inoculum. On attached rape leaves, mycoparasitism was avoided since conidia of the pathogens germinate more quickly than those of the mycoparasite (Tsuneda and Skoropad 1978a,b).

Mycoparasites often do not affect a high proportion of the population of pathogenic fungus unless the humidity and temperature are high although mycoparasitism occurs commonly with world wide distribution (Campbell 1989). Moreover, application of the mycoparasite must coincide with periods when there is a sufficient pathogen biomass to sustain the mycoparasite (Blakeman 1985; Whipps *et al.* 1988). Thus, mycoparasites like *D. filum* might be useful only for slowly developing diseases or for diseases with high economic thresholds (Kranz 1981).

Some mycoparasites, although not normally associated with aerial

surfaces, have been successfully used in this habitat for control of pathogens. Inoculum of *Trichoderma viride* was applied repeatedly onto grapes from the beginning of flowering until three weeks before harvest to control *Botrytis cinerea* infection. The level of the control achieved was only slightly inferior to that obtained by use of a fungicide (reviewed in Blakeman 1988). *B. cinerea* on apples (dry eye rot) was controlled by *T. harzianum* applied in 0.1% malt extract (Tronsmo and Ystaas 1980). *Trichoderma* species have been found to parasitize various groups of fungi (Dennis and Webster 1971); other mechanisms, however, may also be involved in the biocontrol of pathogens (Dubos 1987).

Antibiotic production

Antibiotics are low molecular weight organic compounds produced by microorganisms and are deleterious to the growth or activity of other organisms (Fravel 1988). Although antibiotics have not been recovered from the leaf surface, many antagonistic fungi found on the phylloplane do produce antibiotics in culture, and the roles in biological control have been demonstrated in some cases (Cullen and Andrews 1984b; Fawcett and Spencer 1970; Rai and Singh 1980).

Several fungi which inhabit the phylloplane have been shown to be capable of producing antibiotics. *Aureobasidium pullulans* has been shown to produce a heat-stable antibiotic in culture (Baigent and Ogawa 1960); Strains of *Alternaria* produce substances active against fungi (Lindenfelser and Ciegler 1969) and *Sporobolomyces ruberrimus* produces a fungistatic substance (Yamasaki *et al.* 1951). *Botrytis cinerea* is known to produce at least three antibiotics and it inhibits *Erysiphe polygoni* on clover leaf (Barnes 1971). In addition, culture filtrates of several leaf surface fungi were found to inhibit development of lesions caused by *Alternaria brassicae* on mustard and *Drechslera graminea* on barley leaves (Rai and Singh 1980).

Control of *Venturia inaequalis* on apple seedlings by *Chaetomium globosum* has been ascribed to the production of the antibiotic chetomin by

the antagonist (Cullen and Andrews 1984b). In seven strains of *C. globosum*, antibiotic production in vitro positively correlated with suppression of the pathogen. In another study, treatment of seed with *C. globosum* reduced the number of seed coat microflora, and an unidentified antibiotic was isolated from treated seed (Hubbard *et al.* 1982).

Trichoderma species produce both nonvolatile and volatile antibiotics (Dennis and Webster 1971a,b). One of them was identified as trichodermin, a sesquiterpene antibiotic active against fungi. Only one of 22 isolates tested *in vitro* by Dennis and Webster (1971a) lacked antibiotic activity. A recent study shown that *T. harzianum* effectively controlled *Puccinia arachidis* on groundnut, and a phenol-like compound active against fungi was isolated from cultures of germinating conidia of *T. harzianum* (Govindasamy and Balasubramanian 1989)..

Antibiotic production obtained *in vitro* is not always correlated with effective control *in vivo* (Fravel 1988). Indeed, only rarely have organisms selected for antibiotic production been used successfully (Campbell 1989). Inhibition zones on agar between colonies of candidate antagonists and pathogens may be due to antibiotic production, however, they may also result from staling products from the colony of the pathogen, which easily lead to false interpretation (Fokkema 1973). Antibiotics are generally produced most abundantly on a rich substrate and when growth of the fungus is slow or has ceased because certain essential growth requirements become limiting in the environment (Bu'lock *et al.* 1974; Demain 1972). Nutrient situations *in vivo* may not be similar. It also is possible that antibiotics released to the environment are rapidly broken down (Boudreau and Andrews 1987). Moreover, the antagonist may itself be antagonized by other organisms in the ecosystem and made ineffective with respect to biocontrol (Campbell 1989).

Competition

Clark (1960) has defined competition as an active demand in excess of the immediate supply of a material or condition on the part of two or

more organisms. Competition also was described as "the injurious effect of one organism on another because of the utilization or removal of some resource from the environment" (Paulitz 1990). The latter is more relevant to the concept of biological control.

The majority of necrotrophic plant pathogens have a temporary saprophytic phase on the plant surface before penetration and thus may require exogenous nutrients (Cochrane 1966; Abawi *et al.* 1975). Extensive growth of an antagonist may deplete available nutrients for the pathogen and even take up nutrients leached from the pathogen (Blakeman and Brodie 1977), thus suppressing pathogen and resulting in control of disease. Occurrences of competition for nutrients on the phylloplane have been proved directly and/or indirectly by a number of investigators (Blakeman 1978). Blakeman and Brodie (1977) found that when leaves of beetroot planted in a growth room were wetted for 24 h in advance, there was a considerable increase in number of natural epiphytes, and those epiphytes took up nearly 80% of available amino acids and strongly inhibited germination of *Botrytis cinerea* conidia. The amount of inhibition was related to the amount of amino acid uptake. Fokkema *et al.* (1983) demonstrated that, although infections by *Cochliobolus sativus* and *Septoria nodorum* were stimulated by the presence of aphid honeydew, such stimulation is never observed in the field due to rapid removal of this nutrient by the saprophytes. Similarly, stimulatory effects of pollen on *Drechslera sorokiniana* on rye leaves (Fokkema 1973) and on *Phoma betae* on sugar-beet leaves (Warren 1972) were reduced by the presence of an abundant microflora. Also, Fokkema *et al.* (1975) showed that a reduction in saprophyte population on rye leaves after application of benomyl resulted in an increase in infection by a benomyl-insensitive strain of *C. sativus*. Competition for nutrients was considered as the main source of inhibition rather than antibiotic production.

Enhancement of biological control efficiency

Enhancing the activity of antagonists

Activity of an antagonist could be the most important factor in its success in controlling a plant disease and thus its ultimate acceptance for commercial application. Various approaches have been employed in enhancing activity of antagonists for biological control (Cullen and Andrews 1984a; Baker and Scher 1987; Baker 1990).

Providing appropriate chemical adjuvants such as nutrients and additives is a way of promoting colonization and development of the applied antagonist. Different nutrient additives mixed with *Trichoderma harzianum* spore suspension had different results when they were used to control dry eye rot of apple (Tronsmo 1986). Efficiency of biocontrol may also be influenced by the timing of nutrient application. When nutrients and spores of *Sporobolomyces roseus* were applied 5 days before inoculation of wheat leaves with the pathogen *Cochliobolus sativus*, disease was reduced, while no disease control was obtained if nutrient was applied 1 day before inoculation of the pathogen (Bashi and Fokkema 1977).

Genetic manipulation of an antagonist can be used to improve its virulence to the pathogen or its general vigour in the environment. Strains with elevated production of antibiotic could be selected from large populations of wild-type or mutagenized cells using simple bioassays (Burnett 1975) although such use with antagonistic fungi has been rarely reported. Several researchers have used mutants of antagonistic fungi to enhance their resistance to commonly used fungicides (Papavizas *et al.* 1982; Cullen and Andrews 1984c; Katan *et al.* 1984). Some mutants of *Trichoderma harzianum* induced by ultraviolet radiation were tolerant to benzimidazole fungicides and even more effective than wild strain in suppressing damping-off (*Pythium ultimum*) of peas (Papavizas *et al.* 1982). Efficiency of biological control can also be increased by improving resistance of biocontrol agents to certain environmental factors such as temperature (Tronsmo and Ystaas 1980) to meet the requirements of current

agricultural practices. Some newly developed biotechnologies have been and will more commonly be used in improvement of biological control agents (Baker 1990; Howell 1990).

Integration of biological control with other controls

Attempts have been made to control disease on the phylloplane using sprays with fungicides in combination with compatible antagonistic fungi. *Sphaerotheca fuligena* on cucumber is attacked by the mycoparasite *Ampelomyces quisqualis*. The mycoparasite alone did not give plants complete protection from the pathogen. However, when plant were sprayed with the fungicide triforine at one-third of the recommended rate and spore suspension of the mycoparasite, the yield increased 50% over that of the non-sprayed control and was even higher than in plots sprayed with the normal rate of fungicide (Sundheim and Amundsen 1982). Also, combining of *Fusarium lateritium* with the fungicide benomyl gave better control of dieback disease of apricot caused by *Eutypa armeniacae* than either fungicide or the antagonist alone (Carter 1983). In this integrated control, benomyl gives immediate protection and *Fusarium* a longer lasting effect. Biological control may also be integrated with cultural control methods such as alternating the spacing of crop plants (Burge 1988).

Sclerotinia sclerotiorum and white mold of beans

Sclerotinia sclerotiorum (Lib.) de Bary

This fungus is an omnivorous plant pathogen with world-wide distribution. It has been reported to attack 361 host species in 64 families (Purdy 1979) and new hosts are constantly being added to this record (Phillips 1987). In North America, the fungus incites diseases on more than 150 crops (Haas and Bolwyn 1973).

The sclerotia are the perpetuating structures of *S. sclerotiorum* and they can survive for 2-5 years in soil (Adams and Ayers 1979; Steadman

1983; Coley-Smith and Cooke 1971; Cook, *et al.* 1975). Sclerotia overwinter on or in the soil, then may germinate preceding the growth of crop plants. However, they can and often do survive on various weeds if crops are not available (Steadman 1983, Abawi and Grogan 1975). Germination of sclerotia can be myceliogenic (or hyphal), where only individual hyphae or hyphal aggregates are formed, and carpogenic, where fruit bodies arise from the sclerotium (Coley-Smith and Cooke 1971; Purdy 1979). However, carpogenic germination (to produce apothecia from which ascospores are released) is one of the key events for epidemics of the disease to occur (Abawi and Grogan 1975; Boland and Hall 1987). According to Sutton and Deverall (1983), the ways in which the fungus infects plants have been studied since the pioneering work of de Bary in 1886 and the principal modes could be summarized as: a. mycelium from germinating sclerotia infects the stem base; b. ascospores released from apothecia germinate at wound sites and penetrate the tissue; or c. ascospores germinate on dead or senescent flowers, leaves and organic matter in contact with the host and the mycelium which develops in these nutrient bases then infects and colonizes intact tissues. The last mode apparently is the most important one for epidemics of the diseases caused by this fungus.

Epidemiology of white mold

White mold is the disease caused by *S. sclerotiorum* on beans. Infection of bean plants can occur any time after seedling emergence (Purdy 1979) but diseases generally appear during and after flowering of the crop (Abawi and Grogan 1979). Mycelium from sclerotia has been reported to infect beans (Natti 1971 and Tu 1989). Other studies, however, indicated that this occurs rarely under natural condition and is unimportant in epidemics of white mold (Abawi and Grogan 1975; Cook *et al.* 1975; Steadman 1983). Since sclerotia are soilborne, they cannot be involved directly in initiating infection in the aboveground plant canopy. Generally, white mold is initiated by air-borne ascospores ejected from apothecia (Abawi

and Grogan 1975; Abawi *et al.* 1975; Schwartz and Steadman 1978). Only sclerotia in the top 2-3 cm of the soil are functional for production of ascospores because apothecia with stipes longer than 3 cm rarely are produced under field conditions. Ascospore inoculum for epidemics of white mold can be provided from both inside and outside of bean fields (Abawi and Grogan 1975; Boland and Hall 1987; Cook *et al.* 1975). As the primary inoculum, ascospores are blown onto fallen or intact flower petals which adhere to stems or petioles or leaves and infect healthy bean tissues through the flowers (Abawi and Grogan 1975). Ascospores alone can only penetrate young leaves but not fully expanded leaves (Sutton and Deverall 1983). However, recent studies show that ascospores can infect leaves in contact with moist soil (Tu 1989).

The function of flowers in epidemics of white mold is as a food base to provide exogenous energy for ascospores and hyphae to infect host tissues (Abawi *et al.* 1975; Natti 1971; Sutton and Deverall 1983). The availability of a food base is usually a prerequisite for successful infection (Lumsden 1979), and the nutrition of *S. sclerotiorum* during disease development is considered as the most important factor in determining success or failure in the establishment of disease in the host. According to Brown (1936) and Abawi *et al.* (1975), appressoria must be formed for mechanical penetration of plants by germinating ascospores. Purdy (1958) found that a carbon source was essential for formation of appressoria and no infections resulted when the suspending solution lacked a carbon source, unless the inoculation was made at the site of natural or artificial wounds. Similarly, ascospores of *S. sclerotiorum* ejected onto leaf surfaces were able to infect bean leaves only when an energy source was added to the spore suspension. Appressorium formation was closely related to the nutrient level of the medium (Abawi 1975). Furthermore, germination of ascospores and subsequent mycelial growth were stimulated in the presence of bean pollen both in vitro and in vivo (Sutton and Deverall 1983). The importance of a food base in the infection process

also has been indicated in other reports (Lumsden and Dow 1972; Natti 1971).

Ascospores can survive on plant surfaces or on the soil surface for nearly two weeks and will germinate within a few hours when free moisture becomes available (Abawi *et al.* 1975; Singh and Singh 1984; Purdy 1979). On bean petals, ascospores germinated by forming one or two germ tubes within 6 hours of inoculation. Simple, club-shaped appressoria were formed, and direct penetration of the host cuticle by infection pegs occurred within 24 hours. After 72 hours, the infected flowers were completely invaded by *S. sclerotiorum* (Abawi and Grogan 1975). Once a flower is colonized, the mycelium may remain viable for more than a month (Steadman 1983). When leaves were contacted, hyphal strands growing out from infected flowers formed multicellular, cushion-shaped appressoria on the leaf surface. Infection pegs produced by the appressoria penetrate the cuticle directly. Once beneath the cuticle, the fungus grows rapidly throughout the leaf tissue (Abawi and Grogan 1975; Lumsden and Dow 1972) and mycelium will grow out from the surface when moisture is sufficient.

Because asexual spores are not important in the disease cycle and newly produced sclerotia usually exhibit dormancy for carpogenic germination, no secondary infection propagules are available (Abawi and Grogan 1975; Cook *et al.* 1975). Secondary infection occurs when green tissue comes into contact with an infected area. This occurs only to a limited extent and is unimportant in the development of epidemics, especially for snap beans (Abawi and Grogan 1975).

Moisture plays a key role in development of white mold (Grogan and Abawi 1974; Natti 1971; Steadman 1983). Infection occurs only if free moisture is available for a relatively long periods (Abawi and Grogan 1975); 16-24 hours or more than 72 hours of free moisture are required for infection of beans using either moist flowers colonized with actively growing mycelium or dry colonized flowers, respectively (Abawi and Grogan 1979). The optimum temperature for ascospore germination was 25 ^{0}C, and

germination was good at 20 and poor at 10 and 30 °C (Abawi and Grogan 1975). In controlled environments, disease developed from 15-25 °C, most rapidly at 20 °C but not at 30 °C (Boland and Hall 1987). Other factors, such as soil pH, canopy architecture, plant population and planting date etc. also influence epidemics of white mold (Haas and Bolwyn 1972, 1973; Steadman 1983, Abawi and Grogan 1979).

Control of white mold

To date white mold has been difficult to control by cultural means (Sherf and Macnab 1986), and host resistance has been inadequate (Lumsden 1979; Steadman 1983). The most effective general control for the disease is application of fungicides although the efficacy of fungicide treatments is variable. Success in control using fungicides apparently depends upon several factors, including timing of application, development of the disease cycle, environmental conditions, coverage of flowers and plants, and flowering period (Hunter *et al.* 1978; Morton and Hall 1982, 1989). Spraying flowers with fungicide effectively controlled the disease, but no control was obtained when all above ground plant parts except blossoms were covered with the fungicide (Hunter *et al.* 1978). Therefore, fungicide sprays in commercial fields are often applied during flowering periods. Sprays applied at early bloom or full bloom are more effective than at late bloom or at any other time (Hunter *et al.* 1978; Natti 1971; Steadman 1979,1983). A single spray applied at the appropriate time provided acceptable control of white mold, while a single application when disease severity is high (60% or more) is not effective (Steadman 1983). In Morton and Hall's studies (1989), the most effective treatments were one spray at bloom or one spray at full bloom followed by a second spray at late bloom. A number of fungicides can be used for disease control; these include benomyl, thiophanate-methyl, iprodione, PCNB (pentachloronitrobenzene), DCNA (2,6-dichloro-4-nitroanoline) and others (Steadman 1979, 1983; Sherf and Macnab 1986). Benomyl (Benlate^{R} 50W, 1.75-2.25 Kg/ha) and iprodione

(Rovral® 50 W, 1.0 kg/ha) are recommended in Canada (Ministry of Agric. and Food Ontario 1984).

Practical biological control has not been demonstrated for white mold although there have been a series of studies of biological control of *S. sclerotiorum* (Steadman 1979; Ghaffer 1988). Biological control tests have been generally conducted with two goals: to decrease the populations of sclerotia in soil or to suppress infection of plants or disease development. At least 30 species of fungi, bacteria, insects and other organisms have been reported as active against the sclerotia although they were mainly based on laboratory observations or tests (Steadman 1979; Whipps 1987; Phillips 1989). Biological control of *S. sclerotiorum* on the phylloplane has also been reported. Foliar applications of *Coniothyrium minitans* on bean effectively reduced white mold in laboratory experiments but failed in greenhouse or field trials (Trut-
mann *et al.* 1982). *Fusarium lateritium* inhibited ascospore germination and mycelial growth of *S. sclerotiorum* in various media and suppressed infection of lettuce plants when sprayed as macroconidia onto leaves (Sitepu and Wallace 1984). On lettuce leaf disks, several fungi isolated from leaves of lettuce, such as *Alternaria alternata*, *Trichoderma viride*, and *Epicoccum purpurascens* suppressed germ tube elongation, and reduced infection of lettuce by *S. sclerotiorum* in growth chamber tests (Mercier and Reeleder 1987). Co-inoculation of bean petals with ascospores of *S. sclerotiorum* and conidia of either *Cladosporium cladosporioides* or *Alternaria alternata* resulted in suppression of white mold in laboratory and greenhouse trials (Boland and Hunter 1988). Also, others filamentous fungi isolated from bean and rapeseed flowers, such as *Drechslera* sp., *E. purpurascens*, *Fusarium graminearum*, and *F. heterosporum* reduced the percentage of flowers causing the development of white mold lesions on bean plants in the greenhouse (Boland and Inglis 1989).

Epicoccum purpurascens Ehrenb. ex. Schlecht.

Characteristics and distribution

This fungus was first described by Link in 1816 as *E. nigrum*, and the same fungus was described independently by Schlechtendahl as *E. purpurascens* in 1824 (Cannon 1986). Under the rules of nomenclature prior to the 1981 Botanical Congress, *E. purpurascens* was used for this species (Booth 1980). Many other synonyms were also used in some publications (Schol-schwarz 1959). Since the starting date for nomenclature has changed from January 1, 1821 to May 1753 (Hawksworth *et al.*. 1983), *E. purpurascens* should be referred to as *E. nigrum* (Cannon 1986). However, *E. purpurascens* is still popularly used in publications (Pandey *et al.* 1982; Singh 1985; Melgarejo *et al.* 1986; Brown *et al.* 1987; Mercier and Reeleder 1987; Boland and Inglis 1989). *E. purpurascens* is used throughout this thesis.

E. purpurascens has a characteristic appearance. On the surface of the substrate, it is characterised by small black pustules, which are pulvinate sporodochia, covered with conidiophores and conidia. On potato dextrose agar, *E. purpurascens* grows initially with production of a strong yellow, or amber to orange pigment. The culture becomes black with spore formation. More detailed characteristics for the species can be found in works by Booth (1980), Duncan (1974), Ellis (1971), Tuttobello *et al.* (1969), Barron (1968), and Schol-Schwarz (1959).

E. purpurascens is cosmopolitan. In nature, it is very often found on the dying and dead parts of various plants (Domsch and Gams 1972) and it has been isolated from air, animals, foodstuffs, textiles, etc. (Ellis 1971). It distributed world wide, being found in both the temperate and tropical regions of the world (Booth 1980).

Epicoccum has been recovered from the leaf surfaces of many plant species (Dickinson 1967; Godfrey 1974; Last and Warren 1972; Mercier and Reeleder 1987; Norse 1972; Warren 1976). It also has been isolated from

twigs (Melgarejo 1985), soft fruits (Dennis 1976) and flowers (Melgarejo *et al.* 1985; Boland and Inglis 1989). It is generally regarded as a non-parasitic saprophyte, one of the most abundant filamentous saprophytes on the phylloplane (Last and Warren 1972; Dickinson 1976). In his classification of epiphytic fungi found on leaves, Dickinson (1976) placed *Epicoccum* in the group of non-pathogenic epiphytes, and described it as a phylloplane invader, which grows extensively only when conditions are particularly favourable. On leaves of sycamore (*Acer pseudoplatanus* L.), young sporodochia of *Epicoccum* first appeared in July and its frequency increased until after leaf fall. It only began to penetrate the leaf in August, at the time of leaf senescence (Pugh and Buckley 1971). In a study of effects of phylloplane micro-organisms on the senescence of wheat leaves, Jachmann and Fehrmann (1989) inoculated wheat flag leaves with several phylloplane fungi in the greenhouse, and found that unlike *Cladosporium herbarum* and *C. cladosporioides*, *E. purpurascens* developed very slowly and did not affect leaf senescence in terms of reduction of chlorophyll content.

E. purpurascens has been reported as a cause of some plant diseases, which include leaf spots of sunflower (*Helianthus annuus*) (Acimovic 1983), berseem (*Trifolium alexandrinum*) (Gupta and Karwasra 1982) and cassava (*Tapioca*) (Roy 1968); glume blotch of wheat (*Triticum aestivum*) (Goel and Gupta 1977); red blotch of rice (*Oryza sativa*) (Khatua *et al.* 1977) and "red kernel" disease of sweet corn (*Zea mays*) (Wright and Billeter 1974). *E. purpurascens* also has been isolated, together with other fungi, from diseased tissues of certain plants, e.g. from shrivelled seeds of rapeseed (*Brassica campestris* var. *sarson*) (Randhava and Aulakh 1981), core rot of apple (*Malus sylvestris*) (Combrink *et al.* 1985) and leaf spot of oats (*Avena sativa*) (Muller 1964). However, its pathogenicity in these latter cases needs to be confirmed.

Metabolic Products of *E. purpurascens*

E. purpurascens is a pigment-producing mold. Various pigments produced by this fungus have been observed and in some case intensively studied.

As early as 1912, Nauman reported a purple-red pigment produced by *E. purpurascens* growing on rice. According to Burge *et al.* (1976), the pigment was soluble in ethanol and methanol, weakly soluble in water, and insoluble in less polar solvents. It showed a weak absorption band at 448-468 nm. "In solution, the pigment turned yellow upon the addition of acid and red upon the addition of base. On standing, the purple-red pigment became brown-red pigment". The latter pigment no longer changed colour when treated with acids or bases, and it was shown that it was the same brown-red pigment (a weak band at 461-491 nm) as that obtained from older cultures of the fungus. The solubility of the brown-red pigment was similar to the purple-red one.

When cultured in yeast extract medium, *Epicoccum* produce brown polymeric "humic acids", consisting of phenolic substances and amino acids, especially older culture. The production and properties of humic acids by *E. purpurascens* have been studied by Martin *et al.* (1967) and Burge (1973).

Four carotenoid pigments have been isolated from the dark-red mycelium of *Epicoccum*, identified as β-carotene, γ-carotene, rhodoxanthin and torularhodin (Gribanovski-Sassu and Foppen 1967; Foppen and Gribanovski-Sassu 1968). Production of carotenoids by ultraviolet-induced mutants has been studied (Gibanovski-Sassu *et al.* 1970). The mutants showed differences from the wild-type strain in color of mycelium, growth (dry weight in liquid medium) and production of total carotenoids.

Bamford *et al.* (1961) isolated a yellow pigment from cultures of *Epicoccum* grown on modified Czapek-Dox and Ranlin-Thom media. The pigment was extracted from the culture filtrate with charcoal, which was then eluted by acetone. The resulting compound was identified as flavipin

(3,4,5-trihydroxy-6-methyl-0-phthalaldehyde), a known metabolite of *Aspergillus flavipes* and *A. terreus* (Raistrick and Rudman 1956). Flavipin has also been isolated by Burge (1973) and Brown *et al.* (1987). Flavipin sublimes (at 140 °C) as small pale-yellow rods. However, its melting point (decomposed) varies from 228-234 °C, in different reports. Those reports include 233-234 °C by Raistrick and Rudman (1956), 228-229 °C by Bamford *et al.* (1961) and 229-231 °C by Burge (1973). In aqueous sodium bicarbonate, flavipin produces a yellow solution, and in sodium hydroxide, it forms a solution which is initially cherry-red but quickly becomes brown. A few reactions of flavipin are useful for its diagnosis (Raistrick and Rudman 1956). Molar absorption spectra of flavipin vary among the studies. The principal peaks of visible and ultraviolet spectra are at wavelengths of 261, 264 and 330 nm (Raistrick and Rudman 1956), 265 and 346 (Burge 1973) and 209 nm (Brown *et al.* 1987). From malt extract agar cultures, alcohol-soluble pigments A and B were isolated (Burge *et al.* 1976). Pigments A and B are dark orange-red solids (yellow in dilute solution). Ethanol solutions of both A and B show a very characteristic absorption maximum at 429 nm and a shoulder at 450 nm although they have different R_f values on TLC plates. Interconversion of the pigments was found in aqueous solution. Pigment A appeared more stable and gave rise to fluorescent products. Pigments A and B are different from the alcohol-soluble pigments described by Naumann (1912) and Foppen (1969). In addition, a golden yellow pigment with pH-dependent fungistatic activity has been reported by Eka (1970b), and two phenolic compounds have been isolated from mycelial extracts of *E. purpurascens* in 0.3 N HCl in methanol and identified as chrysin and p-hydroxybenzoic acid. The former is yellow in colour (Singh 1985).

Various investigators have followed different approaches to study pigment production by *E. purpurascens*. Thus there is a possibility that each may be dealing with a fraction of what may be a single, complex substance (Eka 1970 b).

Besides the pigments, other metabolic products of *E. purpurascens*

have also been investigated. Epicorazines A and B were isolated from fermentation broth of *E. purpurascens*, and separated by preparative TLC. These compounds were isomers with the same epidithiodiketoperazine skeleton, and their difference is related to a *cis-trans* configuration (Baute *et al.* 1978, Deffieux *et al.* 1978a,b; Brown *et al.*). In addition, siderophores (high-affinity, microbial ferric transport molecules) have been isolated from culture filtrates of *Epicoccum*, and studied in detail by Frederick *et al.* (1981a,b).

Coleoptiles of *Avena* have been used to detect growth promoting substances produced by filamentous phylloplane fungi and it was found that culture filtrates of *Epicoccum* produced a 55 % increase in elongation of coleoptile over distilled water controls. Auxin was isolated from an ether extract of the culture filtrates (Buckley and Pugh 1971). However, the culture filtrate of *E. purpurascens* showed strong inhibition of seed germination of *Setaria italica* (Pandey *et al.* 1982).

Some metabolic substances of *Epicoccum* have been found with antibiotic activity. Pigments A and B, renamed as epirodins A and B (Ikawa 1978), greatly restricted growth of *Bacillus megaterium* (Burge *et al.* 1976) and *Saccharomyces cerevisiae* (Ikawa 1978), and epicorazines A and B showed strong inhibition of *Staphylococcus aureus* (Baute 1978) as well as certain soil-borne plant pathogenic fungi (Brown *et al.* 1987). Flavipin strongly inhibited conidial germination of *Botrytis allii* Munn but was only weakly antibacterial (Bamford *et al.* 1961; Eka 1970b; Raistrick and Rudman 1956) and not effective in inhibiting other fungi tested by Bamford *et al.*(1961). Moreover, inhibition of germination of *Phytophthora cinnamomi* zoospores and *Pythium intermedium* oogonia by flavipin has also been reported (Brown *et al.* 1987). Both spore germination and mycelial growth of *Macrophomina phaseolina* and *Colletotrichum capsici*, the pathogens of fruit rot of cucurbits, were inhibited by culture filtrates of *E. purpurascens*, in which chrysin and p-hydroxybenzoic acid were thought to be active compounds (Singh 1985).

Influence by environmental factors

After 16 solid and 11 liquid media were tested, Eka (1970a) reported that certain complex media, e.g those containing malt extract or yeast extract, supported better growth and pigmentation than most synthetic media. Bonnell and Levetin (1981) found that nutrient requirements varied among strains of *E. purpurascens*. Starch was the preferred carbon source for a mycelial strain and glucose for a sporulating strain. From studies on red pigment production, Naumann (1912) concluded that maltose and starch were the most favourable carbohydrates and the production of the pigment was dependent on the presence of Mg^{++} and NO_3^-. However, Schol-Schwarz (1959) did not find any influence of Mg^{++} and NO_3^-. Growth and pigmentation were also influenced by the amount of the culture medium and the size of the culture vessel used. Large volumes of culture medium were not suitable for growth and pigmentation under certain conditions (Eka 1970a).

A series of transfers of various strains of *E. purpurascens* to oatmeal agar at different pH values showed that the pigmentation of mycelium, as well as that of the agar, was the most noticeable on alkaline oatmeal (pH 8.85), somewhat less on normal (6.85) and still less on acid oatmeal (3.1), that is, declining in intensity with decreasing pH (Schol-Schwarz 1959). Similarly, Eka (1970a) found that pigmentation in both mycelium and medium increased with increasing pH. However, growth as well as pigmentation were rather poor at pH 9. The optimum pH values for growth and pigmentation were 5.0 to 6.6 and 5.4 to 7.0, respectively.

An investigation using spore trap over a pea field indicated that recovery of *Epicoccum* spores was favoured by moderate temperatures and low relative humidity (Verma and Kamal 1982). *In vitro*, the optimum temperatures for growth and pigmentation of *Epicoccum* were found to be from 23 to 28 °C (Eka 1970). *Epicoccum* conidia are able to germinate at 92% RH (Webster and Dix 1960 and Hudson 1971).

The type and intensity of light and duration of exposure are

important for *Epicoccum* in sporulation and pigmentation although the fungus grows well both in light and in the dark (Eka 1970a). The influence of light on pigmentation seems to vary with the pigment. Eka (1970a) found that there was very little or no pigment formed in the dark, while better pigmentation appeared when the culture were exposed to light. Also, sunlight and fluorescent tubes were superior to incandescent bulbs for induction of pigment, and both growth and pigmentation were inhibited by very high light intensities (Eka 1970a). However, the production of red pigment was induced by very bright daylight (Schol-Schwarz 1959). In contrast, the production of carotenoids and ergosterol were inhibited by light (Gribanovski-Sassu and Foppen 1968). Ponnat *et al.* (1972) described *E. purpurascens* as a species with an absolute requirement for light in order to sporulate. However, this may be true for certain strains but not all. Kilpatrick and Chilvers (1981) found that only two of thirty-six isolates they examined failed to form mature conidia in the darkness, and about one-third of examined isolates produced abundant spores in total darkness within just one week. Moreover, in the colonies which did not sporulate within a week sporulation was successfully induced by intermittent exposure to white light above 4000 lux or 'black light' high in the near ultraviolet. Conidial germination of this fungus can be stimulated by treatment of lower doses of gamma and X-rays (5 and 1 Krad) (Salama *et al.* 1977).

Antagonistic activity and the use in biological control

Antagonism between *E. purpurascens* and a number of plant pathogenic fungi has been observed and its potential for biological control has been studied in some cases. *E. purpurascens* reduced infection of wheat by *Cochliobolus sativus* (*Helminthosporium sativum*) as successfully as *Trichoderma viride*. Both production of antibiotics and direct parasitism were demonstrated and were responsible for disorganization of the pathogen mycelium (Campbell 1956). Growth of *Rhizoctonia solani* was inhibited *in*

vitro by *E. purpurascens*, and hyphae of *R. solani* was found to be tightly coiled around and to be penetrated by the hyphae of the antagonist when cultures intermingled (Chand and Logan 1984; Wu 1976). Parasitism of *Fusarium culmorum* by *E. purpurascens* also has been observed (Wu 1976). Brown *et al.* (1987) discovered that mycelia of *Phytophthora* spp. and *Pythium* spp. antagonized by *E. purpurascens* were stunted and swollen, and hyphae of the antagonist coiled and penetrated those of the two pathogens where opposing cultures intermingled. Also, *E. purpurascens* was a strong antagonist *in vivo* against *Macrophomina phaseolina* and *Colletotrichum capsici*, and other pathogens of fruit rot of cucurbit that were tested, including *Botryodiplodia threobromae*, *C. lagenarium*, *Colletotrichum* state of *Glomerella cingulata*, *Curvularia ovoidae*, *Curvularia* state of *Cochliobolus lunatus*, *Drechslera* state of *Cochliobolus specifer* and *Sclerotium rolfsii* (Singh 1985). In studies of interactions between pathogenic and saprophytic fungi isolated from soybean roots and seeds, Manandhar *et al.* (1987) found that in dual-culture tests, *Epicoccum* was one of the most active antagonists toward the pathogens, which included *Cercospora sojina*, *Colletotrichum truncatum*, *Macrophomina phaseolina*, *Phomopsis sojae*, and *Septoria glycines*. In addition, various formulations of *E. purpurascens* (i.e. mycelial suspensions, culture filtrates and mycelial extracts) provided excellent control to *Gleosporium psidii*, the cause of guava anthracnose (Singh 1985). In Kumar and Singh's study (1983), culture filtrates of *E. purpurascens* showed strong inhibition of radial growth of *Alternaria solani*, the incitant of early blight of potato. *E. purpurascens*, as a component of the resident microflora of cabbage, reduced the incidence of infection of cabbage leaves by *Alternaria brassicicola*, especially if the antagonists were pre-inoculated onto the leaves (Pace and Campbell, 1974). *Epicoccum* is also a resident of peach twigs and flowers, and it inhibited *Monilinia laxa*, the causal organism of dry wilt of twigs and flowers on peach trees (*Prunus persica*), both *in vitro* and *in vivo* under laboratory conditions (Melgarejo and M.-

Sagasta 1984; Melgarejo *et al.* 1985). Under field conditions, treatments with *Epicoccum* resulted in a significant reduction of *M. laxa* infection in both spring and fall (Melgarejo *et al.* 1986). An isolate of *E. purpurascens* from the leaf surface of lettuce reduced germ tube elongation of *Sclerotinia sclerotiorum* ascospores on leaf discs of lettuce and greatly decreased infection of lettuce by *S. sclerotiorum* in growth chamber tests (Mercier and Reeleder 1987). Boland and Inglis (1989) examined several fungal isolates from bean and rapeseed flowers for antagonists of *S. sclerotiorum,* and found *E. purpurascens* was one of the most disease-suppressive fungi.

E. purpurascens has several attributes beneficial to candidate biocontrol agents. *Epicoccum* spp. have pigmented multicellular conidia, such features are considered as protective mechanisms against desiccation and strong sunlight (Nicot 1960). Pugh and Buckley (1971) found that *Epicoccum* appeared to resist high levels of ultraviolet light (253.7 nm) and a high survival rate of spores was obtained when spores on PDA plates were exposed to ultraviolet light for 35 min. Conidia of *Epicoccum* can germinate at a relative humidity as low as 92%, and under favourable humidity (100% RH) grows faster than the secondary colonizers of the plant surface, and had a shorter latent period before germination (0-3 h) than competitors (Hudson 1971). The ability to utilize cellulose is often regarded as essential for saprophytic fungi. *Epicoccum* was one of the fungi with cellulolytic activity (Siu, 1951). Also, cellulase and β,1-3 glucanase activities were detected in cultures of *E. purpurascens* grown on media containing hyphal wall material from *Phytophthora* or *Pythium* spp. as the sole carbon source (Brown et al. 1987). The lack of any requirement for exogenous nutrients for germination of *Epicoccum* may also be considered as a beneficial attribute (Hudson 1971).

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3. Selection of Strains of *Epicoccum purpurascens* for Tolerance to Fungicides and Improved Biocontrol of *Sclerotinia sclerotiorum*

Introduction

Efficiency of biocontrol agents can be increased by screening for more effective isolates, or by genetically improving the original strain (Baker 1986). Cullen and Andrews (1984b) selected more effective strains of *Chaetomium globosum* Kunze for control of the apple scab pathogen, *Venturia inaequalis* (Cooke) Winter. Papavizas *et al.* (1982) selected strains of *Trichoderma* Pers. for tolerance to benzimidazole fungicides by irradiation of conidia with ultraviolet (UV) light; some resulting strains were more effective than the original cultures in suppressing damping-off caused by *Pythium ultimum* Trow. Selection or improvement of strains with respect to tolerance to fungicides and high production of antifungal compounds is important in improving effectiveness of biocontrol agents (Cullen and Andrews 1984a).

An iprodione-tolerant strain of *Epicoccum purpurascens* Ehrenb. ex Schlecht. (syn. *E. nigrum* L.) was effective in controlling white mold of bean *(Phaseolus vulgaris* L.), caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, under greenhouse and field conditions (Zhou and Reeleder 1989). Resistance to only iprodione limits the utilization of *E. purpurascens* since other fungicides are also used in control of white mold. Moreover, strains with greater *in vitro* inhibition of *S. sclerotiorum* should be more effective in controlling white mold of beans and diseases of other crops. Thus, the objectives of this study were to produce and select strains of *E. purpurascens* which were tolerant to several commonly used fungicides and to test their inhibition of *S. sclerotiorum in vitro* and *in vivo*.

Materials and Methods

Preparation of fungicide-amended medium

Potato dextrose agar (PDA; Difco, Detroit MI) or malt agar (MA; Difco, Detroit MI) was prepared and autoclaved at 121°C and 104 kPa for 15 min before it was placed in a 50°C water bath. Fungicide was first suspended in 95% ethanol, then 1 ml of the suspension was added to 100 ml of medium. Appropriate amounts of fungicides were added to the 95% ethanol to obtain the desired active ingredient concentrations for the experiments described below. Fungicides used were: iprodione (Rovral 50 WP, May & Baker Canada Inc., Mississauga, Ont.); benomyl (Benlate 50 WP, E.I. DuPont de Nemours & Co. Inc., Wilmington, DE); mancozeb (Manzate 200 80WP, E.I. DuPont de Nemours and Co. Inc., Wilmington, DE); and vinclozolin (Ronilan 50 WP, BASF Wyandotte Corporation, Parsippany, NJ). The fungicide-amended medium was mixed using a magnetic stirrer and dispensed (10 ml) into 100 x 15 mm standard plastic culture dishes, and left overnight in a laminar flow hood to accelerate evaporation of ethanol. Fungicide-free control media were amended with 1 ml of ethanol.

Generation of initial fungicide-tolerant strains of *E. purpurascens*

The wild-type isolate of *E. purpurascens* (MACF-032) was recovered from a lettuce leaf (Mercier and Reeleder 1987) and stored on MA slants at 4°C. Conidia were collected from cultures growing on MA at room temperature (20-24°C), and adjusted to 10^6 conidia/ml of distilled sterile (DS) water. An aliquot of the conidial suspension was placed in the bottom of a plastic petri dish (9 cm diam.) and exposed to UV irradiation (range of wavelength 200-280 nm, peak at 254 nm, 70 amps; Minerallight lamp, Model C81, UVP Inc., San Gabriel, CA) for 8-10 min. The lamp was 20 cm above the surface of the suspension. This exposure time resulted in a killing rate of 80% of conidia. The treated conidial suspension was transferred to fungicide-amended MA, at a rate of 0.25 ml suspension for each dish. Media contained either iprodione at 5, 10, 15 or 20 µg/ml, or mancozeb at 10, 20, 30 or 40 µg/ml. After 7 to 14 days at room temperature,

individual colonies were selected, and assessed for size, sporulation and production of pigmentation. Selected colonies were transferred to fungicide-free medium. Conidial suspensions prepared from these cultures were placed on medium amended with a higher concentration of fungicide. Further selections of surviving colonies then were made. Cultures were stored on MA slants at 4°C.

Assessment of *in vitro* inhibition of *S. sclerotiorum* by *E. purpurascens*

A procedure similar to that of Fokkema (1973) was used. Ten to 15-day-old PDA cultures of *E. purpurascens* and 5 to 7-day-old PDA cultures of *S. sclerotiorum* were used as sources of inoculum. A disk of *E. purpurascens* (5 mm in diam.) was placed 20 mm from the edge of a PDA dish (100 x 15 mm containing 15 ml of medium). Five days later, a disk of *S. sclerotiorum* was placed 50 mm away from the *Epicoccum* disk and cultures were incubated in the dark for an additional 7 days at 20°C. The width of the inhibition zone between the two colonies then was recorded. The percentage of inhibition of radial growth of *S. sclerotiorum* was calculated by comparing radial growth of the colony directly opposite the *E. purpurascens* colony with radial growth of that part of the colony not adjacent to *E. purpurascens*. Four replicate dishes were prepared for each strain.

Selection of strains of *E. purpurascens* for fungicide tolerance and increased inhibition of *S. sclerotiorum*

E. purpurascens (strain M-20-A) was cultured on MA and placed under near UV light (fluorescent lamp with two 15W Blacklight Blue F15T8 tubes, GTE Products Corporation, Danvers, MA) to promote sporulation (Kilpatrick and Chilvers 1981) for 10-20 days. Conidia then were washed from the culture with DS water and 2 ml of the conidial suspension were transferred to wheat seed (WS) medium (Zhou and Reeleder 1989) for production of mass inoculum. The conidia from the WS medium were removed by immersing seeds in DS water, stirring with a glass rod and filtering the suspension through double layers of cheese cloth to remove mycelium and seeds. Conidia then were washed two times with DS water and centrifuged for 20

min. Conidial suspensions (10^6 conidia/ml DS water) were applied with a pump sprayer onto the surface of fungicide-amended (iprodione or vinclozolin at concentrations of 10, 50, 100, 500, 1500 and 2000 µg/ml with six dishes per concentration) MA. Conidial germination and colony growth were assessed during the first 24-72 h after application. Single-conidium colonies were selected and transferred to fungicide-free PDA, then assessed as described previously for their ability to inhibit *S. sclerotiorum*. Cultures superior in inhibition of *S. sclerotiorum*, and in sporulation and growth rate were transferred to media amended with higher, or the same, concentrations of fungicide. These procedures were repeated at least three times. The resulting cultures then were used to select for resistance to the other fungicide (iprodione or vinclozolin), using similar procedures.

In vitro* comparisons of strains of *E. purpurascens

Fungicide tolerance assessment:

Three characters were used: percentage of germination, length of germ tube, and radial growth. For the germination test, conidia of *E. purpurascens* were removed with DS water from WS medium, washed twice by centrifugation for 20 min and suspended in DS water at 10^6 conidia/ml. Two percent malt extract (ME; Difco, Detroit, MI) was added to the conidial suspension for some treatments (Table 3.3). Conidial suspensions with or without ME were mixed with equal amounts of fungicide suspensions so the final suspension contained 5 x 10^5 conidia/ml, 1% ME and a fungicide concentration of 0, 10, 50, 100, 500, or 1000 µg/ml. A completely randomized design with three replications was used. Germination was assessed on a ceramic ring slide (76 x 51 x 2.3 mm slide with 12 raised ceramic rings, I.D. 14 mm; Clay Adams, Division of Becton, Dickinson and Company, Parsippany, NJ). A 30 µL conidial suspension was added to each ring, and slides were placed in a closed plastic box with moistened filter paper, and incubated at 20°C without light for 6 h. Conidial germination was stopped by adding one or two drops of lactophenol in cotton blue.

Germination then was observed microscopically (x250). For each replicate ring, at least 100 conidia were observed for germination and the germ tube lengths of 20 germlings were determined. A conidium was considered germinated when it had a germ tube longer than its radius. For the radial growth test, strains were cultured on fungicide-free MA for 8-10 days. The fungicide-amended and control (no fungicide) MA dishes were centrally seeded with a disk (5 mm diam.) from these cultures. Media were prepared as previously described. Cultures were incubated at 20 ± 2°C and the radii of colonies were measured at intervals of 2 days.

Inhibition test:

The procedures were as described previously.

Growth and sporulation:

Six strains of *E. purpurascens* were cultured on MA for 10 days and then disks (5 mm in diam.) were transferred to the center of fresh MA dishes (100 x 15 mm with 15 ml medium). Radial growth was measured at 3, 6, 10 and 14 days after seeding dishes. Sporulation was assessed by classifying each culture into one of four groups: -, no conidia; +, less than 1/4 colony covered with conidia; ++; more than 1/4 but less than 1/2 covered with conidia; +++, more than 1/2 but less than 3/4 covered with conidia; and ++++, more than 3/4 covered with conidia.

Evaluation of ability of strains of *E. purpurascens* to control *S. sclerotiorum* in the greenhouse

Beans (cv. Strike) were produced as described previously (Zhou and Reeleder 1989). Five strains (Table 3.2) of *E. purpurascens* were cultured on WS under near UV light for 20 days. Conidia were washed out of culture flasks with DS water, suspended in 0.01% Tween 80 (J.T. Baker Chemical Co., Phillipsburg, NJ), and adjusted to 10^5 conidia/ml (Appendix 1.3). Ascospores of *S. sclerotiorum* were produced as described previously (Mercier and Reeleder 1987) and suspensions were adjusted to 10^6 ascospores/ml phosphate buffer (0.01 M, pH 6.0) with 0.01% Tween 80. Each *E. purpurascens* strain was considered as a treatment. Control (no *Epicoccum*)

plants were sprayed with 0.01% Tween 80. A completely randomized block design with three replicates was used. Plants were held in a mist chamber and spray applications were done as previously described (Zhou and Reeleder 1989). *E. purpurascens* was applied when 80% of the plants had at least one open flower and again 4 days later. All plants (including controls) were inoculated with ascospores of *S. sclerotiorum* 24 h after the second application. The number of lesions on each plant was determined 6 days after inoculation with ascospores. The percentage of diseased plant tissue (leaves and stems), percentage of pods with rot and fresh weight of pods in each treatment (3 plants) were recorded 14 days after inoculation.

Data calculation and analyses

Except for the tests involving shortwave UV treatment and initial strain selections on fungicide-amended agar, all experiments were carried out at least twice. The data from *in vitro* tests were analyzed using the F-test and treatment means were separated using Duncan's multiple range test (SAS Institute Inc. 1987). To calculate percentage inhibition in the tolerance tests, data were used in the formula [100 x (N-T)/N] (where N is the value for non-fungicide control treatment and T is the value for the appropriate concentration of fungicide). These percentage values were normalized using an arcsine transformation and then analyzed by the F-test. Treatment means were separated by Duncan's multiple range test or an LSD value was calculated (P=0.05). EC_{50} (effective concentration required for 50% inhibition) values were obtained using the Probit procedure (SAS Institute Inc. 1987) with percentage inhibition data. Data from greenhouse trials for the number of lesions were analyzed with the Friedman analysis of variance and the associated multiple comparison procedure (Daniel 1978). Other variables from greenhouse trials were analyzed with the F-test and separated by Duncan's multiple range test.

Results

Generation of fungicide-tolerant strains

Following treatment with shortwave UV light, and culture on fungicide amended media, several new strains were obtained. They were designated according to the source of the culture: R-10-A, R-10-B, and R-10-C (from media with 10 µg iprodione/ml), and M-20-A and M-30-C (20 and 30 µg mancozeb/ml, respectively).

In vitro inhibition *S. sclerotiorum* by *E. purpurascens*

All tested strains of *E. purpurascens* were capable of inhibiting radial growth of *S. sclerotiorum* on PDA. Strain M-20-A exhibited greater radial growth inhibition of *S. sclerotiorum* than the wild-type isolate (Table 3.1). All new strains were superior to the wild-type isolate with respect to the width of the inhibition zone. M-20-A appeared to produce more diffusible pigmented compounds than other strains and it was used as the source from which additional strains were selected.

Selection of strains of *E. purpurascens* for higher fungicide tolerance and increased inhibition of *S. sclerotiorum*

When conidia of strain M-20-A were sprayed onto fungicide-amended MA, 4.3% (1.2 - 7.6%) of the conidia germinated on plates with 500 µg iprodione/ml and 22.4% (14.9 - 32.8%) germinated on plates with 1000 µg vinclozolin/ml after 24 h at 20°C. Following evaluations of fungicide tolerance and inhibition of *S. sclerotiorum* on fungicide-free agar, sporulation ability on WS medium was recorded. Several strains were selected using this procedure. Strain R4000 was first recovered from MA with 1000 µg iprodione/ml and then transferred to MA with 2000 µg iprodione/ml. It sporulated very well on WS medium. Strains 2-A and 7-A were recovered from MA with 1000 µg vinclozolin/ml and transferred to MA with 1500 µg vinclozolin/ml. Subsequently, conidia of these isolates were sprayed onto and recovered from MA with 500 µg iprodione/ml, followed by transfer to MA with up to 1000 µg iprodione/ml. Strain 2-A was a poor inhibitor of *S. sclerotiorum* (Table 3.2). Strain 16-B was

Table 3.1. Width of inhibition zone (IZ) and percent inhibition of radial growth of *Sclerotinia sclerotiorum* in the presence of *Epicoccum purpurascens*[x]

Strain of *E. purpurascens*	Radial growth inhibition (%)[y]	Width of IZ (mm)[z]
M-20-A	45.0 a	5.3 a
R-10-B	42.3 ab	3.8 ab
M-30-C	41.5 ab	3.0 b
R-10-A	37.1 b	3.2 b
R-10-C	37.5 b	2.8 b
Wild-type	37.6 b	1.1 c

[x]Data were collected 6 days after seeding culture dishes with *S. sclerotiorum*. Values listed are means from pooled data of two trials (total of 8 replicates per strain) and are not significantly different from others in the same column if followed by the same letter (Duncan's multiple range test, P=0.05).

[y]Percent inhibition was calculated by 100 x (R1-R2)/R1, where R1 is maximum radius of the colony of *S. sclerotiorum* and R2 is the radius of that part of the colony of *S. sclerotiorum* directly opposite the colony of *E. purpurascens*.

[z]Width of inhibition zone (IZ) is the minimum distance between the edges of the two fungal colonies.

Table 3.2. Comparison of strains of *Epicoccum purpurascens* for inhibition of *Sclerotinia sclerotiorum in vitro* and *in vivo*

Strains[t]	*In vitro* [v]		*In vivo* [w]			
	Inhibition (%)	Width of IZ (mm)	Number of lesions[x]	Diseased tissue (%)[y]	Pod rot (%)[z]	Yield (g)[z]
Wild-type	52.4 a	1.9 a	-	-	-	-
2-A	49.7 a	1.6 a	6.7 ab	41.7 b	32.3 ab	30.6 b
M-20-A	54.4 a	3.4 b	5.3 ab	52.2 a	34.7 a	33.2 b
R4000	55.0 a	3.8 bc	3.0 b	15.2 d	19.7 bc	54.3 a
7-A	53.8 a	4.9 c	4.0 b	31.1 c	8.3 c	50.6 a
16-B	54.9 a	7.6 d	3.0 b	16.1 d	9.7 c	55.7 a
(Control[u])			9.0 a	54.4 a	43.0 a	33.6 b

[t]All strains, except the wild-type and M-20-A, were derived from M-20-A.

[u]Control plants were not sprayed with *E. purpurascens* but were sprayed with *S. sclerotiorum*.

[v]The test was carried out on PDA and data were collected six days after inoculating dishes with *S. sclerotiorum*. Percentage of inhibition was calculated using the formula 100 x (R1-R2)/R1; where R1=maximum radium of the colony of *S. sclerotiorum* and R2=the radius of that part of the colony of *S. sclerotiorum* directly opposite the colony of *E. purpurascens*. Width of IZ (inhibition zone) is the minimum distance between the two fungal colonies. Values in a column are pooled means of two trials (eight replicates) and are not significantly different if followed by the same letter (Duncan's multiple range test, P=0.05).

[w]The tests were carried out in the greenhouse. Applications of *E. purpurascens* spores were made when more than 80% of plants had at least one open flower and again four days later. Controls were treated with

(Table 3.2 continued)

0.01% Tween 80. Ascospores of *S. sclerotiorum* were applied to all plants one day after the second application of *E. purpurascens*. Data were collected from three plants in each treatment and values are means of three replicates.

[x]Data collected six days after inoculation with *S. sclerotiorum*. Values followed by the same letter are not significantly different. (Freidman analysis of variance and associated multiple comparison test; experimentwise error rate = 0.75).

[y]Percentage of leaf and stem area diseased, assessed 14 days after inoculation. Values in a column followed by the same letter are not significantly different according to Duncan's multiple range test, P=0.05.

[z]Values in a column followed by the same letter are not significantly different according to Duncan's multiple range test, P=0.05.

first recovered from MA with 500 µg iprodione/ml, then sprayed onto and recovered from media with 1000 µg vinclozolin/ml, followed by transfer to media with up to 1500 µg vinclozolin/ml.

Comparison of six strains of *E. purpurascens*

Fungicide tolerance

The six strains of *E. purpurascens* were significantly different in tolerance to iprodione and vinclozolin (Table 3.3). Strain R4000 had, overall, the highest tolerance to iprodione of all strains when all characters were considered. Strain 2-A had greater tolerance than the wild-type isolate, M-20-A or 16-B, when percentage of conidial germination was compared. For radial growth, 2-A, R4000, 7-A and 16-B were not significantly different from one another, but did possess higher tolerance than the wild-type or M-20-A.

When tested on vinclozolin, strain 2-A had more tolerance than the wild-type isolate or M-20-A, when all characters were considered. Strains R4000 and 16B had more tolerance than the wild-type isolate in terms of conidial germination but not for radial growth. Tolerance ofstrain 7-A was also greater than the wild-type isolate for all characters except length of germ tube in the treatment without ME.

E. purpurascens generally had more tolerance to vinclozolin than to iprodione. Tolerance to benomyl was similar for all strains, however, the tolerance for a given strain to benomyl varied with the character measured. For conidial germination, EC_{50} values ranged from 792 to 1714 µg/ml, whereas those for radial growth EC_{50} values were less than 10 µg/ml. Addition of ME to the spore suspension generally did not result in increased tolerance to fungicides (as measured by germination and germ tube length) although tolerance did increase in some fungicide-strain combinations (Table 3.3). These were R4000 in iprodione, M-20-A and 7-A in vinclozolin (for percentage of conidial germination), and 7-A in vinclozolin (for length of germ tube). However, the overall percentage of conidial germination in treatments with ME was significantly increased

TABLE 3.3. Fungicide tolerance (EC_{50}) in selected strains of Epicoccum purpurascens[a]

	Spore Germination						Length of Germ Tube						Radial growth		
	Iprodione		Vinclozolin		Benomyl		Iprodione		Vinclozolin		Benomyl		Iprodione	Vinclozolin	Benomyl[a]
Strain	M[y]	O[y]	M	O	M	O	M	O	M	O	M	O			
Wild-type	10 a	10 a	786 a	664 ab	1550	910	<10 a	<10 a	306 a	246 a	601	447	28 a	402 a	<10
M-20-A	21 ab	20 ab	1365* b	231* a	1556	1168	11 a	12 a	323 a	176 a	497	559	20 a	347 a	<10
2-A	274 c	99 bc	1708 b	887 bc	1714	873	37 a	35 a	906 b	921 b	666	700	291 b	994 b	<10
R4000	1415* d	753* c	2279 b	1134 c	837	1407	699 b	824 b	979 b	1033 b	486	531	378 b	470 a	<10
7-A	116 bc	52 ab	1635* b	650* bc	1145	792	21 a	45 a	964* b	414* a	595	421	428 b	740 b	<10
16-B	25 ab	-	2468 b	1169 c	981	1095	24 a	-	939 b	1006 b	521	561	277 b	493 a	<10
Pr > F	0.0001	0.0001	0.0001	0.0001	0.8627	0.7129	0.0001	0.0001	0.0001	0.0001	0.1963	0.2455	0.0001	0.0001	-

[a]Data used for analysis of variance were percentages of inhibition calculated by the formula 100 x (N-T)/N; where N=value of measurement for control (no fungicide) treatment, T=value of measurement from different concentrations of fungicide treatment (10, 50, 100, 500 and 1000 µG/mL). Values in each column followed by the same letter are not significantly different (Duncan's multiple range test, P=0.05). Values were obtained with the PROBIT procedure using data of percentage of inhibition. EC_{50}: Effective concentration for 50% inhibition.

[b]M indicates that 1% malt extract was added to the spore suspension; O indicates no malt extract added * indicates that differences between M and O for that fungicide-strain combination are significant (*) based upon percentage of inhibition data (LSD, P=0.05).

[c]No growth of any strain occurred in cultures with 10 µG benomyl/mL.

compared to those without ME (Appendix 1.1). Similarly, with some exceptions, increases in germ tube length occurred when ME was added.

In vitro* inhibition of *S. sclerotiorum

Strains did not differ in inhibition of radial growth of *S. sclerotiorum*. However, strains M-20-A, R4000, 7-A and 16-B produced significantly wider zones of inhibition than the wild-type isolate (Table 3.2). The best strain appeared to be 16-B, followed by 7-A. The inhibition abilities of strains R4000 and M-20-A were not as great, but they were significantly better than the wild-type isolate. Strain 2-A exhibited low inhibition, similar to that of the wild-type isolate.

Radial growth and sporulation

Radial growth varied significantly among strains (Table 3.4). The wild-type isolate, R4000 and 7-A grew faster than 2-A and M-20-A. Strain 16B grew more slowly than the wild-type but faster than 2-A and M-20-A. The wild-type sporulated very poorly and only a few sporodochia were obtained from a PDA culture held under near UV or strong white light (300 $\mu E/m^2/s$) for more than one month. R4000 sporulated very well on both agar media (PDA and MA) and WS medium. For strains M-20-A, 2-A and 16-B, spores were produced on agar media but sporulation on WS medium was erratic.

Evaluation of strains of *E. purpurascens* for control of *S. sclerotiorum* in the greenhouse

Although no significant differences were found among strains regarding the number of lesions, differences were noted for other parameters (Table 3.2). For percent diseased tissue, percent pod rot, and yield, treatments with strains 16-B, R4000 and 7-A were significantly different from the control (Table 3.2). Correlations between tests *in vitro* and *in vivo* were indicated by their correlation coefficient. The correlation coefficient between width of inhibition zone and percentage of diseased plant tissue was -0.5361 ($p=0.0394$). The wild-type isolate was not used in this experiment since an insufficient number of spores was produced in culture.

Table 3.4. Comparison of radial growth and sporulation in strains of *Epicoccum purpurascens*.

Strains	Radial growth (mm/day)[y]	Sporulation[z]
R4000	2.6 a	++++
7-A	2.5 ab	+++
Wild-type	2.5 ab	-
16-B	2.3 b	++
M-20-A	2.0 c	++
2-A	1.5 d	+

[y]Means of three replicates. Radial growth per day was calculated using the formula: $(R_{10} - R_3)/7$ where R_{10} is radial growth after 10 days and R_3 = radial growth after 3 days. Values in a column followed by the same letter are not significantly different, Duncan's multiple range test (P=0.05).

[z]Sporulation was rated 10 days after seeding of PDA medium, -, no sporulation observed; +, less than 1/4 of the colony covered with conidia; ++, more than 1/4 but less than 1/2 covered with conidia; +++, more than 1/2 but less than 3/4 covered with conidia; and ++++, more than 3/4 covered with conidia.

Discussion

These studies show that it is possible to obtain strains of *E. purpurascens* with improved biocontrol characteristics. Five new strains differed from the wild-type in various respects. M-20-A, recovered after irradiating the wild-type with shortwave UV light, was ameliorated in sporulation and inhibition ability although its tolerance to iprodione and vinclozolin was not great. However, improvement in sporulation made further studies feasible and made it possible to consider utilizing *E. purpurascens* as a biocontrol agent in field trials (Zhou and Reeleder 1989). High levels of tolerance to fungicides were obtained by selection following culturing on fungicide-amended media. After exposure to shortwave UV light, strains recovered from fungicide-amended media (R-10-A, R-10-B, R-10-C, M-20-A and M-30-C) did not have high tolerance to iprodione or mancozeb. Strains with much higher tolerance to iprodione were obtained during the second selection when higher concentrations of fungicides were used (Table 3.3). In fact, strains with high tolerance to iprodione were obtained from not only M-20-A but also R-10-B, although all strains used here for further studies were from M-20-A. However, some strains were obtained from R-10-B cultured on media with 2000 µg iprodione/ml. Tolerance to iprodione and vinclozolin varied greatly among the tested strains but in all cases resistance to benomyl was low, based upon results of radial growth assays. This is probably because benomyl-amended media were not used in the selection procedures and indicates that, in *E. purpurascens*, tolerance to iprodione or vinclozolin does not confer tolerance to benzimidazoles. The ranking of strains with respect to fungicide tolerance varied with the character being considered.

The EC_{50} values obtained for conidial germination data generally were higher than those from germ tube and radial growth data. However, there was more variation in spore germination data than in radial growth data. In addition, some strains exhibited decreased germination of conidia over time. Thus radial growth was a more reliable measure of inhibition than

conidial germination, although periods of incubation for radial growth must be considered carefully since growth rates will vary with the age of the culture. The addition of ME to spores did not affect the percentage of inhibition of conidial germination but it did affect the percentage of germination. Increase in germination following addition of nutrients resulted in more uniform and reproducible responses to the fungicides. Thus, careful consideration should be given to experimental protocols used in assessing biocontrol agents for fungicide tolerance. Fungicide tolerant strains obtained during these studies have been maintained on fungicide-free media, with frequent transfer, for over 2 years without any loss in tolerance. In this case, fungicide tolerance appears to be a stable trait.

The ability to inhibit *S. sclerotiorum* also was improved for the new strains. R4000, 16-B and 7-A produced wider inhibition zones and exhibited improved performance in control of white mold in the greenhouse than either the wild-type or M-20-A. Improved disease control in the field may be obtained using strains with greater *in vitro* inhibition ability. Continued selection may yield further improvements in this character. Increased widths of inhibition zones are likely due to increased production of antifungal compounds by *E. purpurascens*. Some of these compounds are pigmented and pigment production could be used as a preliminary screen for production of antifungal products by *E. purpurascens*. Previous results have suggested that biocontrol of *S. sclerotiorum* by *E. purpurascens* is related to pigmented antifungal compounds (Mercier and Reeleder 1987). There was some correlation between inhibition zones and *in vivo* greenhouse tests, although the correlation coefficient was not very high. This also has been noted elsewhere (Cullen and Andrews 1984b) and it is clearly important to consider other factors such as plant colonization ability when selecting strains for use in the field.

Inhibition of radial growth was not always associated with width of the inhibition zone and, in some cases, the relationship between these two

parameters changed over time for particular strains. These inconsistencies cannot be readily explained but have been reported for other antagonists (Fokkema 1973). The width of the inhibition zone seems to be a more reliable predictor of *in vivo* performance than radial growth inhibition. Fokkema (1973) pointed out that inhibition zones do not necessarily arise from the production of antifungal products by the antagonist. Thus, reliance upon inhibition zone width may not be justified unless there is other evidence from *in vivo* or other tests suggesting that antibiosis is a factor in the activity of a particular antagonist. This is the case for *E. purpurascens* (Mercier and Reeleder 1987).

Greenhouse trials resulted in levels of control similar to those in field trials (Zhou and Reeleder 1989). Integrated control of plant diseases with a fungal agent and a fungicide becomes feasible when strains with high fungicide tolerance are available. With this combination, less chemical and fewer sprays may be required for adequate disease control (Dubos *et al.* 1982). Better disease control may be obtained than when either component is used alone (Carter and Price 1974, 1975). Although our previous field data (Zhou and Reeleder 1989) suggested that combining *E. purpurascens* and iprodione did not provide increased control, it is possible that the combinations of other fungicides and strains tolerant to those fungicides may improve control of *S. sclerotiorum*. Development of strains with improved biocontrol characteristics is a key to the practical use of these microorganisms in disease management. However, other factors such as plant colonization ability and formulation of the biocontrol agent for use in the field are important issues that need to be addressed.

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Connecting Text

Five new mutant strains of *E. purpurascens* were selected. M-20-A, recovered after irradiating the wild type with shortwave UV-light, was improved in sporulation and ability to inhibit *S. sclerotiorum*. However, its tolerance to iprodione and vinclozolin was not improved. Strains R4000, 16-B and 7-A, selected from M-20-A, inhibited *S. sclerotiorum in vitro* more than either the wild type or M-20-A, and exhibited improved control of white mold of bean in the greenhouse compared with M-20-A. Strain R4000 had the highest tolerance to iprodione and the highest degree of sporulation, and was used in the further studies.

E. purpurascens showed some activity in the suppression of *S. sclerotiorum in vitro* and in the greenhouse (Section 2 and 3). There have been no previous reports of *E. purpurascens* or other antagonists controlling white mold under field conditions. A microorganism may be an outstanding antagonist *in vitro* or *in vivo* under controlled conditions but may not show activity in nature. Evaluation in the field is a crucial step in development of a biocontrol agent. Also, integrating biological control with chemical control may result in better disease control than either of them used alone. Therefore, the next section describes the evaluations of *E. purpurascens* conidia, iprodione, and their combination to control white mold of snap bean under greenhouse and field conditions.

4. Application of *Epicoccum purpurascens* Conidia to Control White Mold of Snap Bean

Introduction

White mold, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most common and destructive diseases of bean (*Phaseolus vulgaris* L.) in temperate regions (Steadman 1983). Epidemics usually are initiated by ascospores (Steadman 1983) that land on senescing or dead flower petals. After colonizing the flower, mycelium can infect adjacent pods, leaves or stems. Thus, white mold epidemics generally occur during or after flowering of the crop (Steadman 1983).

Hunter *et al.* (1978) reported that spraying blossoms with fungicide effectively controlled the disease, but no control was obtained when all aboveground plant parts except blossoms were covered with fungicide. This suggests that white mold can be controlled by protecting blossoms from colonization by ascospores of *S. sclerotiorum*. Colonization of blossoms by organisms antagonistic to *S. sclerotiorum* could suppress epidemics of white mold (Mercier and Reeleder 1987a). Such biocontrol agents could augment or replace fungicides used in management of this disease.

Epicoccum purpurascens Ehrenb. ex Schlecht. (syn: *E. nigrum* Link) is a saprophytic fungus commonly found on plant surfaces (Mercier and Reeleder 1987b). It inhibited elongation of germ tubes of ascospores *in vitro* and reduced infection of lettuce by *S. sclerotiorum* in growth chamber tests (Mercier and Reeleder 1987a). Our objective in these studies was to determine the efficacy of *E. purpurascens* in control of white mold of snap bean under greenhouse and field conditions. A portion of these results has been reported previously (Zhou and Reeleder 1987).

Materials and Methods

Conidial production

Conidia of an isolate of *E. purpurascens*, recovered from the surface of a lettuce leaf (Mercier and Reeleder 1987b), were treated with short-wave ultraviolet irradiation. Irradiated conidia were placed on an iprodione-amended medium and strains that grew were assayed for tolerance to iprodione. One of the resulting strains (R4000), tolerant to 2000 µg/ml iprodione (compared to 10 µg/ml for the original isolate), was used in all experiments and was maintained under sterile mineral oil on potato dextrose agar (PDA) (Difco, Detroit, MI) slants at 8 °C.

For all experiments, conidia of *E. purpurascens* were obtained from 14 to 20 day-old cultures grown on a wheat seed medium held at 22 ± 2 °C. To prepare the medium, cleaned wheat seeds were soaked in water for 12 hr at 40 °C (or boiled for 20 min), then 30-40 ml of the seeds in a 125-ml Erlenmeyer flask were autoclaved at 121 °C, 104 kPa, for 1 hr. After flasks had cooled, conidia from PDA slant cultures or wheat seed cultures were added to the flasks.

Ascospores of *S. sclerotiorum* (isolate MACF-152) were produced and maintained as described previously (Mercier and Reeleder 1987a).

Preparation of Spore Suspensions

Conidia of *E. purpurascens* were washed from wheat seed cultures with distilled water containing 0.01% Tween 80 (J.T. Baker Chem. Co., Phillipsburg, NJ). Mycelium and wheat seeds were removed by filtering through three layers of cheesecloth. For field tests, the filtrate was adjusted to 1 x 10^6 conidia/ml with distilled water containing 0.01% Tween 80. For greenhouse tests, conidia were washed twice by centrifugation before the concentration was adjusted to 1 x 10^6 conidia/ml. For some treatments, suspensions of *E. purpurascens* were amended with 1% malt extract (Difco, Detroit, MI), 1% potato dextrose broth (Difco, Detroit, MI) and/or iprodione (Rovral 50WP) before application.

Ascospores of *S. sclerotiorum* (10^6 ascospores/ml) were suspended in phosphate buffer (0.01 M, pH 6.0) containing 0.01% Tween 80. The concentrations of *E. purpurascens* conidia and *S. sclerotiorum* ascospores were determined using a hemacytometer.

Greenhouse Tests

Snap beans (cv. Strike) were seeded in Pro-Mix BX (Les Tourbirès Premier Ltée, Rivière du Loup, Quebec) in pots 130 mm diameter to provide 10 replications of one plant per pot for each treatment. Plants were fertilized with a 0.1% NPK (20-20-20) solution every second week after emergence. Greenhouse temperatures were maintained at 24 ± 2 °C with a 14 hr photoperiod. Treatments consisting of conidial suspensions of *E. purpurascens* and/or nutrient solutions (see Table 4.1 for treatment combinations) were applied when all plants had at least one open flower and again four days later. Tween 80 (0.01%) was added to all treatments as a wetting agent.

Plants were inoculated with ascospores of *S. sclerotiorum* 24 hr after the second treatment. For each inoculation, plants were sprayed with a hand-held pump sprayer until runoff (about 15 ml per plant, applied over the entire surface), permitted to air-dry for 1 hr, and then were placed in a plastic mist chamber in a greenhouse. Plants were misted each night from 8 p.m. to 8 a.m. with an electric atomizer; plastic sheets were removed from the sides of the chamber to permit air circulation during the day. This regime was continued until disease symptoms appeared. No mist was provided after that time and plastic sheets were removed from the sides of the chamber. The number of lesions and the percentage of diseased leaf and stem area on each plant were determined six and 14 days, respectively, after application of ascospores. The experiment was performed twice.

Field trials

A randomized complete block design with four replications of each treatment was used for field trials in 1987 and 1988. Snap beans (cv.

Strike) were seeded at a rate of 20 seeds per meter of row with 0.5 m between rows. Each plot consisted of four rows, 3.2 m long. Treatments were applied during the flowering period (which begin 36 to 41 days after planting) and, except where indicated, all plants were inoculated with ascospores of *S. sclerotiorum* one day after the last treatment. All inoculations were performed with a hand-held compressed air sprayer in the evening (7 to 10 p.m.), 2-3 hr after 1 hr of sprinkler irrigation. Approximately 1.5 l of suspension was used for each plot, and care was taken to ensure that flowers and buds were covered although stem and leaf tissue was also sprayed.

During the first week after inoculation, plots were sprinkler-irrigated for 30 min every second day to ensure high levels of disease. The number of lesions on 20 plants in each plot was determined six days after inoculation. Pod weight, disease incidence, disease severity (percentage of leaf and stem area diseased), and the percentage of infected pods were determined at harvest from two 2-m sections of each of the two middle rows of each plot. All trials were located at the Macdonald College Research Station, Ste-Anne-de-Bellevue, Quebec, on land that was cropped to lettuce in 1985 and 1986. The lettuce plants had been artificially inoculated with *S. sclerotiorum* in both years.

In 1987, two trials with similar treatments were seeded on 1 June and 2 July and harvested on 6 August and 3 September, respectively. The treatments used in one trial are listed in Table 4.2. The first treatments were applied when 70% of plants had at least one open flower. In 1988, two trials with similar treatments were seeded on 7 and 28 June and harvested on 10 and 31 August, respectively. The treatments of one trial are listed in Table 4.2. The first treatments were applied when all plants had at least one open flower. In each year, one trial contained a treatment where *E. purpurascens* was applied three times without subsequent inoculation with ascospores of *S. sclerotiorum*.

In 1988, 30-40 senescing flowers were collected from each plot and

at least 10 of these were examined under a stereomicroscope (30X) for the presence of sporulating colonies of *E. purpurascens*. Petals were then cleared with chloral hydrate (Bruzzese and Hasan 1983) and observed under a compound microscope (200X). Samples of leaves also were collected and examined similarly.

Data analyses

Data from the greenhouse trials for the number of lesions were analyzed using the Kruskal-Wallis test (Daniel 1978). Treatment means were separated using Dunn's Multiple Comparison Procedure (Daniel 1978). Similar data from the field trials were analyzed with the Friedman analysis of variance and the associated multiple comparison procedure (Daniel 1978).

Percentage data (disease incidence, disease index and percentage of pods with white mold) were arcsine-transformed before ANOVA when the range of percentages among treatments was greater than 40 (Little and Hills 1978). Yield data were not transformed before ANOVA. Treatments were compared using Duncan's Multiple Range Test (P = 0.05) (SAS 1987).

Results

Greenhouse tests

Typical symptoms of white mold appeared four days after inoculation with ascospores of *S. sclerotiorum*. All treatments containing *E. purpurascens* significantly reduced the number of white mold lesions per plant compared to the water control (Table 4.1). Addition of nutrients to conidial suspensions of *E. purpurascens* and treatment with nutrients alone had no significant effect on the number of lesions. When applied alone, malt extract (1%) and potato dextrose broth (1%) appeared to reduce the percentage of leaves and stems with disease symptoms but these effects were not significant. Treatment with *E. purpurascens*, however, resulted in

Table 4.1. Effect of application of *E. purpurascens* and nutrient solutions on the development of white mold on greenhouse-grown snap beans.

Treatment[v]	Number of plants with symptoms[w]	Number of lesions[x]	Diseased tissue[y] (%)	Pod rot[z] (%)
1% Malt extract	9	6.5 a	59.0 b	15.9
E. purpurascens + 1% malt extract	7	1.3 b	10.0 c	10.9
1% Potato-dextrose broth	10	5.4 a	41.5 b	17.6
E. purpurascens + 1% potato-dextrose broth	5	0.9 b	12.0 c	9.0
E. purpurascens	8	1.2 b	17.5 c	7.8
Water	10	6.5 a	81.0 a	26.6

[v]Treatments were applied when all plants had at least one open flower and again four days later. Tween 80 (0.01%) was added to all applications. Ascospores of *Sclerotinia sclerotiorum* were applied to all plans 1 day after the second application of each treatment.

[w]10 plants were observed.

[x]Data were collected 6 days after inoculation with ascospores of *S. sclerotiorum*. Values followed by the same letter are not significantly different by the Kruskal-Wallis test ($p>0.0001$) and Dunn's Multiple Comparison Procedure (experimentwise error rate = 0.75).

[y]Percentage of leaf and stem area diseased, assessed 14 days after inoculation. Values in a column followed by the same letter are not significantly different by Duncan's Multiple range test ($p = 0.05$). Percentage data were arcsine-transformed prior to analysis.

[z]Percentage of pods with white mold among all 10 plants in each treatments.

significantly less diseased tissue than treatment with water or nutrients. *E. purpurascens* was observed to colonize senescent petals. Both trials provided similar results.

Field experiments

In 1987, plants that received four applications of *E. purpurascens* in 1% malt extract and those that received two applications of *E. purpurascens* combined with iprodione had significantly fewer white mold lesions than plants in control plots (Table 4.2). Treatment with *E. purpurascens* plus 1% malt extract (two or four times), *E. purpurascens* plus iprodione (two times), and iprodione (0.5 kg a.i./ha) alone (one time) significantly reduced disease incidence, disease index and the percentage of pods with white mold. However, two applications of *E. purpurascens* in 1% malt extract and one application of iprodione alone at 0.25 kg a.i./ha had less effect on the percentage of pods with mold. Yield was not significantly affected in one trial (Table 4.2) although in the other trial (Appendix 1.2) applications of *E. purpurascens* in 1% malt extract (three or five times), iprodione (0.5 kg a.i./ha) alone (two times), and iprodione combined with *E. purpurascens* (three times) did significantly increase yields.

Typical white mold symptoms also appeared in plots where plants were treated with *E. purpurascens* but not inoculated with *S. sclerotiorum* ascospores (Appendix 1.2). Disease in these plots was assumed to be the result of natural infection. Although these plots had disease ratings similar to those of plots that received *E. purpurascens* and/or iprodione followed by *S. sclerotiorum*, yield did not differ significantly from the control.

Table 4.2. Effect of application of *E. purpurascens* and iprodione in the field on development of white mold of snap bean caused by *S. sclerotiorum*

Treatment[w]	Dates of treatment	Number of lesions[x]	Disease incidence[y] (%)	Disease index[y,z] (%)	pod rot[y] (%)	Yield[y] (tons/ha)
1987 (2 July)						
Control		26 a	78.2 a	27.0 a	12.2 a	16.65 a
E. purpurascens + 1% Malt Extract	10,16 Aug.	13 ab	60.9 b	17.0 b	5.2 bc	17.23 a
E. purpurascens + 1% Malt Extract	6,10,14 17 Aug.	8 b	51.7 b	12.1 b	4.0 c	18.35 a
Iprodione (0.5 kg a.i./ha)	10 Aug.	15 ab	43.5 b	14.3 b	4.4 c	16.85 a
E. purpurascens + Iprodione (0.25 kg a.i./ha)	10,16 Aug.	7 b	40.8 b	10.1 b	3.5 c	17.30 a
Iprodione (0.25 kg a.i./ha)	10 Aug.	17 ab	61.2 b	15.3 b	6.7 b	16.35 a
1988 (June 28)						
Water	6,9,12 Aug.	24 a	82.1 a	27.7 a	24.0 a	13.97 b
1% Malt Extract	6,9,12 Aug.	16 a	73.8 ab	21.3 ab	17.3 b	14.02 b
E. purpurascens	6,9,12 Aug.	9 ab	61.8 bc	16.7 bc	12.1 bc	16.15 ab
E. purpurascens + 1% Malt Extract	6,9,12 Aug.	4 b	52.1 c	13.0 bc	7.5 cd	17.45 a
Iprodione (0.5 kg a.i./ha)	8,12 Aug.	6 b	48.5 c	11.2 c	5.4 d	16.86 a
E. purpurascens + Iprodione (0.25 kg a.i./ha)	8,12 Aug.	5 b	57.7 bc	15.3 bc	9.2 cd	17.95 a
E. purpurascens + 1% Malt Extract	8,12 Aug.	12 ab	62.3 bc	20.9 ab	11.2 cd	14.05 b

[w]Control plants (1987) were inoculated only with *Sclerotinia sclerotiorum*. Water treatment (1988 control treatment) was distilled water containing 0.01% Tween 80. Tween 80 (0.01%) was added to all suspensions of *E. purpurascens*. Ascospores of *S. sclerotiorum* were applied to all plots on 18 August in 1987 and on 13 August in 1988.

[x]Number of lesions was recorded from 20 plants in each plot on 24 August in 1987 and on 19 August in 1988. The data were analyzed using the Freidman analysis of variance. Values followed by the same letter are not significantly different (multiple comparison experimentwise error rate = 1.05).

[y]Values for each year in a column followed by the same letter are not significantly different (Duncan's Multiple Range Test, P = 0.05). Percentage data of disease incidence was arcsine-transformed before analysis.

[z]Disease severity classes: 0 = 0%, 1 = 1-10%, 2 = 11-30%, 3 = 31-50%, 4 = 51-75%, 5 = 76-100% of stem and leaf area diseased. Disease index=$[\Sigma S_i n_i/5N]x100$ (i=0, 1, 2,...5), where S is the appropriate disease class, n = number of diseased plants in the same class, and N = number of plants rated.

In 1988, plants in plots that received *E. purpurascens* in 1% malt extract (three times), iprodione (0.5 kg a.i./ha)(two times), and iprodione (0.25 kg a.i./ha) plus *E. purpurascens* (two times) had significantly lower values for the number of lesions, disease incidence, disease index, and percentage of pods with white mold than those received only water (Table 4.2). *E. purpurascens* alone (three times) had a similar efficacy except in the number of lesions. Two applications of *E. purpurascens* in 1% malt extract resulted in significantly lower values than the control only with respect to disease incidence and the percentage of pods with white mold. Treatment with 1% malt extract alone provided some reduction in the percentage of pods with white mold; however, those effects were significantly less than that achieved with three applications of *E. purpurascens* in 1% malt extract. Yields in plots receiving *E. purpurascens* in 1% malt extract (three times), iprodione (two times), and iprodione plus *E. purpurascens* (two times) were significantly higher than those of the control plots. Results of both trials were similar. When *E. purpurascens* was applied three times but not followed by inoculation with *S. sclerotiorum*, disease ratings were similar to those obtained for the same treatment in 1987 except that yield was significantly higher than in the control plot (Appendix 1.2).

Colonies of *E. purpurascens* with conidia and sporodochia were readily detected with the stereomicroscope on all samples of senescing flowers from plants treated with *E. purpurascens*. In most cases, colonies completely covered the petal surface. Colonization by *E. purpurascens* did not appear to be inhibited on flowers that received mixtures of *E. purpurascens* and iprodione, although quantitative measurements were not made. No colonies of *E. purpurascens* were observed on healthy leaves, although many conidia could be seen. Flowers from plots where *E. purpurascens* was not applied rarely possessed colonies of *E. purpurascens*.

Discussion

Results from both greenhouse and field experiments indicate that *E. purpurascens* can reduce disease incidence and disease index of white mold of bean. At least one of the treatments containing *E. purpurascens* resulted in yields significantly higher than those in control treatments in three out of four field trials.

Conidia of *E. purpurascens* can be found on the surface of plants in early stages of growth but usually are more frequent on older or newly-dead plant tissue (Dickinson 1967). The fungus appears to establish itself and sporulate quickly on senescent or dead plant tissue. This ability plus the production of antifungal compounds (Brown *et al.* 1987) makes *E. purpurascens* an attractive choice for control of *S. sclerotiorum* on beans and other crops (Mercier and Reeleder 1987a).

No previous studies have shown that *E. purpurascens* or other antagonists control white mold of bean under field conditions. *E. purpurascens* also may be useful for controlling grey mold of beans caused by *Botrytis cinerea*, which also infects bean blossoms before invading other parts of the plant (Nelson and Powelson 1988).

The effects of malt extract in promoting disease control did not appear to be very great in this study; however, other nutrients may prove to be useful additives to antagonist suspensions (Morris and Rouse 1985). The isolate of *E. purpurascens* used in these studies was tolerant to iprodione and observations of petals collected from the field suggest that it colonized petals well in the presence of iprodione. More critical studies are needed, but tolerance to fungicides appears to be a useful characteristic for biocontrol agents. Tolerant strains may be combined with a fungicide to provide increased control of the pathogen (either by eliminating saprophytic microflora competing with the antagonist, or through the chemical's direct effects on the pathogen) and may allow a biocontrol agent for one pathogen to be packaged with a chemical targeted

for a second pathogen. In our studies, combining *E. purpurascens* with iprodione did not appear to provide levels of control greater than those provided by either agent alone, indicating that there were no additive or synergistic effects.

Although *E. purpurascens* was effective in the field in this study, much work needs to be done before it can be offered as an alternative or supplement to chemical control. In these studies, two to four applications of *E. purpurascens* were required to provide control similar to that provided by fewer applications of iprodione. *E. purpurascens* was applied before *S. sclerotiorum* to give *E. purpurascens* some opportunity to colonize flowers before arrival of ascospores, but this may not always be the case under conditions of natural infection. Although *E. purpurascens* generally is considered to be a nonpathogen, some reports have shown that it can act as a weak pathogen on certain plants (Geol and Gupta 1979). Moreover, *E. purpurascens* has been reported as an allergen and culture extracts have been shown to be toxic to mice (Hensel *et al.*1974; Ohtsubo *ea al.* 1978). Nevertheless, *E. purpurascens* appears to have good potential as a biocontrol agent, and strain selection to overcome these problems should be a major goal. The usefulness of additives, such as nutrients and other compounds, to promote growth and survival of *E. purpurascens* needs to be investigated. The ability of bean stems, leaves, and flower buds to act as sources of spore populations for colonization of senescing flowers should be assessed. Other microorganisms may also be potential antagonists (Mercier and Reeleder 1987a), and they merit more extensive testing, either alone or in combination with *E. purpurascens*. When coupled with the development of techniques to increase the rate of destruction of soilborne sclerotia (Anas and Reeleder 1989), the approach outlined here may result in integrated control of white mold of bean and similar diseases.

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Connecting Text

An iprodione-tolerant strain of *E. purpurascens*, R4000, was evaluated for its efficacy in controlling white mold under greenhouse and field conditions. Application of conidial suspensions of *E. purpurascens* reduced disease incidence and disease index of white mold and two to four applications provided disease control similar to that resulting from applications of the fungicide iprodione. However, efficiency of the disease control varied from trial to trial, and the factors which affect the efficacy were not clear. Since control of white mold resulted from protection of bean flowers from infection of ascospores of *S. sclerotiorum*, colonization of flowers by *E. purpurascens* may be one of important elements in the biological control. Knowledge of the colonization may be very useful for modification of application procedures leading to more efficient control of white mold. Also, it is essential to understand effects of biocontrol agents on crops before they can be used in practice. The following section contains experiments designed for determination of the effects of flower age and conidial suspension additives on colonization of bean flowers by *E. purpurascens* and evaluation of the effects of *E. purpurascens* on flower abscission, pod abortion, pod yield, and senescence of leaves.

5. Colonization of Bean Flowers by *Epicoccum purpurascens*

Introduction

Epicoccum purpurascens Ehrenb. & Schlecht. generally is considered a saprophytic fungus (Booth 1980), although it is pathogenic on certain plants (Geol and Gupta 1979; Khatua *et al.* 1977). It possesses antagonism to a number of plant pathogens, including *Sclerotinia sclerotiorum* (Lib.) de Bary (Boland and Inglis 1989; Booth 1980; Mercier and Reeleder 1987). In nature, the fungus is found commonly on older or newly dead plant tissue but also can be recovered from the surface of young plant tissue (Dickinson 1967). Although *E. purpurascens* may colonize leaf surfaces early in the growing season, it does not penetrate leaf tissue until senescence has occurred (Pugh and Buckley 1971). While leaf saprophytes may accelerate senescence and thereby reduce crop yield (Fokkema 1981; Smedegaard-Petersen and Tolstrup 1986), *E. purpurascens* does not appear to affect leaf senescence (Jachmann and Fehrmann 1989).

Epidemics of white mold of snap bean (*Phaseolus vulgaris* L.), generally are initiated by ascospores of *S. sclerotiorum*. Hyphae arising from germinated ascospores colonize senescing bean flowers prior to invasion of adjacent plant tissues (Abawi *et al.* 1975). Protection of flowers with fungicides (Hunter *et al.* 1978) or antagonistic microorganisms such as *E. purpurascens* (Boland and Inglis 1989; Zhou and Reeleder 1989) reduces flower colonization by *S. sclerotiorum*, thus reducing disease incidence. Application of conidia of *E. purpurascens* effectively suppresses white mold under both greenhouse and field conditions (Zhou and Reeleder 1989). Colonization of flowers and production of antifungal compounds by *E. purpurascens* are important elements in this control (Boland and Inglis 1989; Mercier and Reeleder 1987).

An antagonist must be selected not only for its activity against the

pathogen but also for its capacity to survive adverse environmental conditions and maintain itself at effective population levels (Blakeman 1988). Knowledge of the behavior of *E. purpurascens* on bean flowers and leaves is important because it may lead to modification of application procedures and more efficient control of white mold. Also, it is essential to understand effects of biological agents on crops before antagonists can be used in practice. Thus, our objectives were to determine the effects of flower age and conidial suspension additives on the colonization of bean flowers by *E. purpurascens* and evaluate the effects of *E. purpurascens* on flower abscission, pod abortion, pod yield, and senescence of leaves.

Materials and Methods

Snap bean (cultivar Strike) and strain R4000 of *E. purpurascens* (Zhou and Reeleder 1990) were used throughout this study. Beans were planted in 130-mm-diameter plastic pots containing Pro-Mix BX (Les Tourbières Premier Ltée, Rivière du Loup, Quebec) and were fertilized every 2 wk with 0.1% NPK (20-20-20) solution (Peters Fertilizer Products, W. R. Grace and Co., Fogelsville, PA). Conidia of *E. purpurascens* were produced on a wheat seed medium prepared as described previously (Zhou and Reeleder 1989). Concentration of the suspension was adjusted to 10^6 conidia per ml in 0.01% Tween 80 (J. T. Baker Chemical Co., Phillipsburg, NJ). Flowers sampled in these studies were dissected immediately upon removal, and only banner (standard) petals were used for observations and measurements. Based on preliminary observations, these petals were assumed to represent the flower as a whole. Flowers were placed in one of three classes where emerging flowers were those having unopened petals, with the banner petal extending at least 5 mm past the tip of the sepal but still unopened and other petals not yet visible; newly opened flowers were those with the banner petal opened, all other petals visible and at least partly opened, with petals white and turgid; and senescent flowers

were those where all petals were yellowish and flaccid but not dry, and all petals were still attached to the flower.

Behavior of *E. purpurascens* on bean flowers.

Three plantings of bean, sown at 5-day intervals, were held on a growth bench (day/night temperatures of 22/19 C), with a 14-h photoperiod and a light intensity of 275 $\mu E/m^2/s$. There were 10 plants per date. When 70% of the flowers on the oldest plants (45 days from planting) were open, all flowers except those that were senescent were removed. On the second group of plants (40 days), all but newly opened flowers were removed, and on the youngest plants (35 days), only emerging flowers were left. Conidial suspensions then were sprayed on the plants with a hand-held pump sprayer, with or without addition of 1% malt extract (ME; Difco Laboratories, Detroit, MI), until run-off (about 15 ml per plant). Suspensions were applied to the entire plant. Plants were air-dried for 1 h, then placed in a dew chamber (100% relative humidity [RH], 23 C) for 48 h. Two or more flowers per plant were sampled for various measurements at 4, 8, 12, 24, and 96 h after spraying. Banner petals were cleared with concentrated chloral hydrate solution (2 g/ml) for 24 h, then stained with lactophenol-cotton blue. Entire petals were used for open flowers, and only the exposed parts of the petal were used for emerging flowers. Each petal was treated as an experimental unit in a completely randomized design. Cleared petals were mounted on glass slides with clear lactopheno[1] and observed under a microscope. The number of conidia on the petals at 4 h was estimated by determining the number of conidia in a microscope field (200X). These values were used to calculate the number of conidia per mm^2. Five randomly selected fields were observed on each petal to obtain an average. Similarly, at least five fields were observed per petal to obtain values for percentage of conidial germination and length of germ tubes, although, for these latter characters, only a subsample of six petals per treatment was examined. Where more than one germ tube was present, the longest germ tube was measured. To assess

colonization by *E. purpurascens*, the whole petal surface first was observed, and two representative fields were recorded photographically (100X). Photographs (labelled on the back) were randomized, then rated as follows: 0, 0% of surface covered with mycelium; 1, less than 10%; 2, 10-30%; 3, 31-50%; 4, 51-75%; and 5, more than 75% of surface area covered.

A similar experiment was designed to test the effects of Tween 80, Gelatin, and Malt extract on colonization of emerging flowers. Bean plants were produced as described previously. When one to two flowers per plant were open, open flowers and small (incompletely developed) inflorescences were removed, leaving only emerging flowers. Conidial suspensions of *E. purpurascens* plus the additives then were sprayed on plants, as described previously. Plants not receiving any application served as controls. Plants were placed in a greenhouse mist chamber for 4 days, with mist applied each night for 12 h and with the chamber open during the day (Zhou and Reeleder 1989). A completely randomized design with five plants per treatment was used. Newly opened flowers from treated plants were removed 4 days after application; senescent flowers were removed 6 days later. Four flowers per plant were sampled each time, with each flower treated as the experimental unit. Colonization of banner petals was recorded photographically through a dissecting microscope (38X). Photographs (labelled on the back) were randomized and rated for colonization where: 0, no conidia; 1, less than 25% area covered by conidia in sporodochia; 2, 25-50% area covered; 3, more than 50 but less than 75% area covered; and 4, 75% or more of the area covered.

Effects of *E. purpurascens* on beans.

Plants were produced as described previously and arranged in a randomized complete block design with four blocks on a growth bench (day/night temperatures of 22/19 C with a 14-h photoperiod). Treatments were conidia (10^6 conidia per ml) alone; conidia in 1% malt extract; malt extract alone; and an unsprayed control. Applications were made when more

than 80% of the plants had at least one open flower and repeated 4 days later. The method of application was as described previously. Treated plants were placed in dew chambers (23 C, 100% RH without light) for 48 h before they were returned to the growth bench. After the first flower opened, plants were observed every third day. Number of inflorescences and number of flowers on each inflorescence were recorded when inflorescences were completely developed (no new flower buds present). Inflorescences and flowers (including emerging flowers with the banner petal extended at least 5 mm from the sepal) were counted and marked with an oil-base paint touching the peduncle of the inflorescence and sepals of the flower (Wiebold *et al.* 1981). The number of pods greater than 1 cm long also was determined, and each was marked with paint by touching the tip of the pod. The apex leaflet of the first trifoliolate leaf on each plant was similarly marked and observed for leaf senescence, expressed as the yellowish proportion of leaf. Pods were removed when at least 80% of them were judged ready to harvest commercially (70 days after planting and 20 days after the last application). Number of pods and fresh weight of pods per plant were recorded at harvest. Pods were dried in a 65-C oven for 96 h to obtain dry weight measurements. Percentage of abscised inflorescences was calculated with the formula: ([number of inflorescences developed - number of inflorescences at harvest]/number of inflorescences developed) X 100; percentage of abscised flowers was calculated with ([number of flowers - number of pods developed]/number of flowers) X 100, and percentage of abscised pods with ([number of pods formed - number of pods harvested]/number of pods formed) X 100 (Wiebold *et al.* 1981).

Effects of *E. purpurascens* on chlorophyll content of leaves and behavior of *E. purpurascens* on leaves.

Beans (produced as described previously) were planted in a greenhouse (16-26 C) and inoculated twice with suspensions of *E. purpurascens* or 0.01% Tween 80 (control). The time and methods for application and management after application were as described previously (Zhou and

Reeleder 1989). Plants were arranged in a completely randomized design with three replicate plants per treatment. The first trifoliolate leaf of each plant was used for measurement of chlorophyll content, and samples were taken every 5 days following the second application. The method of Hiscox and Israelstam (1979) was used to measure chlorophyll content. On each sampling date, three leaflets per treatment were cut into small pieces. A 100-mg subsample was placed in a test tube with 100 ml of dimethyl sulphoxide (Fisher Scientific Co., Fairlawn, NJ). Tubes then were placed in a 65-C water bath for 2 h. Light absorption of the resultant extract was measured immediately with a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, NY) at 663 and 645 nm for chlorophyll a and b, respectively. Chlorophyll content was calculated with an equation used by Arnon (1949), and total chlorophyll (a + b) was expressed as milligrams chlorophyll per gram leaf fresh weight. Five 9-mm disks were cut from each sampled leaf and observed under the microscope after clearing with chloral hydrate. Leaf disks also were sampled from relatively young leaves (at the top of the plant) and the first true leaf of the plant, which had begun to senesce at the time of sampling.

Data analyses.

Data for number of conidia on flowers (Fig. 5.1), area of flower surface colonized by *E. purpurascens* (Table 5.2), and sporulation of *E. purpurascens* (Fig. 5.2) were analyzed by the Kruskal-Wallis test and means were separated with Dunn's multiple comparison procedure (Daniel 1978). Percent data (arcsine-transformed, Table 5.1) and data on germ tube length (Table 5.2), pod weight, and chlorophyll content were subjected to analysis of variance and differences among treatments were determined by Duncan's multiple range or LSD tests (SAS 1987). All experiments were performed at least twice. Where variances were homogenous, data from trials of an experiment were pooled before analysis.

Results

Behavior of *E. purpurascens* on bean flowers.

After beans were inoculated with conidial suspensions of *E. purpurascens*, banner petals from emerging flowers had fewer conidia than those of newly opened or senescent flowers. Addition of ME increased the number of conidia on emerging flowers, but not on newly opened or senescent flowers (Fig. 5.1). Four hours after application, percent conidial germination varied from 6.1 - 42.9% (Table 5.1). Germination was greatest on flowers where ME had been added to the conidial suspension, with values for senescent flowers being greater than those for emerging flowers. Without added ME, percentages of germination among the flower groups were not significantly different. At 8 and 12 h, trends were similar. With ME, higher values were found on newly opened flowers than on emerging flowers at 8, but not at 4 or 12 h. Similar relationships were found for germ tube length (Table 5.2). However, at 4 h, no differences in germ tube length among the various flower groups were apparent, regardless of whether ME was added or not. Differences were apparent in the 8-h sample. With ME, the longest germ tubes were present on senescent flowers, followed by newly opened flowers, then emerging flowers. Without ME, germ tubes on emerging flowers were shorter than on newly opened or senescent flowers. With or without ME, a conidium initially produced one germ tube, but, by 8 h, two or more germ tubes generally were present on conidia found on open and senescent flowers. Such additional germ tubes usually were not formed on emerging flowers. Measurements could not be made at 12 or 24 h due to intertwining of elongated germ tubes. Conidiophores and immature conidia were present in clusters (sporodochia) at 24 h.

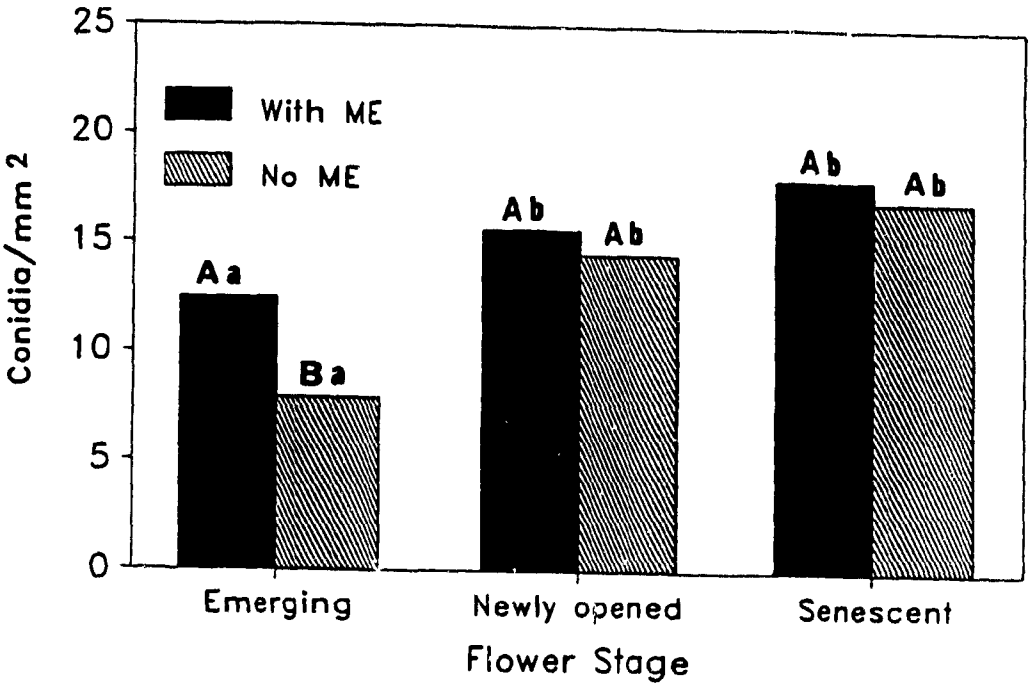

Fig. 5.1. Number of conidia of *Epicoccum purpur scons* present on bean flowers following spray application with and without malt extract (ME). Bean flowers were sampled 4 h after application with *E. purpurascens*. Columns represent means of 20 petals. Means were separated using Dunn's multiple comparison procedure. Upper case letters at the top of each column represent comparisons of ME treatments with No-ME treatments (P = 0.05) within each flower age. Lower case letters represent comparisons among flower age groups (P = 0.15). For each type of comparison, means with the same letter are not significantly different from one another.

Table 5.1. Percent germination of conidia of *Epicoccum purpurascens* on bean flowers in the presence (ME) and absence (No ME) of malt extract in inoculum[x]

	Time after application (h)					
	4		8		12	
Flower stage	ME[y]	No ME	ME	No ME	ME	No ME
Emerging	24.7 a*	6.1 a	61.8 a*	40.8 a	86.4 a*	65.5 a
Newly opened	33.1 ab*	11.2 a	73.4 b	58.2 ab	88.8 ab*	76.7 a
Senescent	42.9 b*	15.9 a	84.9 c*	68.9 b	94.9 b*	80.2 a

[x]Bean flowers were sampled 4, 8, and 12 h after application with *E. purpurascens*. Means were separated by Duncan's multiple range test (columns) or the LSD test (rows; paired ME and No ME values). Values (means of six petals) in a column followed by the same letter are not significantly different (P = 0.05). For paired ME and No ME values in each row, the value indicated by "*" is significantly greater than the other value (P = 0.05).

[y]The conidial suspension contained (ME) or did not contain (No ME) 1% malt extract.

Table 5.2. Germ tube growth from conidia of *E. purpurascens* on bean flowers

	Length of germ tube (µm)[x]				Amount of flower surface covered by mycelium[z]	
	4 h		8 h			
Flower stage	ME[y]	No ME	ME	No ME	ME	No ME
Emerging	27.3 a*	8.6 a	66.3 a*	48.9 a	2.5 a*	1.6 a
Newly opened	30.9 a*	14.7 a	106.1 b	105.7 b	2.9 b	2.7 b
Senescent	25.5 a*	11.9 a	130.5 c*	111.4 b	3.7 c	3.5 c

[x]Bean flowers were sampled 4 and 8 h after application with *E. purpurascens*. Means were separated by Duncan's multiple range test (columns) or the LSD test (rows; paired ME and No ME values), respectively. Values (means of six petals) in a column followed by the same letter are not significantly different (P = 0.05). For paired ME and No ME values in each row, the value indicated by "*" is significantly greater than the other value (P = 0.05).

[y]The conidial suspension contained (ME) or did not contain (No ME) 1% malt extract.

[z]Bean flowers were sampled 24 h after application with *E. purpurascens*. Rating system: 0, 0%; 1, less than 10%; 2, 10-30 %; 3, 31-50%; 4, 51-75%; and 5, more than 75% area covered by mycelium of *E. purpurascens*. Means were separated with Dunn's multiple comparison procedure. Values in a column followed by the same letter are not significantly different (F = 0.15). Values in rows followed by "*" are significantly greater than the other paired value (P = 0.05). Values are pooled means of 44 petals.

Colonization of banner petals by *E. purpurascens* varied with the flower stage (Table 5.2). A greater area of senescent flowers was covered by mycelium compared with newly opened flowers or emerging flowers. Mycelial coverage was greater with newly opened flowers than with emerging flowers. Addition of ME increased mycelial coverage on emerging flowers, but not on other stages. Within 96 h, emerging flowers became fully opened flowers, on which a few sporodochia of *E. purpurascens* could be seen. Flowers that were newly opened at the start of the experiment were senescent by this time, and sporodochia covered most of the surface. Flowers that were senescent at the start of the experiment were now dried up, and much of the tissue was filled with conidia of *E. purpurascens*. Conidia of *E. purpurascens* applied to emerging flowers germinated and resulting germ tubes elongated as the flowers aged (Fig. 5.2). A few conidia were observed on five of the 40 flowers from unsprayed control plants at 4 and 10 days. Addition of ME and gelatin resulted in increased sporulation at 10 days, but not at 4 days.

Effects of *E. purpurascens* on beans.

There were no significant differences among treatments with respect to fresh and dry pod weights or abscission of inflorescences, flowers, or pods (Appendix 1.4).

Development of leaf senescence was followed until 20 days after harvest of pods (90 days after planting). The proportion of yellowed leaves (data not shown) was not affected by treatment with *E. purpurascens*. Percentage of yellowed area varied from 33.3-46.7% at harvest. Addition of ME appeared to maintain the green color of the leaves.

Effects of *E. purpurascens* on chlorophyll content of leaves.

Chlorophyll contents of leaves sprayed with *E. purpurascens* were not significantly different from control leaves, although means for leaves treated with *E. purpurascens* tended to be greater.

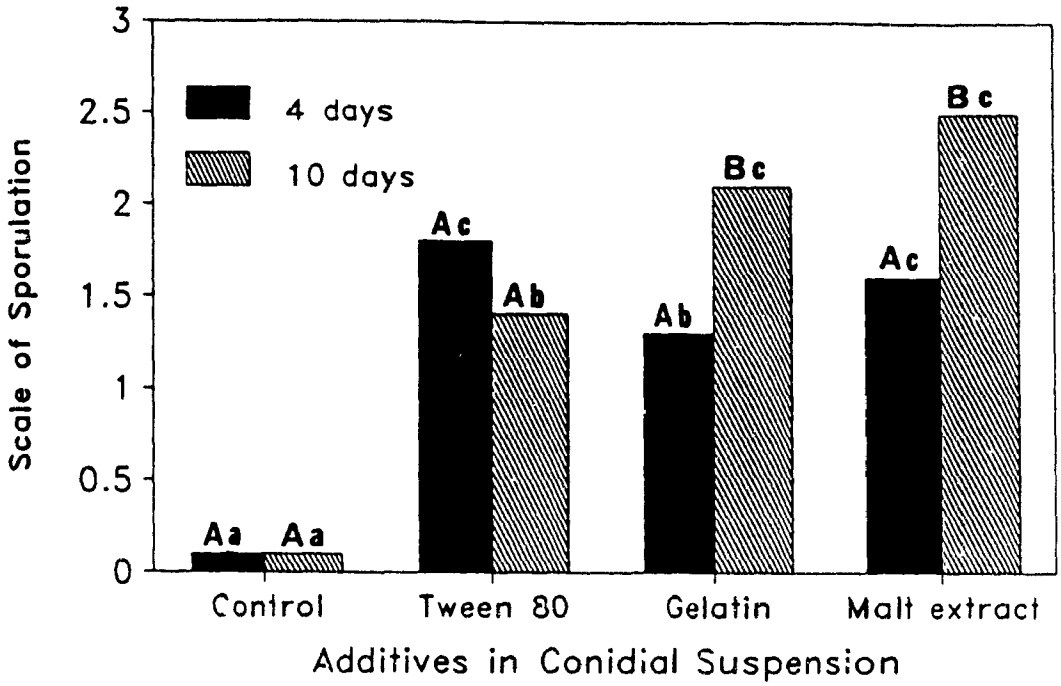

Fig. 5.2. Sporulation of *Epicoccum purpurascens* on bean flowers. Columns represent pooled means of 40 petals. Scale for assessing the amount of sporulation: 0, no conidia; 1, less than 25% area covered by conidia in sporodochia; 2, 25-50% area covered; 3, more than 50 but less than 75% area covered; and 4, more than 75% area covered. Means were separated by Dunn's multiple comparison procedure. Upper case letters at the top of each column represent comparisons of sample times ($P = 0.05$). Lower case letters represent comparisons of additives ($P = 0.30$). For each type of comparison, means with same letter are not significantly different from one another.

Observations of *E. purpurascens* on leaves.

On disks from younger leaves, one-third to one-half of the conidia germinated with a single hypha. Hyphae usually did not elongate much on the surface of epidermal cells and were <100 to a few hundred micrometers long (data not shown). Additional germ tubes were not produced. No penetration of leaf cells was observed. Similar phenomena were observed on disks from leaves used for chlorophyll measurements. On disks from older leaves (yellowed but not necrotic), hyphae were longer and more branched than those on young leaves. However, hyphae were still relatively short and no conidiophores or conidia were formed. On necrotic spots on leaves, sporodochia of *E. purpurascens* containing mature and immature conidia were observed.

Discussion

Colonization and growth of *E. purpurascens* were affected by the age of the tissue to which the conidia were applied. The number of conidia per mm^2 of flower was greater on newly opened or senescent flowers than on emerging flowers. In addition, conidial germination was greater on senescent petals than on younger tissue. These factors contributed to high rates of colonization of senescent flowers by *E. purpurascens*. It is likely that variations in conidial adherence, germination, and germ tube elongation mainly resulted from physical and chemical differences among the flower stages. Available nutrients leached or exuded from flowers (Brown 1922; Mitchell 1968), or received from other sources, may explain differences in growth, because quantities and compositions of eachates may vary with age of the tissue being leached (Blakeman 1972; Stenlid 1958). As with tests in vitro (Zhou and Reeleder 1990), addition of ME greatly improved conidial germination of *E. purpurascens* on flowers. Also, ME stimulated an increase in mycelial coverage of emerging flowers. This, however, did not occur on newly opened or senescent flowers. Increases in germination and rates of growth following addition of nutrients may indicate that there is a limitation of nutrients available

to *E. purpurascens* on emerging flowers.

Conidia of *E. purpurascens* were able to survive on emerging flowers and eventually colonize the flowers. Colonization, however, was not as extensive as when conidia were applied directly to older flowers. Nevertheless, it may be possible to apply conidia of *E. purpurascens* to earlier growth stages of beans to help protect flowers from infection by *S. sclerotiorum*. This may increase efficiency of white mold control by *E. purpurascens* because ascospores of *S. sclerotiorum* infect young flowers of bean when artificially inoculated (Abawi *et al.* 1975). However, additives to the spray suspension will have to include nutrients selected to assist *E. purpurascens* in the colonization of emerging flowers. Although MF is of use in this regard, additional nutrients may be required.

During these studies, plants used as controls were placed on a greenhouse bench together with plants treated with *E. purpurascens*. However, flowers on control plants were colonized only rarely by *E. purpurascens*, indicating that movement of conidia of *E. purpurascens* between plants was not great even though there was considerable air movement in the greenhouse during this period. In previous field experiments (Zhou and Reeleder 1989), we observed that plants not treated with *E. purpurascens* rarely had flowers colonized by this fungus, even when plants in adjacent plots had been inoculated with *E. purpurascens*. Although *E. purpurascens* releases conidia violently (Meredith 1966), it seems doubtful that conidia of *E. purpurascens* on leaves and stems could provide enough inoculum to efficiently colonize flowers on the same plant. Limited movement of conidia among plants may reduce the efficiency of biocontrol in the field, but it also may reduce chances for spread of *E. purpurascens* outside of the treated crop.

Application of *E. purpurascens* to plants under growth chamber conditions did not have deleterious effects. Yields and percentages of abscised flowers and pods were similar in all treatments. These results are similar to findings from earlier field and greenhouse studies (Zhou and Reeleder 1989). As with previous studies on wheat (Jachmann and Fehrmann 1989), application of *E. purpurascens* did not accelerate

senescence of bean leaves or affect chlorophyll content. This may be because *E. purpurascens* did not penetrate cells of leaves until tissue was in an advanced state of senescence. The fungus also may produce auxin (Buckley and Pugh 1971). However, it should be noted that, whereas snap beans are harvested when pods are fresh, dry beans are harvested when seed is mature. It is not known if *E. purpurascens* will accelerate senescence of bean leaves during maturation of bean seeds and affect seed quality, although this seems unlikely. Also, higher concentrations of *E. purpurascens* may result in harmful effects on plant growth, particularly if concentrations of these dark-colored conidia are high enough to interfere with light reception by leaves. Promotion of early colonization of young flowers by adding other nutrients to spray suspensions may decrease yield if pod formation is affected.

Superior performance in bioassays (Boland and Inglis 1989;Mercier and Reeleder 1987), effective suppression of white mold in greenhouse and field trials (Zhou and Reeleder 1989), and the availability of improved strains (Zhou and Reeleder 1990), together with the results presented here, indicate that *E. purpurascens* has potential as a biological control agent. Knowledge of the mode of antagonism of *S. sclerotiorum* by *E. purpurascens* would provide useful additional information for development of formulation techniques and further improvements in efficiency of disease control.

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Connecting Text

Emerging flowers, newly opened flowers, and senescent flowers of bean were sprayed with conidial suspensions of *E. purpurascens*. It was found that the number of conidia per square millimeter of tissue surface was greater on newly opened or senescent flowers than on emerging flowers and germination of conidia was higher on senescent petals than on younger tissue. Addition of malt extract to conidial suspensions improved conidial germination on flowers and increased mycelial coverage on emerging flowers. The investigation of effects of *E. purpurascens* on bean plants showed that application of *E. purpurascens* did not accelerate senescence or affect chlorophyll content of bean leaves, and did not affect yield or percentage of abscised flowers or pods. This information together with previous studies (sections 3 and 4) indicate that *E. purpurascens* has a potential as a biological control agent. However, the mode of action of suppression of *S. sclerotiorum* by *E. purpurascens* and the mechanisms of white mold disease control have not been elucidate. The knowledge of mechanisms of biological control is very important for enhancement of biocontrol. The next section investigates these mechanisms.

6. Interactions between *Sclerotinia sclerotiorum* and *Epicoccum purpurascens*

Introduction

Plant pathogens can be controlled by the introduction of antagonistic micro-organisms onto plant surfaces (Trutmann *et al.* 1982; Zhou & Reeleder 1989). Suppression of pathogens by antagonists can occur in various ways: parasitism, competition for nutrients or space, mechanical obstruction and/or production of toxic or inhibitory metabolites (Fravel 1988; Jayapal & Balasubramanian 1988). *Epicoccum purpurascens* Ehrenb. ex Schlecht. (syn. *E. nigrum* Link) has been studied as an antagonist to a number of plant pathogenic fungi (Campbell 1956; Chand & Logan 1984; Wu 1976; Brown *et al.* 1987; Mercier & Reeleder 1987; Boland & Inglis 1989; Zhou & Reeleder 1989). Its antagonism to *Cochliobolus sativus* (Ito & Kuribayashi) Drechsler ex Dastur was due to both parasitism and production of antibiotics (Campbell 1956). Parasitism also was responsible for inhibition of *Rhizoctonia solani* Kühn and *Fusarium culmorum* (W.G.Sm.) Sacc. (Chand & Logan 1984; Wu 1976). Antifungal compounds such as flavipin and epicorazines A and B have been extracted from culture filtrates of *E. purpurascens* (Bamford *et al.* 1961; Brown *et al.* 1987).

Sclerotinia sclerotiorum (Lib.) de Bary causes a wide range of plant diseases including lettuce drop and white mold of beans. *E. purpurascens* was shown to inhibit germ tube elongation of ascospores on leaf disks of lettuce and reduced infection of lettuce in growth chamber tests (Mercier & Reeleder 1987). This inhibition was thought to be due to production of antifungal compounds by *E. purpurascens*. Epidemics of white mold often are initiated by ascospores that land and germinate on senescing bean flowers. Subsequent mycelial growth spreads to adjacent green tissue (Abawi & Grogan 1975). *E. purpurascens* reduced radial growth of *S. sclerotiorum* on potato dextrose agar plates (Zhou & Reeleder 1990) and

application of *E. purpurascens* conidia to beans effectively controlled white mold in field trials (Zhou & Reeleder 1989). Mechanisms of biological control, however, have not been elucidated. Knowledge of these mechanisms is important for enhancement of biocontrol. The objective of this study was to elucidate the roles of antifungal compounds and nutrients in interactions between *S. sclerotiorum* and *E. purpurascens* and in control of white mold.

Materials and Methods

Snap bean (*Phaseolus vulgaris* L., cv. Strike) was utilized throughout this study. *E. purpurascens* strain R4000 (Zhou & Reeleder 1990) and *S. sclerotiorum* isolate MACF - 152 were used. Conidia of *E. purpurascens* were obtained from 15-20 day old cultures produced on wheat seed medium (Zhou & Reeleder 1989) and ascospores were produced and maintained as described previously (Mercier & Reeleder 1987). Media used in this study included malt agar (MA; Difco, Detroit, MI), potato dextrose agar (PDA, Difco), Czapek solution agar (CSA, Difco), 1.5 % water agar (WA; Bacto-agar, Difco) and potato dextrose broth (PDB, Difco).

Preparations of medium-covered slides

Clean glass microscope slides (75 x 25 mm) were soaked in 95% ethanol, then removed and flamed using a bunsen burner. Slides then were immersed in a petri dish containing sterile molten medium and placed on a sterile paper towel. After cooling, agar on the underside of the slides was removed and slides were placed in petri dishes lined with moistened filter paper.

Dual cultures

E. purpurascens and *S. sclerotiorum* were cultured on PDA at 20°C for 10 and 6 days respectively. A small square (2 x 2 mm) of each fungal culture was used as inoculum. *E. purpurascens* was transferred to one end of each medium-covered slide and *S. sclerotiorum* to the other, such that the squares were separated by 4 cm. Five slides of each medium were

inoculated with *E. purpurascens* 3 days prior to inoculation with *S. sclerotiorum* and another five slides were inoculated with both fungi simultaneously. Slides inoculated with *E. purpurascens* or *S. sclerotiorum* alone served as controls. Dual culture dishes were set up similarly and fungal culture disks (5 mm diam.) were used as inoculum. Slides were placed in a petri dish with moistened filter paper and held at 20°C in the dark until the fungal colonies were close to or in contact with each other. Incubation periods were 3-4 days on PDA, 4 days on MA, 5-6 days on CSA and 8-10 days on WA. Dual culture dishes were held under the same conditions for 6-12 days depending upon the medium. Interactions occurring on slides and dishes were observed under the microscope. Results were photographically recorded.

Interactions on flower disks

Bean flowers were obtained from plants grown on a growth bench and disks (5 mm in diam.) were cut from banner petals of newly-opened flowers. The petal disks were sterilized with 70% ethanol (Spurr 1979), washed three times with sterile water, then placed on glass slides. Conidia of *E. purpurascens* and ascospores of *S. sclerotiorum* were placed on the opposite edges of the petal disks with a transfer needle. Disks inoculated with a single fungus served as controls. Slides with the inoculated petal disks then were placed in petri dishes lined with moistened filter paper for 48 h. Disks were cleared with concentrated chloral hydrate (2 g/ml) for 24 h before being observed under the microscope.

In vitro ascospore germination in the presence of *E. purpurascens*

Conidia of *E. purpurascens* were washed twice with distilled sterile water by centrifugation and adjusted to 10^6 conidia/ml. Ascospores of *S. sclerotiorum* were removed from the filters used for storage (Hunter *et al.* 1982) with sterile water and adjusted to 10^6 ascospores/ml. Dialysis membrane tubing (Spectrapor membrane tubing, m.w. cutoff 12,000 - 14,000, Spectrum Medical Industries, Inc., Los Angeles) was immersed in double

distilled water then cut into single layer segments (80 x 30 mm, slightly larger than the glass slide). Segments were autoclaved in double distilled water at 121°C for 15 min then rinsed twice using sterile water. Segments of tubing were placed on the surface of each PDA covered slide. A 100 µL conidial suspension (10^6 conidia/ml) of *E. purpurascens* was placed on the surface of the tubing and spread carefully with a glass rod. Tubing treated with 100 µL of sterile water served as a control. Slides were held in petri dishes with moistened filter paper at 20 ± 2°C for 6, 12, 18, 24, 36, 48, 72, and 96 h respectively. Tubing segments then were removed from the slides and the surfaces of the PDA on the slides were examined under the microscope (100x) to be certain that no contamination of the PDA had occurred. Ascospore suspensions of *S. sclerotiorum* (100 µl) with or without addition of 2% PDB then were placed onto the PDA surface of the slides and spread with a sterile glass rod. Percentage of ascospore germination and lengths of germ tubes were determined after 12 h incubation at 20°C. A completely randomized design with three replicates was used. Percentage of inhibition was calculated and data were arcsine-transformed before performing analysis of variance (SAS 1987). Means were separated using the LSD test.

Control of white mold with culture filtrates of *E. purpurascens*

The experiment was conducted in a greenhouse (16-25°C). Production of bean plants was as described previously (Zhou & Reeleder 1989). To obtain culture filtrates, 75 ml sterile water with 0.01 % Tween 80 was added to each flask of 50 ml *E. purpurascens* wheat seed culture (Zhou & Reeleder 1989), and stirred magnetically for 20 min. The liquid then was poured through two layers of cheese cloth. These procedures were repeated four times providing about 300 ml of conidial suspension from each flask. After adjusting the spore concentration as required (Table 6.1), one-half of the suspension was filtered through a vacuum funnel with Whatman No. 1 filter paper. The resulting filtrate then was passed through a sterilization filter unit (0.45 µm, Nalge Co., Rochester, NY) prior to

application to plants.

Plants were sprayed with conidial suspensions and/or filtrates either prior to or after inoculation of plants with ascospores of *S. sclerotiorum*. When treatments were applied before ascospores, the first application was made when more than 80% of plants had at least one open flower. Plants were sprayed again 4 days later, followed by inoculation with ascospores the next day. When treatments were applied following inoculation of plants with ascospores, applications were made 2 h after inoculation with ascospores and then again 2 days later. Plants sprayed with water plus 0.01% Tween 80 served as controls. Maintenance of treated plants, recording of results and data analyses were as described previously (Zhou & Reeleder 1989).

Extracts from culture filtrates

Double distilled sterile water (100 ml) was added to 50 ml of wheat seed culture, and mixed in a blender for 2 min. The resulting suspension was vacuum-filtered (Whatman No. 1) to remove conidia, mycelium and other solid materials. The filtrates then were transferred to a separatory funnel and extracted three times with ethyl acetate (J. T. Baker Chemical Co., Phillipsburg, NJ) with a ratio of filtrate to ethyl acetate of 1:0.8 (v/v). The organic phases were pooled, transferred to a round bottom flask, and dried down with a rotary evaporator at 35°C. The extract was removed by rinsing the flask three times with about 3 ml methanol. The rinsates were pooled and transferred to a screw-cap tube and dried down completely under flowing nitrogen. The dry material was redissolved using 5 ml of methanol for each 50 ml of wheat seed culture. Extraction of sterile wheat seed medium (without *E. purpurascens*) provided control materials. The extracts were tested for their activities against *S. sclerotiorum* by two means: **a) ascospore germination** - Known volumes of extracts from filtrates were dried down under nitrogen and redissolved in the same amount of double distilled sterile water by sonicating for 2 min. An equal volume of methanol was treated similarly and served as an

additional control. Various dilutions of extracts (From 0 - 0.5) were prepared by adding sterile water, then combined with an ascospore suspension in 2% PDB to provide for final concentrations of 10^6 ascospores/ml in 1% PDB. Ascospore germination assessment was carried out using ceramic ring slides (Clay Adams, Division of Beckton, Dickinson and Co., Parsippany, NJ). Suspensions (40 µl) were placed in each ring using a completely randomized design with three replicates of each extract dilution. After incubating in a plastic container lined with water-moistened filter paper at room temperature for 12 h, ascospore growth was stopped by adding a drop of formaldehyde to each ring. Percent germination and length of germ tubes then were recorded. **b) mycelial growth** - This test was conducted on PDA-covered slides. A block (2 X 2 mm) cut from a 6 day-old PDA culture of *S. sclerotiorum* was transferred to one end of the slide. Two days later a well (5 mm in diam.) was cut from the opposite end (4 cm from inoculum). Extracts (20 µL) from wheat seed medium or from culture filtrates were placed in the well. Wells containing methanol served as controls. Methanol in the extracts was permitted to evaporate and then slides were placed in humid chambers at room temperature for 2 additional days. At this time, *S. sclerotiorum* on the control slides had grown past the well. The width of the inhibition zone around the well was measured and the hyphae were observed microscopically. Data were analyzed with the F-test, followed by mean separation with the LSD test (SAS 1987). Percent data were arcsine-transformed.

Germination of conidia and ascospores on petal disks

Petal disks were prepared as previously described. Preparations of spore suspensions of *E. purpurascens* and *S. sclerotiorum* were as described previously for the *in vitro* test, however, Tween 80 (0.01%) was added to both suspensions. Disks were sprayed with conidial suspensions of *Epicoccum*, ascospore suspensions or mixtures of the two (1:1) using a chromatographic sprayer. The treated disks were placed on glass slides

and incubated in petri dishes lined with water-moistened filter paper at room temperature for 4 or 8 h. Germination of conidia and/or ascospores was recorded after the disks had been cleared with concentrated chloral hydrate for 24 h. Data were analyzed with the F-test, followed by orthogonal contrast comparisons (SAS 1987).

Effects of flower colonization by E. purpurascens on development of white mold

Bean plants were placed on a growth bench (day/night temperatures of 24 and 22°C, respectively, with a 14 h photoperiod) until at least 70% of the flowers on each plant were open (45 days after seeding). All emerging and senescent flowers were removed, leaving only newly-opened flowers on the plant. The plants were sprayed with conidial suspensions of *E. purpurascens* prepared as previously described, or with 0.01% Tween 80 as a control, then were placed separately in two dew chambers for 48 h (23°C 100% RH) before being returned to the growth bench. Flowers colonized by *E. purpurascens* and those on control plants were removed after an additional 48 h. Half of the flowers from each group were placed on a metal screen in a petri dish then autoclaved at 121°C for 15 min. Autoclaved and non-autoclaved flowers, previously colonized by *E. purpurascens*, and similar non-colonized flowers from control plants, were randomly attached with small pieces of label tape (Shamrock, Bellwood, Ill.) onto leaflets of 40 day-old bean plants from which all flowers had been removed previously. Two flowers (one colonized by *E. purpurascens*, and the other a related control) were attached to each leaflet. Each leaflet was considered as an experimental unit. Healthy leaves in the middle of the plant canopy were used. Plants were inoculated with a suspension of ascospores (10^6 ascospores/ml + 0.01% Tween 80) then air-dried for 1 h. Plants then were divided into two groups having equal representation of each treatment. The two groups of plants were sprayed again with either 2% PDB or 0.01% Tween 80. All spray applications were made with a hand-held pump sprayer with the spray directed mainly towards

attached flowers although the entire foliage was sprayed. The amount applied was controlled so that plants received as much as possible but spray did not run off. Data were recorded after the plants had been incubated in a dew chamber (20°C 100% RH) for 3 days. Numbers and diameters of lesions associated with flowers were determined. Percent inhibition of lesion size was calculated using the formula: Inhibition (%) = 100*(C-T)/C, where C was lesion size on leaflets with control flowers and T was lesion size on leaflets with *E. purpurascens*-colonized flowers. For data on number of lesions, the McNemar test was used to separate treatment means (Daniel 1978). Data for inhibition of lesion size were analyzed using ANOVA and the LSD test (SAS 1987).

All experiments were carried out at least twice, and data from trials of an experiment were pooled, where possible, prior to analysis.

Results

Hyphal interactions between *E. purpurascens* and *S. sclerotiorum*

Colonies of *E. purpurascens* grew more rapidly and were more dense on PDA and MA than on CSA and WA. Red to red-brown droplets were observed in the middle and edge of the colonies. Some droplets adhered to hyphae, other were free in the medium. More droplets were observed in colonies on PDA and MA than on CSA, and none were seen on WA. Hyphal tips of *E. purpurascens* were colourless or light yellow. *S. sclerotiorum* also grew more rapidly and had more dense mycelium on PDA and MA than on other media. Hyphal tips of *S. sclerotiorum* in control cultures were colourless.

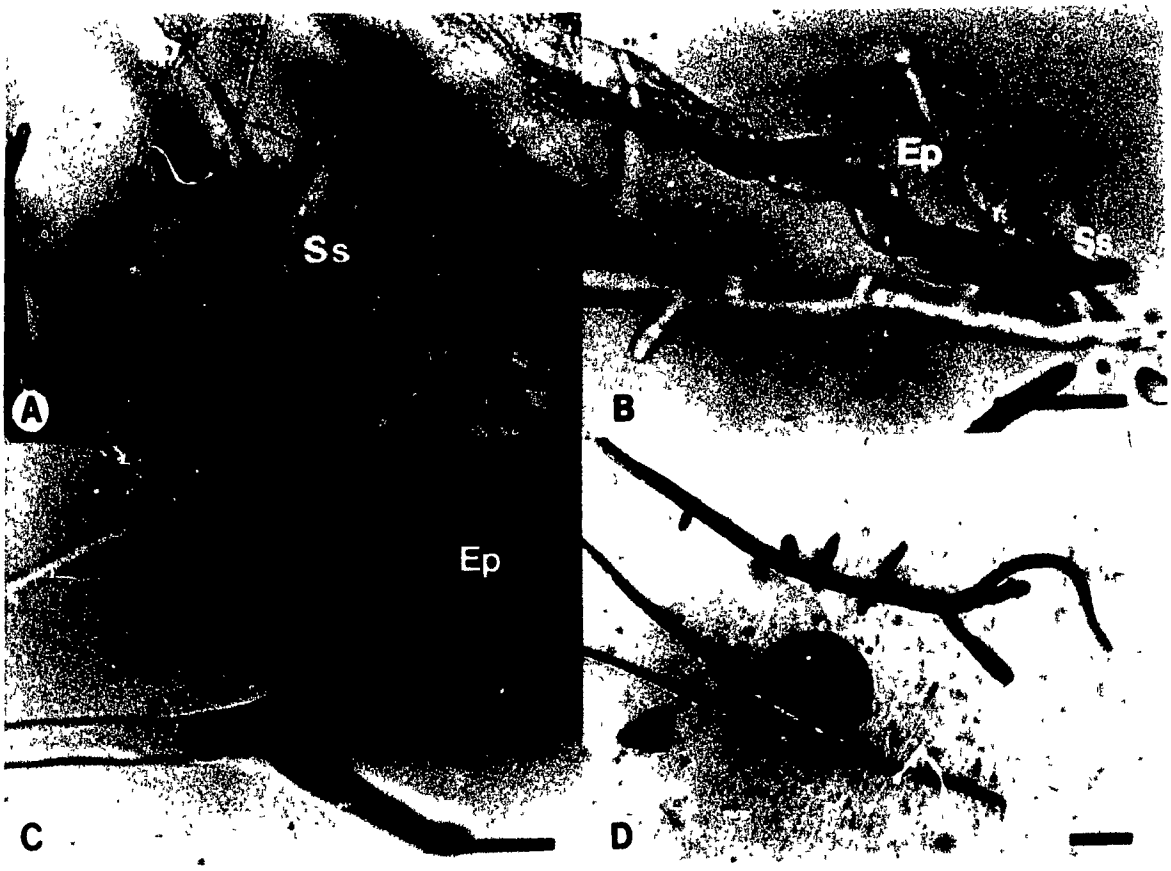
In dual cultures, occurrence of inhibition zones was dependent upon the agar medium used and whether the two fungi were seeded onto the dual culture dish simultaneously or with the 3 day interval. When *E. purpurascens* was seeded 3 days in advance of *S. sclerotiorum* in PDA and MA dual cultures, an inhibition zone (1-5 mm) formed between the two colonies. When cultures of the two fungi were started at the same time on

PDA and MA, no inhibition zone was present and colonies appeared to have met. On CSA and WA, inhibition zones were absent, regardless of time of seeding of *E. purpurascens*. On CSA, *S. sclerotiorum* grew over the colony of *E. purpurascens*.

Areas on slides where colonies of the two fungi met or grew close together were observed for hyphal interactions. On PDA, MA and CSA, hyphal tips of *S. sclerotiorum*, generally did not contact hyphae of *E. purpurascens* but became orange to red-brown in colour, were malformed in shape (Fig. 6.1 A), and had increased branching. Branches were shorter and thicker than on control slides. Yellow or orange droplets were observed in many *Sclerotinia* hyphae and some hyphae with droplets had several (2-5) empty or partially empty cells near the hyphal tips. Cytoplasm of the hypha between these empty cells and the tip was disorganized and clumped. In the area of hyphal contact, hyphae of *S. sclerotiorum* occasionally were enmeshed by coiling hyphae of *E. purpurascens* (Fig. 6.1 B). Hyphal contact between *S. sclerotiorum* and *E. purpurascens* (Fig. 6.1 C) and the growth of hyphae of *E. purpurascens* inside hyphae of *S. sclerotiorum* also was observed but was infrequent. Hyphal tips of *S. sclerotiorum* in contact with *E. purpurascens* became malformed (Fig. 6.1 B & C) and red to orange in colour.

On petal disks, both fungi were observed growing in the interior tissues of the disk, as well as on the surface. For interior growth, hyphae of both fungi were found in the same area, but no direct contact was observed. On the surface, hyphae of the two fungi generally were found growing in close proximity in areas where the colonies met. On some disks, aggregated hyphae (consisting of 2-4 hyphae) of *E. purpurascens* were observed growing into a colony of *S. sclerotiorum*. Hyphae of *S. sclerotiorum* were found occasionally with highly-branched tips, which were malformed and had dense cytoplasm, similar to hyphae observed *in vitro*.

Figure 6.1. **A.** Hyphae of *S. sclerotiorum* (Ss) in the area near the colony of *E. purpurascens* (Ep). **B.** Hyphae of *S. sclerotiorum* surrounded by coiling hyphae of *E. purpurascens*. **C.** Hyphae of *E. purpurascens* in contact with hyphae of *S. sclerotiorum*. **D.** Hyphae of *S. sclerotiorum* in the area near the well to which extracts from culture filtrates of *E. purpurascens* were added. The horizontal bars = 50 μm.


Ss
A
Ep
B
Ep
C
D

Ascospore germination in the presence of *E. purpurascens*

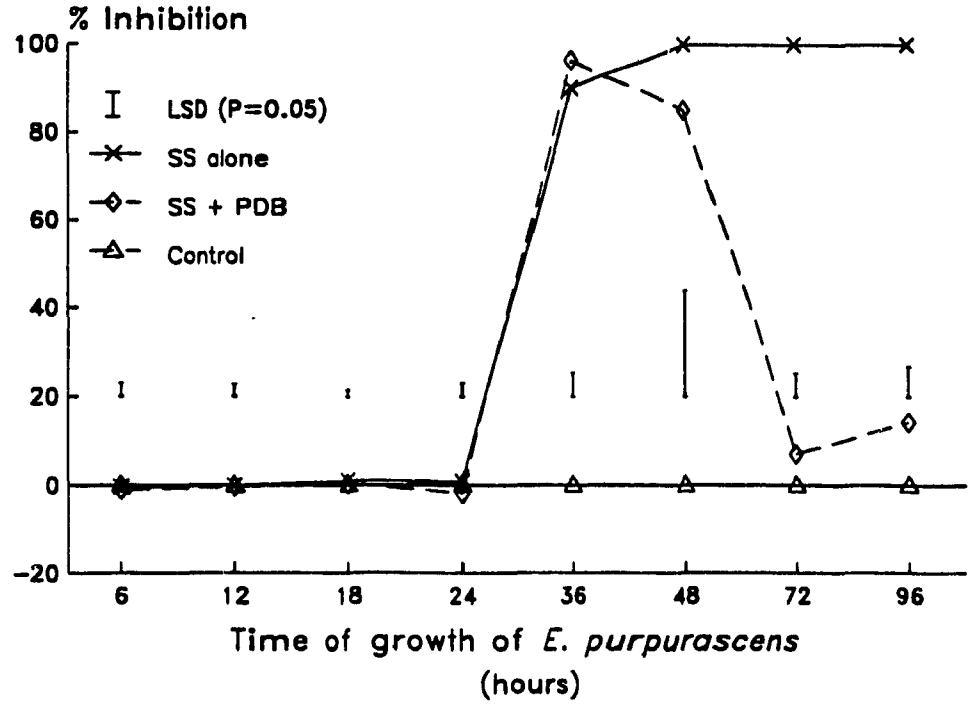
Six hours after conidia of *E. purpurascens* were applied to the tubing surface on PDA covered slides, more than 90% of conidia had germinated. Colour of the tubing surface and PDA surface did not change until 12 h after application of *E. purpurascens*. By 18 h, the membrane surface had become pink to yellow in colour. These colours were observed also on the PDA surface after the tubing with *E. purpurascens* was removed. Newly-formed conidia were observed on the membrane tubing. Colonies of *E. purpurascens* fully covered the tubing segment 36 h after application and colours on both the tubing segments and the PDA medium were darker than previously. Pigment intensity and numbers of conidia both increased as the incubation period was increased.

When ascospore suspensions were applied without PDB, germination of ascospores was significantly inhibited on the slides on which *E. purpurascens* had been grown for 36 h or more, compared to control slides and slides where *E. purpurascens* was present for shorter periods (Fig. 6.2 A). Similar results were found for elongation of germ tubes (Fig. 6.2 B), except that significant inhibition of germ tube elongation was found on the slides where *E. purpurascens* had been grown for only 24 h, although the inhibition was not as strong as that for longer time periods.

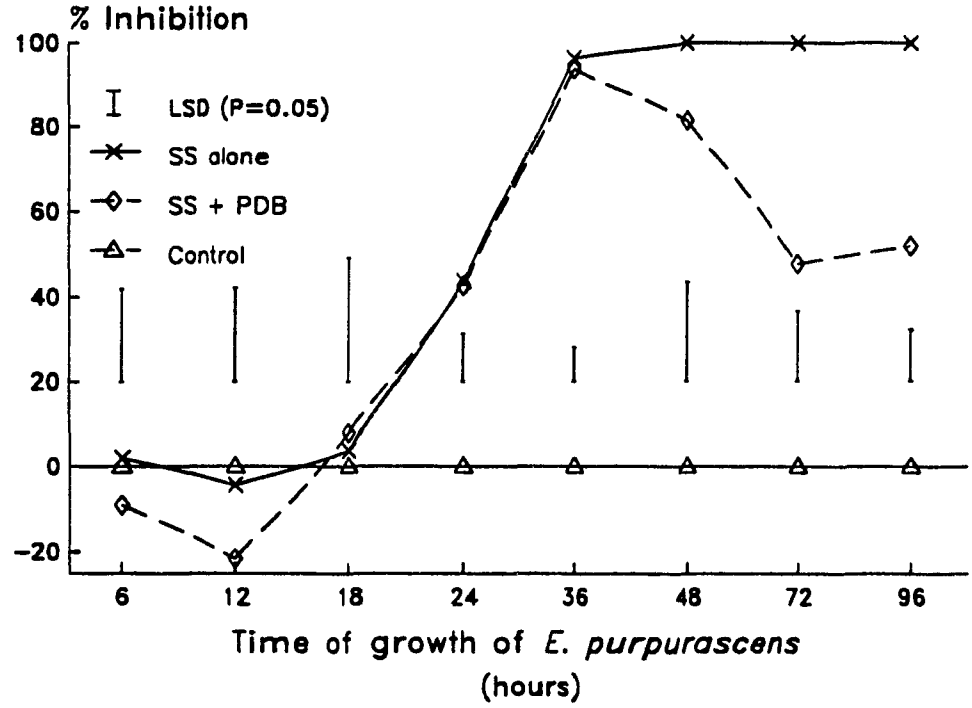
When ascospores were applied with 2% PDB, significant inhibition of ascospore germination was found only on slides where *E. purpurascens* had been grown for 36 and 48 h (Fig 6.2 A). Significant inhibition of elongation of germ tubes was obtained from the slides on which *E. purpurascens* had been grown for 24 h or more (Fig. 6.2 B). However, unlike the treatment where ascospores were applied without PDB, less inhibition was found on slides on which *E. purpurascens* had been grown for 72 and 96 h as compared to 36 and 48 h (Fig. 6.2 A). In treatments where the germination of ascospores was inhibited, ascospores and their germ tubes were found to be light red to brown in colour. This was especially the case for slides where no germination of ascospores occurred. No colour change was observed in ascospores or germ tubes on control slides.

Figure 6. 2. Ascospore germination of *S. sclerotiorum* in presence of *E. purpurascens*. Ss: *S. sclerotiorum;* Vertical bars are LSD values (p=0.05). A. Percent germination. B. Length of germ tube. Control of white mold with culture filtrates of *E. purpurascens*

A.


% Inhibition
100
80
60
40
20
0
-20
LSD (P=0.05)
SS alone
SS + PDB
Control
6
12
18
24
36
48
72
96
Time of growth of E. purpurascens
(hours)

B.


% Inhibition
100
80
60
40
20
0
-20
LSD (P=0.05)
SS alone
SS + PDB
Control
6
12
18
24
36
48
72
96
Time of growth of E. purpurascens
(hours)

Control of white mold with culture filtrates of *E. purpurascens*

Treatments containing conidial suspensions of *E. purpurascens* significantly reduced the number of white mold lesions per plant compared to the control (Table 6.1). In terms of percentage of diseased tissue, values for culture filtrates of *E. purpurascens* were lower than those for the control, but they were not as low as for treatments with conidial suspensions of *E. purpurascens*. Application of conidia or filtrates before inoculation with ascospores significantly increased yield, however, yields of conidial suspension treatments were much higher than those of filtrate treatments.

Extracts of culture filtrates

Ascospore germination was inhibited when combined with one-half or one-fourth dilutions of extract from culture filtrates of *E. purpurascens* (Fig. 6.3). No inhibition was observed for one-tenth or one-twentieth dilutions. For germ tube length, inhibition was found for all dilutions of the filtrate extract although less diluted extracts provided greater inhibition. Extracts from sterile wheat seed medium caused slight inhibition at some concentrations (0.5 and 0.05, Fig. 6.3). For the mycelium growth test, inhibition zones (2-5 mm) were found around the wells containing extract from culture filtrate but not from sterile wheat seed filtrate or the methanol control. Some hyphae around the inhibition zone were malformed or lysed (Fig. 6.1 D) and became dark yellow to orange in colour.

Germination of conidia and ascospores on petals

Four hours after inoculation, more conidia of *E. purpurascens* had germinated than ascospores of *S. sclerotiorum* although percentages of germination for both fungi were similar at 8 h (Table 6.2). Germ tube length of conidia of *E. purpurascens* were 2.6 times longer than those for ascospores of *S. sclerotiorum* at 4 h and 3.4 times longer at 8 h. In addition, mycelial growth of both *E. purpurascens* and *S. sclerotiorum* showed inhibition in dual culture compared to growth rates when applied alone. However inhibition of *S. sclerotiorum* was greater (23.4%) than inhibition of *E. purpurascens* (12.5%).

Table 6.1. White mold control by conidia and culture filtrates of *E. purpurascens*

Treatment[v]	Date of treatment[w]	Number of lesions[x]	Diseased tissue (%)[y]	Yield (g)[y]
Water	19,23 Jan.	12.4 a	100.00 a	1.0 a
E. purpurascens culture filtrate(10^6 conidia/ml)	24,26 Jan.	9.0 a	84.44 b	4.1 ab
E. purpurascens culture filtrate(10^7 conidia/ml)	19,23 Jan.	7.3 ab	72.22 bc	9.2 bc
E. purpurascens culture filtrate(10^6 conidia/ml)	19,23 Jan.	6.4 ab	63.33 c	14.4 c
E. purpurascens conidia (10^6 conidia/ml)	19,23 Jan.	1.0 b	8.33 d	54.2 d

[v]Tween 80 (0.01%) was added to all applications.

[w]Beans were planted on 8 Dec. 1989. The first applications (19 Jan.) were made when 80% of plants had at least one open flower. All plants were inoculated with ascospores of *S. sclerotiorum* on 24 Jan. Treatment applied on this date were made two hours after inoculation with ascospores.

[x]Numbers of lesions per plant were determined 6 days after inoculation and analyzed using Freidman analysis of variance. Values (means of 3 blocks) followed by the same letter are not significantly different (multiple comparison test, experimentwise error rate = 0.3).

[y]Percentage of leaf and stem area diseased and weights of fresh pods, respectively. Values (means of 3 blocks) in a column followed by the same letter are not significantly different by Duncan's multiple range test (p=0.05). Percentage data of disease tissue were arcsine-transformed before analysis.

Figure 6. 3. Ascospore germination of *S. sclerotiorum* in the presence of sterile extracts of culture filtrates of *E. purpurascens*. EPWS: extract from culture filtrate of *E. purpurascens* on wheat seed medium; WS: extract from sterile wheat seed medium. Vertical bars are LSD values (p=0.05) for percent germination (left) and length of germ tube (right).

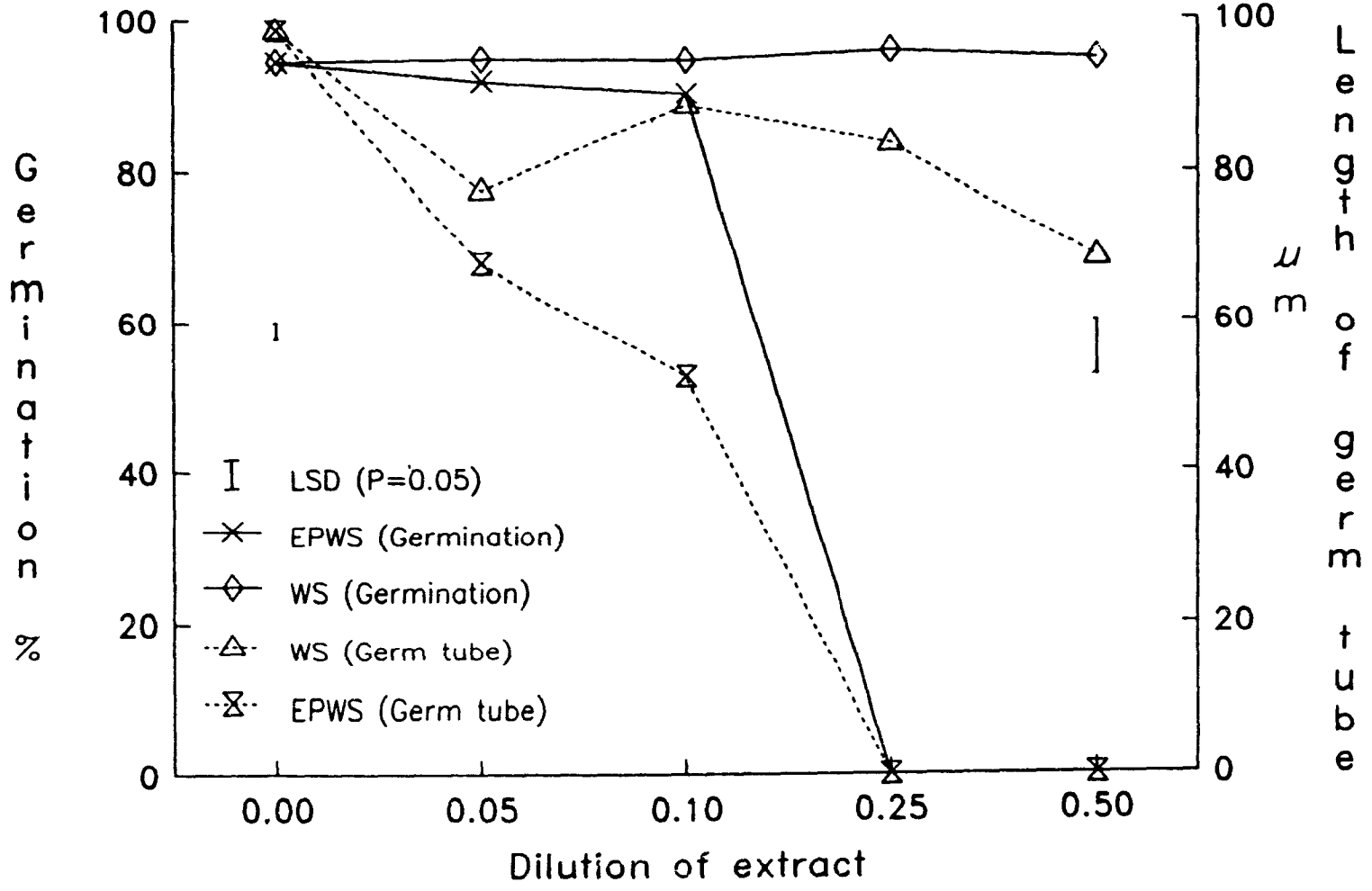

Germination %
Length of germ tube
µm
100
80
60
40
20
0
0.00
0.05
0.10
0.25
0.50
Dilution of extract
LSD (P=0.05)
EPWS (Germination)
WS (Germination)
WS (Germ tube)
EPWS (Germ tube)

Table 6.2. Germination of conidia and ascospores on petal disks of bean flowers[y]

Fungi	Time	Germination (%)		Germ tube (um)	
		Alone[z]	Combin.[z]	Alone	Combin.
	(h)				
E. purpurascens	4	85.7 a	89.5 a	45.4 a*	37.8 a
S. sclerotiorum	4	80.8 b	78.7 b	17.8 b	14.3 b
E. purpurascens	8	90.6 a	89.8 a	167.1 a*	146.2 a
S. sclerotiorum	8	87.9 a	87.0 a	55.9 b*	42.8 b

[y]Values in the table are means of 10 disks, pooled from two trials. Values for a given time period followed by the same letter in a column (paired data) are not significantly different. Values with a "*" are significantly greater than the other paired data in the row (orthogonal contrast test; p=0.05).

[z]"Alone" indicate that data were recorded from the petal disks sprayed with either of the fungi alone. "Combin." indicate data were collected from the disks sprayed with mixtures of suspensions of the two fungi.

Effects of flower colonization by *E. purpurascens* on development of white mold

All treatments with flowers colonized by *E. purpurascens* resulted in low numbers of lesions on leaves and in significant inhibition of lesion development when compared to controls (Table 6.3). When autoclaved colonized flowers were used, more lesions appeared when 2% PDB was applied than when 0.01% Tween 80 was used. PDB did not have an effect on initiation of white mold when flowers with living *E. purpurascens* colonies were used. When plants received 2% PDB following inoculation with *S. sclerotiorum*, the use of autoclaved colonized flowers resulted in significantly higher numbers of lesions than the use of flowers with living *E. purpurascens* colonies. This difference did not appear when only water was applied. Use of autoclaved colonized flowers plus PDB resulted in less inhibition of lesion development than did non-autoclaved flowers.

Discussion

Contact and penetration of hyphae of *S. sclerotiorum* by *E. purpurascens* occurred only rarely on medium-covered slides and was not observed on petal disks. Malformed hyphae and leakage of cytoplasm of *S. sclerotiorum* were commonly observed in agar cultures where hyphae of the two fungi were in the same region but not in contact. This suggests that *E. purpurascens* secretes compounds influencing membrane permeability (Bamford *et al.* 1961; Brown *et al.* 1987). The pigmented droplets observed in single cultures of *E. purpurascens* and in dual cultures may be a source of such compounds.

The use of dialysis membrane allowed inhibitory compounds secreted by *E. purpurascens* to pass through into PDA. Inhibition of ascospore germination probably was due to antifungal compounds secreted by *E. purpurascens*. However, when PDB was added to the ascospore suspension, inhibition of ascospore germination decreased greatly after 36 h. Spores may be more susceptible to inhibitors when nutrient availability is low (Wicklow 1981) and production of antifungal compounds by fungi may vary

Table 6.3. Effect of autoclaving flowers colonized by *E. purpurascens* on occurrence of white mold on bean leaves

Flowers colonized by *E. purpurascens*	Number of lesions[y]		Inhibition of lesion development (%)[z]	
	PDB	Water	PDB	Water
Autoclaved[x]	7 a* (11)	2 a (12)	81.2 a	94.7 a
Not autoclaved	0 b (12)	1 a (12)	100.0 b	98.7 a

[x]Colonized flowers were autoclaved at 121°C for 15 minutes. All leaves with attached flowers were inoculated with ascospores of *S. sclerotiorum* before 2% PDB or water was applied. Tween 80 (0.01%) was added to all applications. There were 12 leaflets for each treatment; a colonized flower and a non-colonized control flower were attached to each leaflet.

[y]Numbers of white mold lesion were determined 3 days after inoculation of *S. sclerotiorum*. The data were analyzed using the McNemar test. Values followed with the same letter in a column are not significantly different. Values with "*" are greater than the paired value in the row (p=0.05). Numbers in the brackets are values for related non-colonized controls.

[z]Inhibition of lesion development was calculated using the formula: Inhibition (%) = 100 x (C-T)/C (C=size of lesion with control flowers, T=size of lesion with flowers colonized with *E. purpurascens*). Values are means of 12 leaves and those followed with the same letter are not significantly different (LSD, p=0.05).

with nutrient status and age of the fungus (Bu'lock *et al.* 1974). At the points with highest inhibition, accumulation of antifungal compounds may have reached the highest values while available nutrients in PDA may have been at the lowest level. When ascospores were applied without PDB, inhibition remained high since ascospores lacked nutrients and were thus very susceptible to antifungal compounds. When added, PDB may provide a supplemental nutrient source. In addition, compounds accumulated in the medium may break down over time (Wicklow 1981; Boudreau & Andrews 1987). Thus, addition of PDB provided a nutrient source which could be utilized once existing antifungal compounds had degraded sufficiently to reduce inhibition. The observations described here imply that nutrient supply may also be a factor in inhibition of *S. sclerotiorum* by *E. purpurascens*.

Culture filtrates of *E. purpurascens* decreased the percentage of diseased tissue and increased pod yield when applied to bean plants prior to inoculation of plants with *S. sclerotiorum*. Antifungal compounds in the culture filtrate likely were responsible for reductions in disease. However, control of disease by the culture filtrate was poorer than that resulting from application of conidia. This may be due to low concentrations of antifungal compounds in the wheat seed culture. As mentioned earlier, degradation of antifungal compounds over time also may be a factor. After conidia were applied, antifungal compounds will be produced continuously as *E. purpurascens* colonizes the flower tissue. Thus, the degree of inhibition of *S. sclerotiorum* would be expected to be greater than that which occurred when filtrates were applied. Moreover, other mechanisms, such as competition, may be involved in the control of white mold following conidium application.

On PDA or MA, *S. sclerotiorum* grew faster than *E. purpurascens* (data not shown). However, in these studies germ tubes of *E. purpurascens* elongated more quickly than those of *S. sclerotiorum* on flower petal disks. Fast growth is an advantageous attribute in competition between microorganisms (Lockwood 1981). For *E. purpurascens*, this probably is a

consequence of its large and multiple-celled conidia and an ability to utilize more quickly than *S. sclerotiorum* the nutrients available from flower diskes. Hyphal growth of both fungi was reduced when they were sprayed simultaneously onto the disk. A similar phenomenon was noticed elsewhere (Manandhar *et al.* 1987). In our case, however, *S. sclerotiorum* was inhibited much more than *E. purpurascens*.

Colonization of flowers by *E. purpurascens* led to large reductions in white mold incidence. When supplemental nutrients were applied, a higher frequency of white mold was found originating from autoclaved flowers previously colonized by *E. purpurascens* compared to those from flowers with living *E. purpurascens*. Both treatments, however, resulted in less disease than their controls. As with the ascospore germination test, these data indicate that colonization of flowers by *E. purpurascens* results in utilization of nutrients by *E. purpurascens*. As nutrients were consumed, antifungal compounds secreted by *E. purpurascens* accumulated in the flowers. When PDB was supplied to the flowers, it may not only have provided nutrients for ascospores but also increased their tolerance to antifungal compounds (Wicklow 1981). In the case of flowers with living colonies of *E. purpurascens*, consumption of the supplied nutrients by *E. purpurascens* (Bashi 1977) probably stimulated further increases in antifungal compound production and increased the sensitivity of nutrient-deprived hyphae of *S. sclerotiorum* to the compound. Thus, inhibition of germination and growth of *S. sclerotiorum* by *E. purpurascens* was kept at a high level. In contrast, inhibition greatly decreased in the case of autoclaved flowers previously colonized by *E. purpurascens* since *Epicoccum* was neither utilizing PDB nor producing antifungal compounds.

It is doubtful that mycoparasitism is important in the interactions observed here. Although hyphal invasion was observed on medium-covered slides, the frequency of direct contact between the two fungi was very low. The penetration of *S. sclerotiorum* was not seen on flower petal disks of bean or on leaf disks of lettuce (Mercier & Reeleder 1987). It

appears that antifungal compounds are important factors in control of white mold by *E. purpurascens*. Reactions of hyphae and ascospores of *S. sclerotiorum* to crude extracts were similar to those observed in dual culture tests, suggesting that the crude extracts contained inhibitory compounds. However, since *S. sclerotiorum* requires exogenous nutrients for infection of healthy plant tissue (Natti 1971) and an extended period of surface wetness for colonization of bean flowers (Abawi & Grogan 1975), competition for nutrients and also for space may play roles in biocontrol. This appears to be the case for some other antagonists of *S. sclerotiorum* (Boland & Hunter 1988). We observed that the frequency of disrupted and malformed *Sclerotinia* hyphae was less on petals than in dual culture. Thus, effects of the antifungal compounds observed *in vitro* may be of less importance *in vivo*. However, we believe that when *E. purpurascens* has colonized flowers prior to arrival of ascospores the levels of antifungal compounds would be higher than in the petal disks used in our tests. Under such conditions, antifungal compounds may be more important although flowers colonized by *E. purpurascens* likely will have less nutrients available to support growth of *S. sclerotiorum* than non-colonized flowers. More direct tests on competition (Blakeman 1978) between the two fungi may prove useful, as would studies on the effects of nutrients on production of antifungal compounds by *E. purpurascens*.

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Connecting Text

Culture filtrates of *E. purpurascens* decreased severity of white mold of bean and increased pod yield when applied prior to inoculation of beans with *S. sclerotiorum*. Extracts of these culture filtrates inhibited ascospore germination and mycelial growth of *S. sclerotiorum*. Similar inhibition of hyphal growth of *S. sclerotiorum* occurred in dual cultures. Contact and penetration of hyphae of *S. sclerotiorum* by hyphae of *E. purpurascens* occurred rarely on medium-covered slides and was not observed on bean petals. Inhibition of ascospore germination and germ tube elongation occurred on medium-covered slides where *E. purpurascens* had been grown previously. However, the addition of nutrients decreased this inhibition. Colonization of bean flowers by *E. purpurascens* reduced white mold incidence. The degree of disease control was affected by addition of nutrients and whether or not colonized flowers were autoclaved. These results from the previous section indicated that production of inhibitory compounds was the most important factor in control of white mold by *E. purpurascens* and competition for nutrients also played a role in biological control. Thus, control of white mold by *E. purpurascens* may be enhanced by providing appropriate nutrients to promote growth of *E. purpurascens* and to improve its production of compounds inhibitory to *S. sclerotiorum*. The studies described in the next section investigated the effects of various carbon compounds and amino acids on the conidial germination, mycelial growth, sporulation and production of inhibitory compounds by *E. purpurascens*. This study was conducted to identify nutrients that could optimize the performance of *E. purpurascens*.

7. Influence of nutrients on *Epicoccum purpurascens*

Introduction

The activity of biocontrol agents can be enhanced by providing appropriate substrates to protect and/or stimulate their development as antagonists (Baker 1985; Baker & Scher 1987; Burge 1988). The substrates may be 'stickers' or 'spreader-stickers', which can help the antagonists maintain high population levels on the plant surface, or chemical 'triggers' or nutrients, which can initiate and/or stimulate spore germination, promote growth, and regulate production of metabolites, thus improving colonization and competitive abilities of antagonists (Sussman & Halvorson 1966; Cullen & Andrews 1984). Populations of *Sporobolomyces roseus* Kluyver and Van Niel increased when wheat leaves were sprayed with a 2% sucrose plus 1% yeast extract solution (Bashi and Fokkema 1977). Potential antagonists might be selectively stimulated by the timely application of the appropriate nutrients if the nutritional requirements of phylloplane microbes were better understood (Cullen & Andrews 1984). In this regard, application of particular amino acids to bean leaf surfaces resulted in alterations in the composition of bacterial communities, which reduced infection of leaves by the pathogen *Pseudomonas syringae* van Hall (Morris & Rouse 1985). Knowledge of the nutritional requirements of biocontrol agents also is important for their mass production which is a requirement for their commercial use.

White mold, incited by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a serious disease on bean (Tu 1984). *Epicoccum purpurascens* Ehrenb. & Schlecht. suppresses white mold development on bean plants (Boland & Inglis 1989; Zhou & Reeleder 1989) and, the disease suppression appears to result from colonization of senescent plant tissue, which denies this substrate to *S. sclerotiorum*. Bonnel and Levetin (1981) found that

nutrient requirements of *E. purpurascens* differ from strain to strain. Production of pigments by *E. purpurascens* is affected by the type of nutrients supplied (Eka 1970). Addition of malt extract increases the percentage of conidial germination *in vitro* (Zhou & Reeleder 1990) and promotes sporulation of *E. purpurascens* on bean flowers (Section 5). However, the efficiency of white mold control does not increase in field trials when malt extract is added to conidial suspensions of *E. purpurascens* (Zhou & Reeleder 1989). The attractiveness of *E. purpurascens* as a biological control agent would be increased if a nutrient 'mix' could be shown to markedly increase its efficiency. The objectives of this study were to determine effects of nutrients on *E. purpurascens* regarding conidial germination, mycelial growth, sporulation and production of antifungal compounds.

Materials and Methods

Maltose, ß-D(-)fructose, L-sorbose, D-mannitol, myo-inositol, erythritol, and carboxymethyl cellulose, which were obtained from SIGMA (St. Louis, MO), dextrose and sucrose, obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ) and, D-xylose and soluble starch, obtained from Fisher Scientific Co. (Fairlawn, NJ), were used as carbon sources. DL-alanine, L-aspartic acid, L-glutamic acid, glycine, DL-histidine, DL-lysine, DL-methionine, L-phenylalanine, obtained from SIGMA, L-(+)-arginine and DL-valine, ontained from Eastman Kodak Co. (Rochester, NY), and DL-tryptophan, obtained from ICN NBCo Biochemicals (Division of ICN Biomedicals, Inc. Cleveland, OH), were the amino acids tested. Media used in the study included corn meal agar (CMA) Czapek solution agar (CSA), malt agar (MA), potato dextrose agar (PDA), yeast extract agar (YA), Czapek solution broth (CSB), 3% malt extract (ME), and potato dextrose broth (PDB), which all were from Difco (Detroit, MI). V8-juice agar (V-8A) was made using 200 ml V8 juice, 3 g $CaCO_3$ and 15 g agar in 1 litre distilled water.

E. purpurascens strains M-20-A and R4000 (Zhou & Reeleder 1990) were used in this study. Conidia of *E. purpurascens* were obtained from wheat seed medium (Zhou & Reeleder 1989,1990). Ascospores of *S. sclerotiorum* (isolate MACF-152) were produced and maintained as described previously (Mercier & Reeleder 1987) . This same isolate also was used in hyphal growth tests. *Ulocladium atrum* Preuss (MACF-127) was cultured on V-8A at room temperature until conidia covered most of each plate (7-10 days).

Media preparation.

With the various carbon and amino acid sources, media were prepared using 10 g carbon source, 2 g amino acid, 15 g Bacto agar (Difco) added to 1 litre of element solution, which contained $MgSO_4$, 0.5 g; KCl, 0.5 g; K_2HPO_4, 1.0 g, and 2 ml micronutrient solution (Stevens 1974). Carbon sources (except soluble starch) and amino acids were dissolved separately in distilled deionized water in a concentration 4 times that required in the final medium and sterilized with a 0.2 µm ultrafilter (Nalge Company, Rochester, NY). Bacto agar (2 times final concentration) was added to the concentrated element solution and autoclaved at 121 °C for 15 min. This solution was placed in a water bath at 75 °C after autoclaving, and was magnetically stirred as the carbon and amino acid sources were added. Solutions of carbon source, amino acid and the element solution were mixed in a flask in a ratio of 1:1:2. Soluble starch was autoclaved with the agar and element solution. Dehydrated commercial media were prepared according to label directions. Each medium (15 ml) was poured into sterile plastic petri dishes (100 x 15 mm).

Conidial germination.

Conidia of *E. purpurascens* strain R4000 from a 15 to 20-day-old wheat seed culture were washed twice with sterile distilled water by centrifugation. Washed conidia were suspended in filter-sterilized sodium phosphate buffer (pH 6.0, 0.01 M) at a concentration of 10^6 conidia/ml. The compounds to be tested were dissolved in the buffer at a concentration of 400 µg/ml for the carbon source and/or 200 µg/ml for the amino acid. The

solutions were filter-sterilized before they were mixed with conidial suspension at 1:1 ratio. The final suspensions contained $5x10^5$ conidia, 200 µg carbon source and/or 100 µg amino acid per ml. Conidial suspensions mixed with sterile water served as controls. Conidial germination tests were carried out on ceramic ring slides (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ) with 30 µl of conidial suspension in each ring. Each test was arranged as a completely randomized design with three replicates. The slides were placed in a closed plastic box lined with moistened filter paper at 20 °C without light for 4-6 h. Conidial germination was stopped by adding a drop of formaldehyde (20%). For each replicate, at least 100 conidia were observed for percentage of germination and lengths of 30 germ-tubes were determined. Conidia were considered to have germinated when they had germ tubes longer then their radii. For conidia with more than one germ tube, only the longest was measured .

Tests on nutrient-amended media.

E. purpurascens strain R4000 was cultured on PDA for 10 days, then a disk (5 mm in diam.) was cut from the edge of the colony. For radial growth and sporulation experiments, a disk was transferred to the center of a petri dish containing the medium to be tested and the petri dishes were held at 20°C. Radial growth was measured after 3, 6 and 10 days and, sporulation was assessed after 10 days by classifying each culture into one of four groups: +, less than 1/4 of the colony covered with conidia; ++, more than 1/4 but less than 1/2 covered; +++, more than 1/2 but less - than 3/4 covered; and ++++, more than 3/4 covered. For tests of inhibition of radial growth of *S. sclerotiorum*, a disk of *E. purpurascens* was placed on the periphery of the dishes. Three days later a disk (5 mm in diam.) cut from a 6 day old culture of *S. sclerotiorum* on PDA was transferred to the opposite periphery of the plate (5 cm away from the disk of *E. purpurascens*). After incubating the cultures for an additional 7 days at 20°C, the maximum radius of the colony of *S. sclerotiorum* (R1), the radius of that part of the colony of *S. sclerotiorum* (R2) directly opposite the

colony of *E. purpurascens* and the width of the inhibition zone between the two colonies were determined. The percent inhibition of radial growth of *S. sclerotiorum* was calculated as 100 x (R1 - R2)/R1. There were four replicate dishes for each medium.

Extracts of culture filtrates.

E. purpurascens (strain M-20-A) was cultured in PDB, CSB, and ME. Medium (100 ml) in a 250 ml Erlenmeyer flask was seeded with 2 ml of conidial suspension of *E. purpurascens* (10^6 conidia/ml). These flasks and flasks with sterile media (as controls) were placed on a rotary shaker (200 rpm) under ambient light at 20 ± 2 °C for 12 days. Cultures were vacuum-filtered onto Whatman No.1 filter paper to remove mycelium. The filtrates were then transferred to a separatory funnel and extracted three times with ethyl acetate (J. T. Baker Chemical Co., Phillipsburg, NJ) (section 6). Dried material of the extracts were redissolved in 5 ml of methanol for each 100 ml of culture filtrate. Extracts of sterile media were obtained using the same procedures to provide control materials.

Thin layer chromatography and spectrophotometry

UV-indicating silica gel (Polygram^R sil G) thin layer chromatography (TLC) plates (20x20 cm; 0.25 mm thickness, Brinkmann) were used to separate extracted compounds. Extracts (25 µl) were applied on 1.5 cm wide TLC lanes. All plates were developed with chloroform/methanol (95:5, v/v) and separated zones were highlighted under short and long UV light. Zones of interest on TLC plates were scrapped off and eluted with chloroform 3 times followed by methanol 3 times in a test tube (silica gel:solvents = 1:4 v/v) with sonication for 5 minutes. Silica gel was removed from the eluates by centrifugation. Pooled eluates for each of zone were dried down and redissolved in methanol up to the same volume that the extract was applied originally on the TLC plate. Eluates of silica gel from unused TLC plates were prepared as additional controls. All extracts and eluates were diluted 100 fold with methanol before their UV/Vis absorbance spectra were determined using a Beckman model DU-40 spectrophotometer (Beckman

Instruments Inc., Irvine, CA).

Assessment of antifungal activity of extracts from *E. purpurascens* culture filtrates.

Antifungal activity of the extracts were first tested directly on TLC plates by bioautography using the procedure described by Homans and Fuchs (1970) with *U. atrum* as the indicator fungus. Conidia of *U. atrum* were washed off from a PDA culture with a salt-sucrose solution (Homans & Fuchs 1970) and the conidial suspension was filtered through one layer of laboratory Kimwipes tissues. The conidial filtrate was sprayed with an air-driven glass spray bottle onto fully dried TLC plates, treated with the extracts as described previously, until a slight greyish colour appeared. The treated plate was held in darkness at room temperature and 100% RH for 3-4 days. During this period, *U. atrum* conidia germinated and resulting hyphae produced masses of brown-black conidia, giving the plate surface a grey background with regions of antifungal activity showing as white regions, where little or no fungal growth occurred.

Tests of mycelial growth of *S. sclerotiorum* were conducted on slides covered with PDA, MA or CSA as described previously (Section 6). A culture disk cut from 6 day old PDA cultures of *S. sclerotiorum* was transferred to one end of the slide and a 5-mm well was cut at the other end, in which 20 µl of extract was placed. Treated slides were then kept under 100% RH at room temperature (20 ± 2 °C) until mycelium of *S. sclerotiorum* grew past the well (3-4 days). The width of the inhibition zone around the well was measured and hyphae around the zone were observed. The effects of extracts from culture filtrate of *E. purpurascens* on ascospore germination of *S. sclerotiorum* were determined by methods described previously (Section 6).

Data analyses.

All experiments were carried out at least twice and the data were analyzed using the F-test. Percentage data were arcsine-transformed before analysis. Duncan's multiple range test and the LSD test were used to separate the means. (SAS 1987).

Results

Conidial germination of *E. purpurascens*.

Except for D-xylose, all compounds tested as carbon sources improved the percentage of conidial germination, although they varied in their promotion of germination (Table 7.1). For elongation of germ tubes, all compounds, except for L-sorbose, resulted in longer germ tubes than the controls. Sucrose, maltose, fructose, and dextrose were more effective in promoting percentage germination and elongation of germ tubes than other compounds. Addition of D-xylose did not affect percentage of germination but did increase elongation of germ tubes, while L-sorbose had positive effects on germination. For amino acids (Table 7.2), percentages of germination were higher with lysine, L-glutamic acid, glycine, DL-tryptophan, DL-alanine, and L-aspartic acid. Similar results were found for elongation of germ tubes. DL-histidine had a positive effect on elongation of germ tubes but did not significantly affect percentage of conidial germination. L-phenylalanine reduced germination. Combinations of sucrose and lysine, dextrose and lysine, maltose and tryptophan, maltose and lysine, sucrose and tryptophan, dextrose and glutamic acid, sucrose and glutamic acid, maltose and glutamic acid, starch and glutamic acid, sucrose and glycine and maltose and glycine were more effective in stimulating conidial germination than control treatments and combinations of starch and lysine, starch and tryptophan, and starch and glycine (Table 7.3). Dextrose and tryptophan, dextrose and glycine, and starch and lysine also promoted conidial germination. Combinations of starch and tryptophan, and starch and glycine had no effect on conidial germination. For elongation of conidial germ tubes, the combination of maltose and lysine was most effective, followed by dextrose and glutamic acid, sucrose and lysine, dextrose and lysine, sucrose and glutamic acid, maltose and glutamic acid, and dextrose and tryptophan. Combinations of maltose and tryptophan, sucrose and tryptophan, starch and glutamic acid, dextrose and glycine, starch and lysine did not affect elongation of germ tubes.

Table 7.1. Effects of carbon source on conidial germination of *Epicoccum purpurascens*

Compounds[x]	Germination (Relative activity)[y]	Length of germ tube (Relative activity)[y]
Sucrose	210 a	201 a
Maltose	198 ab	194 a
β-D(-) Fructose	181 bc	196 a
myo-Inositol	177 bcd	140 c
Erythritol	177 bcd	127 c
Dextrose	168 cde	193 a
Carboxymethyl cellulose	168 cde	160 b
Soluble starch	156 de	144 bc
D-Mannitol	154 de	140 bc
L-Sorbose	149 e	93 d
D-Xylose	109 f	124 c
Control	100 f	100 d

[x] 200 µg/ml was used in all tests. Control was the treatment without any addition of organic compounds to the conidial suspension.

[y] Germination tests were conducted on ceramic ring slides and data were collected 4 h after incubation at 20 °C under moist conditions. Relative activity was calculated using T/C *100 where T is measurement from each treatment and C from control. Values are means of six replicates from pooled data of two trials. Those values followed by the same letter in a column are not significantly different (Duncan's multiple range test, p=0.05). Percentage data were arcsine-transformed before analysis of variance.

Table 7.2. Effects of animo acids on conidial germination of *Epicoccum purpurascens*

Compounds[x]	Germination (Relative activity)[y]	Length of germ tube (Relative activity)[y]
Lysine	214 a	146 a
L-Glutamic acid	199 ab	148 a
Glycine	191 bc	153 a
DL-Tryptophan	174 c	138 a
DL-Alanine	166 cd	118 b
L-Aspartic acid	146 de	122 b
DL-Valine	120 ef	102 c
DL-Histidine	120 ef	137 a
L-(+)Arginine	107 f	96 c
DL-Methionine	92 f	92 c
L-Phenylalanine	54 g	88 c
Control	100 f	100 c

[x] 100 µg/ml was used in all tests. Control was the treatment without any addition of organic compounds to the conidial suspension.

[y] Germination tests were conducted on ceramic ring slide and data were collected 4 h after incubation at 20 C under moist conditions. Relative activity was calculated using (T/C)*100 where T is measurement from each treatment and C from control. Values are means of nine or six replicates from pooled data of two or three trials for percentage of germination and germ tube elongation respectively. The values followed by the same letter in a column are not significantly different (Duncan's multiple range test, p=0.05). Percentage data were arcsine-transformed before analysis of variance.

Table 7.3. Effects of carbon source and amino acids on conidial germination of *Epicoccum purpurascens*

Compounds[x]	Germination (Relative activity)[y]	Length of germ tube (Relative activity)[y]
Suc + Lys	146 a	125 bc
Dex + Lys	145 a	124 bc
Mal + Try	143 ab	96 fgh
Mal + Lys	141 ab	141 a
Suc + Try	141 ab	97 fgh
Dex + Glu	140 ab	129 b
Suc + Glu	138 ab	119 bcd
Mal + Glu	136 ab	119 bcd
Sta + Glu	135 ab	103 efg
Suc + Gly	134 ab	81 ijk
Mal + Gly	134 ab	70 k
Dex + Try	133 abc	114 cde
Dex + Gly	129 bc	108 def
Sta + Lys	107 c	97 ghi
Sta + Try	106 dc	84 hij
Sta + Gly	101 d	75 jk
Control	100 d	100 fg

[x] Concentrations of 200 µg/ml and 100 µg/ml were used for carbon source and amino acids respectively. Dex = Dextrose; Mal = Maltose; Sta = Soluble starch; Suc = Sucrose; Glu = L-Glutamic acid; Gly = Glycine; Lys = DL-Lysine; Try = Tryptophan. Control was the treatment without any addition of organic compounds to the conidial suspension.

[y] Germination tests were conducted on ceramic ring slides and data were collected 4 h after incubation at 20 C under moist conditions. Relative

(Table 7.3 continued)

activity was calculated using (T/C)*100 where T is measurement from each treatment and C from control. Values are means of six replicates from pooled data of two trials. The values followed by the same letter in a column are not significantly different (Duncan's multiple test, p=0.05). Percentage data were arcsine-transformed before analysis of variance.

Combinations of sucrose and glycine, maltose and glycine, starch and tryptophan, and starch and glycine had negative effects on elongation.

Growth and sporulation.

Radial growth of *E. purpurascens* varied from 1.4 mm to 5.8 mm per day among the media tested (Table 7.4). *E. purpurascens* grew most quickly on V-8A, followed by YA, MA, and CMA but most slowly on media with maltose and glutamic acid, dextrose and glutamic acid, and sucrose and glutamic acid. Radial growth on other media tested were intermediary. Sporulation was observed on all media. The best sporulation was observed on V-8A, YA, PDA, maltose with glutamic acid, and dextrose with glutamic acid, while the least sporulation was on CMA, CSA, sucrose with lysine, and starch with lysine. There was no correlation between growth rate and sporulation.

Inhibition of *S. sclerotiorum* by *E. purpurascens* in dual culture.

For inhibition of the radial growth of *S. sclerotiorum*, highest values were obtained on YA, PDA, MA, sucrose with glycine, V-8A, starch with tryptophan, starch with lysine, maltose with lysine, CSA, sucrose with tryptophan, dextrose with tryptophan, and dextrose with glycine (Table 7.4). The least inhibition appeared on starch with glycine, maltose with glycine, dextrose with glutamic acid and sucrose with glutamic acid. The width of the inhibition zone also varied (from 0 to 4.3 mm) among the media. On dextrose with lysine, maltose with lysine, and sucrose with lysine, the inhibition zones between the two fungal colonies were the widest, followed by sucrose with glycine, sucrose with tryptophan, dextrose with glycine, and maltose with glycine. Inhibition zones were not found on V-8A and were very narrow on starch with glycine, CSA and YA. No correlation were found between percentage of inhibition and width of inhibition zone.

Extracts of culture filtrates.

Extracts of filtrates from cultures grown in CSB, ME, and PDB were slightly yellow, light yellow-brown, and yellow-brown, respectively, while extract from sterile CSB was colourless, and the extracts from sterile ME

Table 7.4. Growth and sporulation of *Epicoccum purpurascens* and its inhibition of *Sclerotinia sclerotiorum* on agar media.

Media[w]	Radial growth (mm/day)[x]	Sporulation[y]	Inhibition (%)[z]	Width of IZ (mm)[z]
V-8A	5.8 a	++++	69.9 abc	0.0 g
YA	4.6 b	++++	74.2 a	0.3 g
MA	4.4 bc	+++	71.0 ab	1.8 cdef
CMA	4.4 bc	+	57.6 def	2.0 bcdef
PDA	3.9 cd	++++	71.4 ab	1.6 def
Sta + Gly	3.6 de	++	54.2 f	0.1 g
Dex + Try	3.5 de	++	67.2 abc	2.1 bcde
CSA	3.5 de	+	67.9 abc	0.3 g
Mal + Gly	3.5 de	+++	54.7 ef	2.6 bc
Suc + Try	3.4 def	++	67.9 abc	2.8 b
Sta + Try	3.3 defg	++	69.5 abc	1.3 f
Dex + Gly	3.3 defg	+++	66.0 abc	2.7 bc
Dex + Lys	3.1 efgh	++	64.1 bcde	4.3 a
Suc + Gly	3.0 efgh	+++	70.1 abc	2.8 b
Mal + Try	2.8 fgh	++	63.0 bcde	2.3 bcd
Suc + Lys	2.7 gh	+	63.7 bcde	3.7 a
Sta + Lys	2.6 gh	+	69.3 abc	1.3 ef
Mal + Lys	2.6 gh	++	68.2 abc	4.1 a
Sta + Glu	2.5 hi	+++	65.1 bcd	2.2 bcd
Mal + Glu	1.9 ij	++++	62.0 cdef	2.2 bcd
Dex + Glu	1.7 j	++++	56.2 ef	2.2 bcd
Suc + Glu	1.4 j	+++	62.0 cdef	1.8 cdef

[w] Dex = Dextrose; Mal = Maltose; Sta = Soluble starch; Suc = Sucrose; Glu = L-Glutamic acid; Gly = Glycine; Lys = DL-Lysine; Try = Tryptophan. In the media, concentrations of Dex., Mal., Sta. and Suc. were 10 g/l, Glu., Gly., Lys., and Try. were 2 g/l.

(Table 7.4 continued)

[x] Means of 8 replicates from two trials. Radial growth per day was calculated using the formula: $(R_{10} - R_3)/7$ where R_{10} and R_3 are radial growth after 10 and 3 days respectively. Values in a column followed by the same letter are not significantly different (Duncan's multiple range test, P=0.05).

[y] Sporulation was rated 10 days after inoculation of the media. The rates were: +, less than 1/4 of the colony covered with conidia; ++, more than 1/4 but less than 1/2 covered; +++, more than 1/2 but less than 3/4 covered; and ++++, more than 3/4 covered.

[z] Data were collected 6 days after inoculation of the medium with *S. sclerotiorum*. Percentage inhibition was calculated by 100 x (R1-R2)/R1, where R1=maximum radius of the colony of *S. sclerotiorum* and R2=the radius of that part of the colony of *S. sclerotiorum* directly opposite the colony of *E. purpurascens*. Width of inhibition zone (IZ) is the minimum distance between the edges of the two fungal colonies. Values listed are means of 12 replicates from pooled data of three trials and are not significantly different from others in the same column if followed by the same letter (Duncan's multiple range test, p=0.05).

and sterile PDB were slightly yellow in colour. On TLC plates developed with chloroform and methanol (95:5), extracts from sterile medium filtrates did not show any bands under visible or UV-light, while 8 bands of pigment and fluorescence appeared for the extracts of filtrates from cultures grown in ME and PDB. One band, denoted as AF1 (R_f 0.44), was yellow to slightly brown in colour under visible light. Another band, denoted as AF2 (R_f 0.27), appeared yellow. There were fewer bands for extracts of filtrates from cultures grown in CSB than from ME or PDB cultures. AF1 did not appear on filtrates from cultures grown in CSB, and AF2 was faint. When UV/Vis. absorbance spectra of extracts were determined from 200-500 nm, principal peaks of absorbance were found at 224, 265 and 420 nm for extracts from culture filtrates produced in ME and PDB, and 324 nm for extracts of CSB culture filtrate. Extracts from sterile PDB and sterile CSB did not show any peaks but extracts from sterile ME had a principal peak at 281 nm. Purified AF1 and AF2 from filtrate extracts of cultures produced in ME and PDA were obtained from preparative TLC procedures and their characteristic zones were obtained again after their eluates were applied to TLC plates. The principal peaks of light absorbance were at 209 and 269 for AF1 and 209 for AF2.

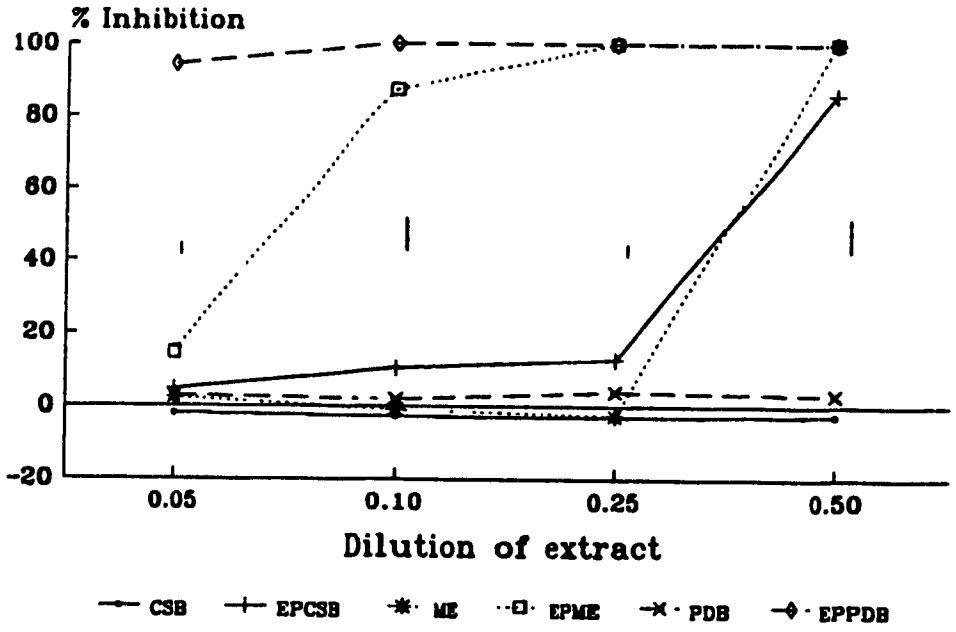
Antifungal activity.

Inhibition of *U. atrum*: After the incubation period, black conidia of *U. atrum* covered most of the TLC plates. On the TLC lanes where extracts from cultures produced in ME or PDB were applied, growth and sporulation of *U. atrum* was inhibited on the region corresponding to the AF1 band, while growth was present but reduced in the region corresponding to the AF2 band. Similar observations were obtained when purified AF1 and Af2 were used. These inhibition zones were not observed with extracts from sterile media and extract from filtrates of cultures grown in CSB.

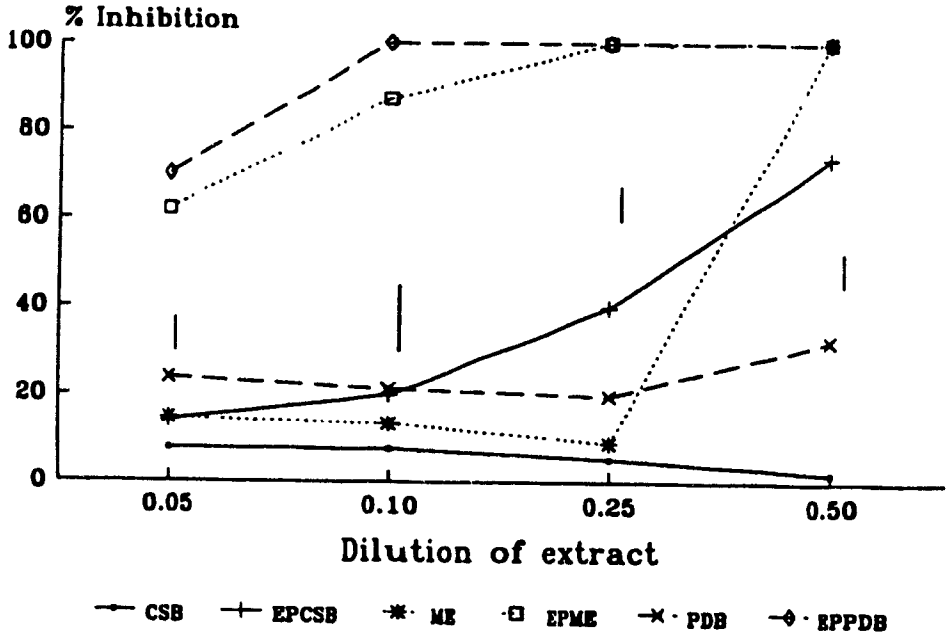
Germination of ascospores of *S. sclerotiorum* was not inhibited by extracts from sterile CSB and PDB compared to the control (Fig.7.1 A). However, extracts from sterile ME inhibited germination of ascospores up

Figure 7. 1. Ascospore germination of *S. sclerotiorum* in presence of extract of culture filtrate of *E. purpurascens*. CSB, ME, and PDB are extracts from sterile Czapek solution broth, sterile malt extract and sterile potato dextrose broth, respectively. EPCSB, EPME and EPPDB are extracts from culture filtrates of *E. purpurascens* grown in CSB, ME and PDB respectively. Vertical bars are LSD values (p=0.05). A. Inhibition of percent germination. B. Inhibition of length of germ tube.

A.


% Inhibition
100
80
60
40
20
0
-20
0.05
0.10
0.25
0.50
Dilution of extract
CSB
EPCSB
ME
EPME
PDB
EPPDB

B.


% Inhibition
100
80
60
40
20
0
0.05
0.10
0.25
0.50
Dilution of extract
CSB
EPCSB
ME
EPME
PDB
EPPDB

to 100 % at concentration of 50% but did not show inhibition at concentration ≤ 25%. Extracts of filtrates of PDB cultures showed the strongest inhibition of germination, followed by extracts of ME-grown cultures. For extracts of CSB-grown cultures, high inhibition of germination was found only at a concentration of 50%. Similar results were observed for elongation of germ tubes. However, elongation of germ tubes was slightly inhibited by extracts from sterile CSB and PDB, or sterile ME at concentrations ≤ 25% (Fig. 7.1 B).

Inhibition of mycelial growth of *S. sclerotiorum*: No inhibition was found on slides treated with extracts of sterile media or extracts of CSB-grown cultures. Inhibition zones (1-2 mm) around wells treated with extracts of PDB-grown cultures were observed. On slides where extracts of ME-grown cultures were added, no inhibition zones appeared but mycelium around the well was less dense than on other areas of the slides. Malformed hyphae and lysed hyphal tips also were observed (Section 6).

Discussion

The influence of nutrients on conidia germination has been reported for many fungi (Wildman & Parkinson 1981; Blakeman 1975). In our study, most of the carbon compounds tested improved conidial germination of *E. purpurascens* but their relative activities varied. Six of eleven amino acids positively affected conidial germination while one showed negative effects. When selected carbon compounds and amino acids were combined, most of the combinations still showed positive effects on conidial germination. However, only seven of 16 combinations positively affected elongation of germ tubes and four of the combinations showed negative effects. This may be due to differences in concentration and pH when the two compounds were combined. Certain other nutrients that affect uptake of carbon or amino acids have been reported elsewhere (Kotyk 1967; Kotyk and Rihova 1972) although in many fungi sugars are stimulators of amino acid uptake (Whitaker & Morton 1971).

Generally *E. purpurascens* grew faster on natural media than on synthetic media. For synthetic media, the rate of growth and sporulation depended mainly on the amino acid. Most fungi utilize amino acids as sources of nitrogen, but not all are used equally well (Evans & Black 1981). Amounts and type of the carbon source affect the utilization of amino acids (Weinhold and Garraway 1966). The limited differences among the carbon compounds in our study may be due to their similar chemical natures. Inhibition of radial growth and width of inhibition zones in dual culture also varied among the media and correlation of these two characters were not strong for some media. Inhibition of radial growth and width of inhibition zone depend upon rate of growth and/or production of antibiotics (Fokkema 1973). Different nutrients in the media may affect not only growth and production of antifungal compound by *E. purpurascens* but also growth of *S. sclerotiorum* and its sensitivity to the antifungal compounds (Wicklow, 1981).

The extracts obtained from ME and PDB-grown culture filtrates had bands on TLC plates similar to ethanol extracts of *E. purpurascens* cultured on malt extract agar (Burge *et al.* 1976). Bands of AF1 and AF2 corresponded to two of the yellow bands (R_f=0.44 and 0.23 respectively) in Burge's study. Those two yellow bands with another band (R_f=0.8) showed inhibitory activity to *Bacillus subtilis*. From the active pigment mixtures, two pigments, isolated and identified as Epirodin A and B, showed inhibition to *B. subtilis* as well as *Saccharomyces cerevisiae* (Ikawa *et al.* 1978). Epirodin A had a very characteristic light absorption maximum at 429 nm, which shifted to 390 nm under low pH (Burge *et al.* 1976). Extracts from ME and PDB grown culture filtrates in our study had a similar principal peak of light absorbance (420 nm) to Epirodin A. Epirodin A therefore may be a component of our extracts. Moreover, Royse and Ries (1978) also found two bands (R_f=0.69 and 0.55) with inhibitory activity to *Cytospora cincta* when fermentation broths from *E. purpurascens* cultures were chromatographed. The two bands were proved to be different

from flavipin although they were unidentified. However, it is difficult to compare the results presented here with those of Royse and Ries (1978) since different solvents were used to develop the TLC plates. Production of flavipin (3,4,5-trihytroxy-6-methanol-0-phthalaldehyde) by *E. purpurascens* was found first by Bamford and his colleagues (1961), and has been confirmed by other investigators (Burge *et al.* 1976; Brown *et al.* 1987). Flavipin was originally isolated from cultures of *Aspergillus flavipes* (Raistrick & Rudman 1956). It is likely our extracts from ME and PDB grown culture filtrates may also contain flavipin since we found a principal peak of light absorbance at 265 nm which is similar to the characteristic peak of flavipin (265 nm, by Burge 1973, 261 and 264 nm by Raistrick and Rudman 1956). AF1 has a similar peak of light absorbance (269 nm) but its R_f (0.44) is different from flavipin (R_f 0.16, by Brown *et al.* 1984). Although antifungal compounds in the extracts were not completely identified, production of antifungal compounds by *E. purpurascens* was strongly influenced by nutrients supplied. Extracts of sterile ME showed inhibition to ascospores of *S. sclerotiorum* at concentration of 50%. Certain substances in the media may be extracted and concentrated in the extract resulting in inhibition of ascospore germination. Thus, sterile media controls are essential when extracts are tested for their biological activities, especially when "natural" media are used.

Different nutrients may affect conidial germination, mycelial growth, sporulation and production of antifungal compounds by *E. purpurascens*. This suggests that, in addition to selection of strains (Zhou & Reeleder 1990), white mold control by *E. purpurascens* may be enhanced by providing appropriate nutrients such as PDB, dextrose and lysine, maltose and lysine, sucrose and lysine, and sucrose and tryptophan. However, information obtained *in vitro* may not be completely dependable as a predictor of behaviour *in vivo*, and effects of nutrients on *S. sclerotiorum* need to be considered. Further characterization and identification of antifungal compounds and the factors affecting their

production may provide useful information for improvement of this biocontrol agent.

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8. Summary and General Discussion

White mold, caused by *Sclerotinia sclerotiorum*, is one of the most destructive diseases of snap bean. Control of white mold on bean by biological measures was the fundamental objective of this research. *Epicoccum purpurascens*, a saprophytic fungus found on the phylloplane of many plants, was chosen as the biological control agent based on previous studies (see review of literature). This thesis presents the results from a series of investigations on the suppression of white mold by *E. purpurascens* and the mechanisms by which control occurs.

Attempts have been made to improve *E. purpurascens* strains with respect to both tolerance to certain commonly used fungicides and to inhibition of *S. sclerotiorum*. Thus, it seem possible to improve performance of *E. purpurascens* by strain selection. Strain M-20-A, recovered after irradiating a wild-type isolate with shortwave UV light, was ameliorated in sporulation and inhibition ability, but its tolerance to iprodione and vinclozolin was not very high. M-20-A was further cultured on media amended with high concentration of iprodione and vinclozolin, and the new strains, R4000, 7-A, 16-B and 2-A were obtained from the second selection. These strains varied in their tolerance to iprodione and vinclozolin but were not tolerant to benomyl. R4000, 7-A and 16-B exhibited improved performance in suppression of *S. sclerotiorum in vitro* as well as in control of white mold in the greenhouse, compared with either the wild type or M-20-A.

The efficacy of *E. purpurascens* in controlling white mold was assessed in greenhouse and field trials. Application of conidial suspensions of *E. purpurascens* reduced disease incidence and disease index of white mold under both greenhouse and field conditions; two to four applications are required to provide disease control similar to that resulting from applications of the fungicide iprodione; combining *E. purpurascens* with iprodione did not produce additive or synergistic

effects on disease control, and addition of malt extract to conidial suspensions did not increase the efficacy of *E. purpurascens* in white mold control.

Investigations of the behavior of *E. purpurascens* on beans showed that colonization and growth of the fungus were affected by the state of the tissue to which conidia were applied. Germination of conidia was greater on senescent petals than on younger tissue. Although conidia of *E. purpurascens* were able to survive on emerging flowers and eventually colonize the flowers, colonization was not as extensive as when conidia were applied directly to older flowers. Movement of *E. purpurascens* conidia among the plants was not extensive. However, addition of malt extract to conidial suspension improved germination on flowers and increased hyphal coverage on emerging flowers. *E. purpurascens* did not penetrate leaves until they were in an advanced state of senescence, although the fungus readily colonized dead leaf tissue.

Effects of *E. purpurascens* on bean plants were also investigated. Application of *E. purpurascens* did not accelerate senescence or affect chlorophyll content of bean leaves and did not affect yield or percentage of abscised flowers or pods.

A low frequency of mycoparasitism by *E. purpurascens* on *S. sclerotiorum* was observed on media covered slides. However, mycoparasitism was not found on flower petal disks. In the dual culture test, malformed hyphae and leakage of cytoplasm of *S. sclerotiorum* commonly appeared on which hyphae of the two fungi were in the same region but not in contact. Inhibition of ascospores was found on medium-covered slides where *E. purpurascens* had been grown for various periods. Culture filtrates of *E. purpurascens* decreased percentage of white mold and increased pod yield when applied to beans in greenhouse trials. Moreover, extracts from *E. purpurascens* culture filtrates inhibited ascospore germination and hyphal growth of *S. sclerotiorum* and the phenomenon was very similar to that seen on *S. sclerotiorum* in dual culture tests. Thus, production of antifungal

compounds seems to be the most important factor in control of white mold by *E. purpurascens*. However, colonization of bean flowers by *E. purpurascens* resulted in a larger reduction in white mold incidence, and the levels of reduction were in part affected by provision of nutrients. Thus competition for nutrients also may be a factor in biological control.

The influence of nutrient supply on *E. purpurascens* was also studied. Different nutrients affect not only conidial germination, growth and sporulation of the fungus but also its inhibition of *S. sclerotiorum* and its ability to produce antifungal compounds.

Overall, results from these studies demonstrate that *E. purpurascens* has great potential as a biological control agent for white mold. The mechanisms of this biocontrol are mainly due to production of antifungal compounds but also in part competition for nutrients. Efficiency of biological control may be increased by using improved *E. purpurascens* strains and/or appropriate additives in conidial suspensions to aid *E. purpurascens* in colonization, sporulation, and production of antifungal compounds.

Although various aspects of *E. purpurascens* has been studied, much more intensive work needs to be done before it can be used as an alternative measure in control of white mold. Colonization of bean petals by *E. purpurascens* to protect flowers from infection by *S. sclerotiorum* is important in white mold control. Besides the state of flower tissues, other factors such as temperature, humidity, fertilization of plants, etc. also may affect colonization. Knowledge on these factors can be useful to improve flower colonization by *E. purpurascens*. In addition, effects on bean plants by *E. purpurascens* were evaluated in the greenhouse and investigations under different environmental conditions may provide more information on this matter and give more confidence in the use this biocontrol agent.

Antifungal compounds produced by *E. purpurascens* play an important role in biocontrol but they were not well identified in this study.

Further identification of the compounds, their pathways of synthesis, and environmental factors affecting synthesis, should be investigated. Toxicity of these antifungal compounds and other metabolic products of this fungus to non-target organisms have to be examined before *E. purpurascens* can be used in agricultural practices.

Mass production and formulation of a biocontrol agent are highly desirable prior to its use in the field. In this study, a wheat seed medium was utilized to produce conidia of *E. purpurascens;* other techniques may produce conidia more effectively and economically. Effects of additives on colonization of flowe · petals and nutrients on germination, growth, sporulation and production of antifungal compounds of *E. purpurascens* have been investigated, however, to establish an appropriate formulation, more chemicals or chemical combinations may need to be tested for their effect on both *E. purpurascens* and *S. sclerotiorum*, not only *in vitro* but also *in vivo*.

E. purpurascens did reduce white mold in the greenhouse and field, but efficiency of disease control in this study was not always high enough to be competitive with chemical control. Improved strains combined with selected additives, and/or with selected fungicides in various concentrations may be included in the field treatments. Fungicide may be applied early in the flowering period to protect emerging flowers before application of *E. purpurascens* at full bloom. When and how many times that *E. purpurascens* needs to be applied during the growing season should also be investigated.

It is likely that the biological control measures developed here may have potential in controlling other diseases that have epidemiologies similar to white mold. However, *E. purpurascens* will be effective only if the pathogens are sensitive to its antifungal compounds and if *E. purpurascens* can colonize plant tissue associated with infection. These diseases may include grey mold of snap bean caused by *Botrytis cinerea* and sclerotinia stem rot of oilseed rape caused by *S. sclerotiorum*. Evidence

that this biocontrol agent may control other diseases in addition to white mold may increase the possibility that *E. purpurascens* could be developed commercially.

9. Claims of Contribution to Knowledge

1. White mold of bean incited by *Sclerotinia sclerotiorum* can be controlled by application of *Epicoccum purpurascens* conidia under field conditions.
2. Some characteristics of *E. purpurascens* can be modified by strain selection. New strains showed differences in hyphal growth, sporulation, tolerance to iprodione and vinclozolin, and ability to inhibit *S. sclerotiorum in vitro* and control white mold in the greenhouse.
3. Colonization and growth of *E. purpurascens* on flowers is affected by age of the petals. Addition of malt extract to conidial suspensions of *E. purpurascens* improved germination of the conidia on flowers and increased hyphal coverage on emerging flowers.
4. Application of *E. purpurascens* conidia to bean plants does not accelerate senescence of, or affect chlorophyll content of, bean leaves, and does not affect bean pod yield or percentage of abscised flowers or pods under greenhouse condition. *E. purpurascens* readily colonizes dead leaf tissue but does not penetrate bean leaves until they are in an advanced state of senescence.
5. Hyphae of *E. purpurascens* can surround, contact or penetrate hyphae of *S. sclerotiorum* in dual cultures. In all the cases, hyphal tips of *S. sclerotiorum* became malformed and red to orange in color.
6. White mold can be reduced by application of culture filtrates of *E. purpurascens* under greenhouse conditions. Extracts of *E. purpurascens* culture filtrates from wheat seed medium, potato dextrose broth or malt extract inhibited ascospore germination and hyphal growth of *S. sclerotiorum*. *S. sclerotiorum* hyphae grown on medium containing the extract became malformed or lysed. Extracts from potato dextrose broth and malt extract also inhibited growth of *Ulocladium atrum* on thin layer chromatography plates.
7. Ascospore germination of *S. sclerotiorum* was inhibited on medium

covered slides where *E. purpurascens* has been grown for certain periods. Addition of nutrients to ascospore suspensions affected the results.

8. Colonization of bean flowers by *E. purpurascens* reduces white mold incidence. The levels of reduction were affected by whether or not supplemental nutrients were applied or if colonized flowers were autoclaved.
9. Conidial germination of *E. purpurascens* can be improved by supplying certain carbon compounds or amino acids. Nutrient supplies affect not only growth and sporulation of *E. purpurascens*, but its ability to inhibit *S. sclerotiorum* and production of antifungal compounds.

10. Appendices

Appendix 1.

Appendix 1.1. Influence of addition of malt extract on conidial germination of *E. purpurascens* in fungicide tolerance test[y]

A. Conidial germination (%)

	Iprodione			Vinclozolin			Benomyl		
Strain	M[z]	O[z]	P > F	M	O	P > F	M	O	P > F
Wild type	17.0	14.0	0.0066	60.0	45.0	0.0114	78.9	73.8	0.1288
M-20-A	25.0	18.1	0.0233	82.1	38.0	0.0001	79.3	68.1	0.0001
2-A	53.0	39.0	0.0095	80.5	62.0	0.0060	74.8	60.2	0.0001
R4000	88.6	53.0	0.0001	94.0	51.5	0.0001	74.0	59.2	0.0001
7-A	44.2	28.4	0.0006	65.7	40.8	0.0001	69.1	61.0	0.0001
16-B	29.2	12.3	0.0001	85.2	72.2	0.0012	80.1	67.3	0.0001

B. Length of germ tube (μm)

	Iprodione			Vinclozolin			Benomyl		
Strain	M	O	P > F	M	O	P > F	M	O	P > F
Wild type	22.3	15.8	0.0001	41.6	36.3	0.1107	53.9	55.4	0.5349
M-20-A	35.3	32.2	0.2095	62.7	47.0	0.0001	80.3	53.1	0.0001
2-A	51.4	48.9	0.5258	81.0	64.8	0.0001	73.1	68.6	0.2783
R4000	91.8	81.9	0.0020	126.7	88.2	0.0001	75.2	46.3	0.0001
7-A	54.9	59.7	0.5794	48.4	39.0	0.0001	62.2	57.1	0.0045
16-B	45.1	37.0	0.1752	104.8	96.3	0.0028	77.8	70.4	0.0002

[y] The methods used for this test were described in section 3. Values in the table are means from different concentrations of fungicides and replicates.

[z] M, 1% malt extract was added to spore suspension; O, no malt extract.

Appendix 1.2. Effect of application of *E. purpurascens* and iprodione on the development of white mold of snap bean in the field

Treatment[w]	Dates of treatment	Number of lesions[x]	Disease incidence[y] (%)	Disease index[y,z] (%)	pod rot[y] (%)	Yield[y] (tons/ha)
1987 (1 June)						
Control		-	70.2 a	21.8 a	22.6 a	17.75 c
E. purpurascens + 1% Malt Extract	13,15,17 July	-	34.8 b	9.0 b	7.9 b	21.41 ab
E. purpurascens + 1% Malt Extract	10,13,15,16 17 July	-	31.0 b	8.4 b	7.1 b	21.29 ab
Iprodione (0.5 kg a.i./ha)	13,15 July	-	30.5 b	6.6 b	6.1 b	23.27 a
E. purpurascens + Iprodione (0.5 kg a.i./ha)	13,15,17 July	-	29.7 b	8.3 b	6.5 b	22.47 a
E. purpurascens + 1% Malt extract (no SS)	13,15,17 July	-	37.5 b	13.8 b	7.8 b	18.51 bc
1988 (June 7)						
Water	19,22,25 July	32 a	78.0 a	22.4 a	14.5 a	17.88 b
1% Malt Extract	19,22,25 July	21 a	70.2 a	18.9 ab	7.7 b	20.12 ab
E. purpurascens	19,22,25 July	13 ab	61.5 a	15.1 bc	6.7 bc	20.11 ab
E. purpurascens + 1% Malt Extract	19,22,25 July	4 b	42.5 b	9.2 c	3.2 bc	22.13 a
Iprodione (0.5 kg a.i./ha)	20 July	18 a	64.5 a	16.4 ab	7.5 b	19.43 ab
E. purpurascens + Iprodione (0.25 kg a.i./ha)	20,24 July	18 a	63.6 a	16.3 ab	7.0 b	20.35 ab
E. purpurascens + 1% Malt Extract (no SS)	19,22,25 July	3 b	38.7 b	9.0 c	1.7 c	21.27 ab

[w]Control plants (1987) were inoculated only with *Sclerotinia sclerotiorum*. Water treatment (1988 control treatment) was distilled water containing 0.01% Tween 80. Tween 80 (0.01%) was added to all suspensions of *E. purpurascens*. Ascospores of *S. sclerotiorum* were applied to all plots except the one indicated with 'no SS' on 18 July in 1987 and on 26 July in 1988.

[x]Number of lesions was recorded from 20 plants in each plot on 1 August in 1988. The data were analyzed using the Freidman analysis of variance. Values followed by the same letter are not significantly different (multiple comparison experimentwise error rate = 1.05).

[y]Values for each year in a column followed by the same letter are not significantly different (Duncan's Multiple Range Test, P = 0.05). Percentage data of disease incidence was arcsine-transformed before analysis.

[z]Disease severity classes: 0 = 0%, 1 = 1-10%, 2 = 11-30%, 3 = 31-50%, 4 = 51-75%, 5 = 76-100% of stem and leaf area diseased. Disease index=$[\Sigma S_i n_i/5N]x100$ (i=0, 1, 2,...5), where S is the appropriate disease class, n = number of diseased plants in the same class, and N = number of plants rated.

Appendix 1.3. Effect of application of *E. purpurascens* at different concentrations on the development of white mold on greenhouse-grown snap beans[v]

Treatment[w]	Number of lesions[x]	Diseased tissue[y] (%)	Pod rot[z] (%)	Yield[z] (G)
Control	7.3 a	85.6 a	70.0 a	16.0 b
E. purpurascens (10^4 conidia/ml)	5.7 ab	55.6 b	44.8 b	35.4 b
E. purpurascens (10^5 conidia/ml)	2.7 abc	14.4 c	9.2 c	62.9 a
E. purpurascens ($5x10^5$ conidia/ml)	1.7 bc	3.3 c	0.9 c	75.0 a
E. purpurascens (10^6 conidia/ml)	1.0 c	3.3 c	0.0 c	66.3 a
Non-application	0.7 c	0.0 c	2.6 c	61.0 a

[v]The experiment was carried out as the greenhouse evaluation experiment described in section 4.

[w]Treatments were applied when all plants had at least one open flower and again four days later. Tween 80 (0.01%) was added to all applications. Ascospores of *Sclerotinia sclerotiorum* were applied to all plants 1 day after the second application of each treatment.

[x]Data were collected 6 days after inoculation with ascospores of *S. sclerotiorum*. Values followed by the same letter are not significantly different. (Freidman analysis of variance and associated multiple comparison test, experimentwise error rate = 0.75).

[y]Percentage of leaf and stem area diseased, assessed 14 days after inoculation. Values in a column followed by the same letter are not significantly different by Duncan's Multiple range test ($p = 0.05$). Percentage data were arcsine-transformed prior to analysis.

[z]Values in a column followed by the same letter are not significantly different by Duncan's Multiple range test ($p = 0.05$).

Appendix 1.4. Effects of *E. purpurascens* on abscission of inflorescences, flowers, pods and on pod yields[v]

Treatment	Inflorescences abscised (%)[w]	Flowers abscised (%)[x]	Pods abscised (%)[y]	Fresh weight of pods (g)	Dry weight of pods (g)[z]
Unsprayed (control)	45.0	28.5	46.0	26.6	3.5
1% malt extract	40.4	26.8	41.9	28.9	4.0
E. purpurascens	43.5	28.3	50.0	24.4	3.6
E. purpurascens +1% malt extract	42.9	24.1	48.2	27.5	3.6
P > F	0.74	0.91	0.56	0.09	0.51

[v]Beans were held on a growth bench (22 and 19 C during day and night, 14 hr photo period) and sprayed with spore suspensions (10^6 conidia/ml) of *E. purpurascens* twice during the flowering period. Values are means of 4 replicates.

[w]Percentage of inflorescences abscised = [(no. of inflorescences developed - no. of inflorescences at harvest)/no. of inflorescences developed] x 100.

[x]Percentage of flowers abscised = [(no. of flowers - no. of pods developed)/no. of flowers] x 100.

[y]percentage of pods abscised = [(no. of pods developed - no. of pods harvested/no. of pods developed] x 100.

[z]Pods were dried in 65 C oven for 4 days.

Appendix 2

Statistical analyses in section 3*

2.1. Width of inhibition zone and percentage of inhibition of S. sclerotiorum in the presence of E. purpurascens (Re:Table 3.1)

S, strains
T, trials
R, replicates

2.1.1 Radial growth inhibition

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	590.7490417	39.3832694	1.24	0.3215
Error	20	635.3703889	31.7685194		
Corrected Total	35	1226.1194306			

R-Square	C.V.	Root MSE	Mean
0.481804	14.03	5.636357	40.15

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	4.5014694	4.5014694	0.14	0.7106
R(T)	4	89.5615444	22.3903861	0.70	0.5979
S	5	319.9300139	63.9860028	2.01	0.1203
S*T	5	176.7560139	35.3512028	1.11	0.3850

Tests of Hypotheses using the Type III MS for R(T) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	4.50146944	4.50146944	0.20	0.6771

* Analyses for the data in Table 3.3 are not included.

2.1.2 Inhibition zone

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	62.2500	4.1500	1.57	0.1714
Error	20	52.8889	2.6444		
Corrected Total	35	115.1389			

R-Square	C.V.	Root MSE	Mean
0.5407	50.91	1.6262	3.19

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	0.6944	0.6944	0.26	0.6139
R(T)	4	3.1111	0.7778	0.29	0.8783
S	5	55.5556	11.1111	4.20	0.0090
S*T	5	2.8889	0.5778	0.22	0.9504

Tests of Hypotheses using the Type III MS for R(T) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	0.6944	0.6944	0.89	0.3982

2.2. Comparison of strains of E. purpurascens for inhibition of S. sclerotiorum in vitro and in vivo (Re:Table 3.2).

S, strains
R, replicates
B, block
T, trials

2.2.1. Radial growth inhibition

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	735.5827	43.2696	0.66	0.8114
Error	30	1954.8460	65.1615		
Corrected Total	47	2690.4287			

R-Square	C.V.	Root MSE	Mean
0.273407	15.12	8.0723	53.36

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	94.7813	94.7813	1.45	0.2372
R(T)	6	442.3574	73.7262	1.13	0.3683
S	5	163.0479	32.6095	0.50	0.7734
S*T	5	35.3958	7.0791	0.11	0.9896

Tests of Hypotheses using the Type III MS for R(T) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
S	1	94.7813	94.7813	1.29	0.3001

2.2.2. Inhibition zone

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	207.6718	12.2159	8.11	0.0001
Error	30	45.1979	1.5065		
Corrected Total	47	252.8697			

R-Square	C.V.	Root MSE	Mean
0.8212	31.76	1.2274	3.86

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	0.4218	0.4218	0.28	0.6006
R(T)	6	10.4895	1.7482	1.16	0.3532
S	5	195.0260	39.0052	25.89	0.0001
S*T	5	1.7343	0.3468	0.23	0.9463

Tests of Hypotheses using the Type III MS for R(T) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	1	0.42187	0.4218	0.24	0.6407

2.2.3 Number of lesions

General Linear Models Procedure

Dependent Variable: RANKY RANK FOR VARIABLE Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	40.3333	8.0667	11.17	0.0004
Error	12	8.6667	0.7222		
Corrected Total	17	49.0000			

R-Square	C.V.	Root MSE	RANKY Mean
0.8231	24.28	0.8498	3.5

Source	DF	Type III SS	Mean Square	F Value	Pr > F
S	5	40.3333	8.0667	11.17	0.0004

Multiple-comparison

Experimentwise error rate = 0.75, Critical range = 8.89

Grouping				
	A	18.0	3	CONTL
	A			
B	A	14.0	3	2A
B	A			
B	A	11.5	3	M20A
B				
B		8.0	3	7A
B				
B		6.0	3	R4000
B				
B		5.5	3	16B

2.2.4 Diseased tissue (%)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	4520.6327	645.8046	29.64	0.0001
Error	10	217.8813	21.7881		
Corrected Total	17	4738.5140			

R-Square	C.V.	Root MSE	Mean
0.9540	13.28	4.6677	35.12

Source	DF	Type III SS	Mean Square	F Value	Pr > F
B	2	74.2995	37.1497	1.71	0.2306
S	5	4446.3332	889.2666	40.81	0.0001

2.2.5 Pod rot (%)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3265.7222	466.5317	9.55	0.0010
Error	10	488.5555	48.8555		
Corrected Total	17	3754.2777			

R-Square	C.V.	Root MSE	Mean
0.8698	28.40	6.9896	24.61

Source	DF	Type III SS	Mean Square	F Value	Pr > F
B	2	230.7778	115.3889	2.36	0.1445
S	5	3034.9444	606.9889	12.42	0.0005

2.2.6 Yield
General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	2298.4336	328.3476	6.03	0.0059
Error	10	544.1756	54.4175		
Corrected total	17	2842.6092			

R-Square	C.V.	Root MSE	Mean
0.8085	17.17	7.3768	42.95

Source	DF	Type III SS	Mean Square	F Value	Pr > F
B	2	236.4933	118.2466	2.17	0.1646
S	5	2061.9403	412.3880	7.58	0.0035

2.3. Comparison of six strains for their radial growth (Re:Table 3.4)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	5.6293	0.3752	7.27	0.0001
Error	20	1.0321	0.0516		
Corrected Total	35	6.6614			

R-Square	C.V.	Root MSE	Mean
0.8450	10.13	0.2271	2.24

Source	DF	Type III SS	F Value	Pr > F
T	1	0.002025	0.04	0.8450
R(T)	4	0.320288	1.55	0.2257
S	5	5.154347	19.98	0.0001
S*T	5	0.152658	0.59	0.7065

Tests of Hypotheses using the Type III MS for R(T) as an error term

Source	DF	Type III SS	F Value	Pr > F
T	1	0.002025	0.03	0.8814

Appendix 3

Statistical analyses in section 4

3.1 Effect of application of *E. purpurascens* and nutrient solutions on the development of white mold on greenhouse-grown snap beans (Re:Table 4.1)

T, treatments:

1, E. purpurascens + 1% malt extract;
2, E. purpurascens + 1% potato dextrose broth;
3, 1% malt extract;
4, 1% potato dextrose broth;
5, Water;
6, E. purpurascens alone

3.1.1 Number of lesions (SAS proc. NPAR1WAY)

Analysis for variable Y classified by variance T

T	N	SUM OF SCORES	EXPECTED UNDER H0	STD DEV UNDER H0	MEAN SCORE
1	10	167.50	305.00	49.94	16.75
2	10	140.00	305.00	49.94	14.00
3	10	462.00	305.00	49.94	46.20
4	10	444.00	305.00	49.94	44.40
5	10	452.00	305.00	49.94	45.20
6	10	164.50	305.00	49.94	16.40

KRUSKAL-WALLIS TEST (CHI-SQUARE APPROXIMATION)
CHISQ = 43.92 DF = 5 PROB > CHISQ = 0.0001

Dunn's multiple comparison procedure (Experimentwise error rate = 0.75)

$|Rj - Rj'| = 15.31$

3.1.2 Diseased tissue (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	5	9.7700	1.9540	10.8	0.0001	0.5004
ERROR	54	9.7549	0.1806			
CORRECT TOTAL	59	19.5250				

C.V	ROOT MSE	MEAN
69.29	0.4250	0.6124

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
T	5	9.7700	10.82	0.0001

3.2. Effect of application of *E. purpurascens* and iprodione on the development of white mold of snap bean in the field (Re: Table 4.2).

B: blocks
T: Treatments

3.2.1. Trial of July 2, 1987

Treatments:
1, Iprodine (0.5 kg/ha);
2, Control;
3, Iprodione (0.25 kg/ha);
4, E. purpurascens + 1% malt extract 2x
5, E. purpurascens + iprodine (0.25 kg/ha) 2x
6, E. purpurascens + 1% malt extract 4x

3.2.1.1. Number of lesions

ANALYSIS OF VARIANCE PROCEDURE (analysis of ranks)

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	5	39.750000	7.950000	4.98	0.0049	0.5803
ERROR	18	28.750000	1.597222			
CORRECT TOTAL	23	68.500000				
		C.V	ROOT MSE		MEAN	
		36.1089	1.263813		3.5000	

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
T	5	39.750000	4.98	0.0049

Multiple comparison test

Experimentwise error rate = 1.05, |Rj-Rj'| = 9.58

Grouping	Total ranks	T
A	22.5	1
AB	16.5	2
AB	15.0	3
AB	14.5	6
B	8.5	4
B	7.0	5

3.2.1.2. Disease incidence (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	8	0.776777	0.097097	3.7	0.0136	0.6651
ERROR	15	0.391048	0.026070			
CORRECT TOTAL	23	1.167825				

C.V	ROOT MSE	MEAN
26.36	0.16146	0.6124

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.126206	1.61	0.2281
T	5	0.650571	4.99	0.0069

3.2.1.3. Disease index (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	8	0.082675	0.010334	2.98	0.0327	0.6136
ERROR	15	0.052055	0.003470			
CORRECT TOTAL	23	0.134730				

C.V	ROOT MSE	MEAN
36.64	0.0589	0.1607

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.009246	0.89	0.4697
T	5	0.073429	4.23	0.0134

3.2.1.4. Pod rot (%) (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	8	0.023996	0.002999	14.97	0.0001	0.8887
ERROR	15	0.003005	0.000200			
CORRECT TOTAL	23	0.027001				

C.V	ROOT MSE	MEAN
23.6251	0.014154	0.0599

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.003038	5.06	0.0129
T	5	0.020957	20.92	0.0001

3.2.1.5. Yield (ton/ha)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	8	44.53000	5.566250	0.66	0.7151	0.2615
ERROR	15	125.74958	8.383305			
CORRECT TOTAL	23	170.279583				

C.V	ROOT MSE	MEAN
16.9115	2.895394	17.1208

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	34.75792	1.38	0.2866
T	5	0.772083	0.23	0.9420

3.2.2. Trial of June 28, 1988

Treatments:

1. Water 3x
2. Malt extract (1%) 3x
3. Iprodione (0.5 kg/ha) 2x
4. E. purpurascens 3x
5. E. purpurascens + 1% malt extract 3x
6. E.+Iprodione (0.25 kg/ha) 2x
7. E. purpurascens + malt extract 2x

3.2.2.1. Number of lesions

ANALYSIS OF VARIANCE PROCEDURE (analysis of ranks)

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	6	79.750000	13.291667	9.71	0.0001	0.7350
ERROR	21	28.750000	1.3690476			
CORRECT TOTAL	27	108.50000				

C.V	ROOT MSE	MEAN
29.2516	1.170063	4.0000

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
T	5	79.7500	9.71	0.0001

Multiple comparison test

Experimentwise error rate = 1.05. |Rj-Rj'| = 14.48

Grouping	Total ranks	T
A	27.0	1
A	23.5	2
AB	17.5	7
AB	16.0	4
B	11.0	3
B	9.5	6
B	7.5	5

3.2.2.2. Disease incidence (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	9	0.823302	0.091478	3.63	0.0096	0.6448
ERROR	18	0.453565	0.025198			
CORRECT TOTAL	27	1.276867				

C.V	ROOT MSE	MEAN
22.8165	0.15874	0.6957

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.145930	1.93	0.1608
T	6	0.677372	4.48	0.0060

3.2.2.3. Disease index (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	9	0.100666	0.011185	4.11	0.0052	0.6726
ERROR	18	0.049002	0.002722			
CORRECT TOTAL	27	0.149668				

C.V	ROOT MSE	MEAN
28.7220	0.052176	0.1816

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.019316	2.37	0.1050
T	6	0.081350	4.98	0.0036

3.2.2.4. Pod rot (data were arcsine-transformed)

ANALYSIS OF VARIANCE PROCEDURE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	9	0.107628	0.011959	8.30	0.0001	0.8058
ERROR	18	0.025938	0.001441			
CORRECT TOTAL	27	0.133566				

C.V	ROOT MSE	MEAN
30.5333	0.037961	0.1243

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	0.007596	1.76	0.1914
T	6	0.100032	11.57	0.0001

3.2.2.5. Yield

ANALYSIS OF VARIANCE PROCEDUR

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F-VALUE	PR > F	R-SQUARE
MODEL	9	139.42120	15.49124	5.81	0.0008	0.7439
ERROR	18	47.992786	2.666266			
CORRECT TOTAL	27	187.413985				

C.V	ROOT MSE	MEAN
10.3482	1.632870	15.7793

SOURCE	DF	ANOVA SS	F-VALUE	PR > F
B	3	66.80721	8.35	0.0011
T	6	72.61399	4.54	0.0057

Appendix 4

Statistical analyses in section 5

4.1 Germination of E. purpurascens on bean flowers (Re:Table 5.1)

A, Stages of flowers: Y, emerging flower; F, Newly-opened flower; O, senescent flower.

I, Sample time, hours after application of E. purpurascens,

N, Addition of malt extract (M) or not (N).

4.1.1 Percentage of germination (data were arcsine-transformed)

4.1.1.1. Overall analysis

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	13.963142	0.821361	37.82	0.0001
Error	90	1.954719	0.021719		
Corrected Total	107	15.917862			

R-Square	C.V.	Root MSE	Mean
0.8772	22.64	0.147374	0.6507

Source	DF	Type III SS	Mean Square	F Value	Pr > F
M	1	1.672291	1.672291	77.00	0.0001
A	2	1.001497	0.500748	23.06	0.0001
I	2	11.056098	5.528049	254.52	0.0001
N*A	2	0.027453	0.013726	0.63	0.5339
A*I	4	0.159874	0.039968	1.84	0.1280
N*I	2	0.016225	0.008112	0.37	0.6894
N*A*I	4	0.029701	0.007425	0.34	0.8490

4.1.1.2 Difference among flower stages (Data were sorted by I and M)

Duncan's Multiple Range Test

I=4 M=M

Alpha= 0.05 df= 15 MSE= 0.011711

Number of Means 2 3
Critical Range 0.133 0.139

Duncan Grouping	Mean	No.	A
A	0.4436	6	O
A			
B A	0.3404	6	F
B			
B	0.2515	6	Y

I=4 N=N

Alpha= 0.05 df= 15 MSE= 0.008117

Number of Means 2 3
Critical Range 0.111 0.116

Duncan Grouping	Mean	No.	A
A	0.1416	6	O
A			
A	0.1125	6	F
A			
A	0.0615	6	Y

I=8 N=M

Alpha= 0.05 df= 15 MSE= 0.0169

Number of Means 2 3
Critical Range 0.160 0.168

Duncan Grouping	Mean	No.	A
A	1.0235	6	O
B	0.8351	6	F
C	0.6692	6	Y

I=8 N=N

Alpha= 0.05 df= 15 MSE= 0.049501

Number of Means 2 3
Critical Range 0.273 0.287

Duncan Grouping	Mean	No.	A
A	0.793	6	O
A			
B A	0.640	6	F
B			
B	0.423	6	Y

I=12 N=M

Alpha= 0.05 df= 15 MSE= 0.011081

Number of Means 2 3
Critical Range 0.129 0.136

Duncan Grouping	Mean	No.	A
A	1.2184	6	O
A			
B A	1.0984	6	F
B			
B	1.0567	6	Y

I=12 N=N

Alpha= 0.05 df= 15 MSE= 0.033005

Number of Means 2 3
Critical Range 0.223 0.234

Duncan Grouping	Mean	No.	A
A	0.941	6	O
A			
A	0.902	6	F
A			
A	0.724	6	Y

* Differences between with and without malt extract are not included.

4.1.2. Length of germ tube

4.1.2.1. Overall analyses

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	131329.0891	11939.0081	129.89	0.0001
Error	59	5422.9709	91.9148		
Corrected Total	70	136752.0600			

R-Square	C.V.	Root MSE	Mean
0.960345	16.61085	9.587219	57.7166197

Source	DF	Type III SS	Mean Square	F Value	Pr > F
N	1	3568.45552	3568.45552	38.82	0.0001
A	2	13643.88427	6821.94214	74.22	0.0001
I	1	99716.49906	99716.49906	1084.88	0.0001
N*A	2	318.54395	159.27198	1.73	0.1857
A*I	2	12029.44966	6014.72483	65.44	0.0001
N*I	1	65.05607	65.05607	0.71	0.4036
N*A*I	2	366.18865	183.09432	1.99	0.1455

4.1.2.2. Difference among flower stage

Duncan's Multiple Range Test

I=4 N=M

Alpha= 0.05 df= 14 MSE= 47.80651

Number of Means 2 3
Critical Range 8.826 9.255

Duncan Grouping	Mean	No.	A
A	30.927	6	F
A			
A	27.288	5	Y
A			
A	25.467	6	O

I=4 N=N

Alpha= 0.05 df= 15 MSE= 81.18693

Number of Means 2 3
Critical Range 11.07 11.61

Duncan Grouping	Mean	No.	A
A	14.667	6	F
A			
A	11.887	6	O
A			
A	8.587	6	Y

I=8 N=M

Alpha= 0.05 df= 15 MSE= 131.9656

Number of Means 2 3
Critical Range 14.11 14.80

Duncan Grouping	Mean	No.	A
A	130.533	6	O
B	106.067	6	F
C	66.273	6	Y

I=8 N=N

Alpha= 0.05 df= 15 MSE= 103.7594

Number of Means 2 3
Critical Range 12.51 13.13

Duncan Grouping	Mean	No.	A
A	111.400	6	O
A			
A	105.733	6	F
B	48.900	6	Y

4.2. Amount of flower surface covered by mycelium (Re:Table 5.2)

S, stages of flowers: 1, emerging flowers; 2, newly opened flowers; 3, senescent flowers.

4.2.1 With malt extract

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums)

S	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	44		1976.50	2926.0	196.37
44.92					
2	44		2776.00	2926.0	196.3763.09
3	44		4025.50	2926.0	196.3791.49

Kruskal-Wallis Test (Chi-Square Approximation)

CHISQ= 36.875 DF= 2 Prob > CHISQ= 0.0001

Dunn's multiple comparison procedure (Experimentwise error rate =0.15)

|Rj - Rj'| = 15.98

4.2.2. Without malt extract

S	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	44		1765.50	2926.0	202.58
40.12					
2	44		2996.50	2926.0	202.5868.10
3	44		4016.50	2926.0	202.5891.27

Kruskal-Wallis Test (Chi-Square Approximation)

CHISQ= 36.875 DF= 2 Prob > CHISQ= 0.0001

Dunn's multiple comparison procedure (Experimentwise error rate =0.15)

|Rj - Rj'| = 15.98

4.3. Number of conidia of *Epicoccum purpurascens* present on bean flowers following spray application with and without malt extract (ME)
(Re:Fig. 5.1)

T, flower stages: 1, emerging flowers; 2, newly opened flowers; 3, senescent flowers.
M, addition of malt extract: M, with ME; N, without ME.

4.3.1 difference among flower stages

M=M

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums)
Classified by Variable T

T	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	22	506.000000	737.0	68.3306441	23.0000000
2	22	723.500000	737.0	68.3306441	32.8863636
3	22	981.500000	737.0	68.3306441	44.6136364

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 16.181 DF= 2 Prob > CHISQ= 0.0003

Dunn's multiple comparison procedure

|Rj-Rj'|= 11.34 (experimentwise error rate=0.15)

M=N

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums) for Variable YT
Classified by Variable T

T	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	22	341.0	737.0	70.5460024	15.5000000
2	22	825.0	737.0	70.5460024	37.5000000
3	22	1045.0	737.0	70.5460024	47.5000000

Average Scores were used for Ties

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 34.752 DF= 2 Prob > CHISQ= 0.0001

Dunn's multiple comparison procedure

|Rj-Rj'|=11.34 (experimentwise error rate=0.15)

4.3.2. Difference between with and with out ME.

T=1

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums)
Classified by Variable M

M	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
M	22	643.0	495.0	40.2119385	29.2272727
N	22	347.0	495.0	40.2119385	15.7727273

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 13.546 DF= 1 Prob > CHISQ= 0.0002

Dunn's multiple comparison procedure
|Rj-Rj'|= 7.6 (experimentwise error rate=0.15)

T=2

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums) for Variable YT
Classified by Variable M

M	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
M	22	529.0	495.0	40.1020501	24.0454545
N	22	461.0	495.0	40.1020501	20.9545455

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 0.71883 DF= 1 Prob > CHISQ= 0.3965

Dunn's multiple comparison procedure
|Rj-Rj'|=7.6 (experimentwise error rate=0.15)

T=3

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums) for Variable YT
Classified by Variable M

M	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
M	22	552.500000	495.0	34.8107842	25.1136364
N	22	437.500000	495.0	34.8107842	19.8863636

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 2.7284 DF= 1 Prob > CHISQ= 0.0986

Dunn's multiple comparison procedure

|Rj-Rj'|= 7.6 (experimentwise error rate=0.15)

4.4. Sporulation of E. purpurascens on bean flowers (Re: Fig.5.2)

T, treatments: 1, unsprayed control; 2, Tween 80;
3, gelatin; 4, malt extract
N, number of flowers (from two trials)

4.4.1 Sample 1 (4 days)

N P A R 1 W A Y P R O C E D U R E
Wilcoxon Scores (Rank Sums)

S	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	40	1273.00	3220.0	243.71	31.82
2	40	4199.50	3220.0	243.71	104.99
3	40	3307.50	3220.0	243.71	82.69
4	40	4100.00	3220.0	243.71	102.50

Kruskal-Wallis Test (Chi-Square Approximation)

CHISQ= 69.853 DF= 3 Prob > CHISQ= 0.0001

Dunn's multiple comparison procedure (Experimentwise error rate =0.3)

$|Rj - Rj'| = 20.3$

4.4.2 sample 2 (10 days)

S	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	40	1457.50	3220.0	244.48	36.44
2	40	3079.50	3220.0	244.48	76.98
3	40	3924.50	3220.0	244.48	98.11
4	40	4418.50	3220.0	244.48	110.46

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ= 63.478 DF= 3 Prob > CHISQ= 0.0001

Dunn's multiple comparison procedure (Experimentwise error rate =0.3)

$|Rj - Rj'| = 20.3$

Appendix 5.

Statistical analyses for data in section 6

5.1. White mold control by conidia and culture filtrates of *E. purpurascens* (Re:Table 6.1).

T, treatments: CONTL, Water; AF6, *E. purpurascens* filtrate (10^6) applied at 24, and 26 Jan.; F6, *E. purpurascens* filtrate (10^6) applied at 19 and 23, Jan.; F7, *E. purpurascens* filtrate (10^7) applied at 19 and 23, Jan.; S6, *E. purpurascens* conidia (10^6).
R, blocks.

5.1.1. Number of lesions.

General Linear Models Procedure

Dependent Variable: RANKY RANK FOR VARIABLE Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	28.66667	7.1667	53.75	0.0001
Error	10	1.33333	0.1333		
Corrected Total	14	30.00000			

R-Square	C.V.	Root MSE	RANKY Mean
0.9556	12.17	0.3651	3.0

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	4	28.6667	7.1667	53.75	0.0001

Multiple comparison:
Experimentwise error rate = 0.3 |Rj - Rj'| = 8.4

Grouping	Mean of Rank	N	T
A	15	3	CONTL
A	12	3	A6
A B	8	3	F7
A B	7	3	F6
B	3	3	S6

5.1.2 Diseased tissue (data were arcsine-transformed)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	3.48700358	0.58116726	40.58	0.0001
Error	8	0.11458266	0.01432283		
Corrected Total	14	3.60158624			

R-Square	C.V.	Root MSE	Mean
0.968186	14.33	0.119678	0.83

Source	DF	Type III SS	Mean Square	F Value	Pr > F
R	2	0.00008416	0.00004208	0.00	0.9971
T	4	3.48691941	0.87172985	60.86	0.0001

5.1.3. Yield

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	5823.045200	970.507533	93.37	0.0001
Error	8	83.152293	10.394037		
Corrected Total	14	5906.197493			

R-Square	C.V.	Root MSE	Mean
0.985921	19.44	3.223978	16.58

Source	DF	Type III SS	Mean Square	F Value	Pr > F
R	2	196.932573	98.466287	9.47	0.0078
T	4	5626.112627	1406.528157	135.32	0.0001

5.2. Germination of conidia and ascospores on petal disks of bean flowers. (Re:Table 6.2)

Treatments: SS, *S. sclerotiorum*; EP, *E. purpurascens*; CSS and CEP, data recorded form the flower discs applied with both SS and EP; 4 and 8, hours after application.

5.2.1. Percent germination

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	1670.54	72.63	3.57	0.0001
Error	56	1179.47	20.35		
Corrected Total	79	2810.01			

R-Square	C.V.	Root MSE	Mean
0.5945	5.23	4.51	86.23

Source	DF	Type III SS	F Value	Pr > F
L	1	22.31	1.10	0.2995
R(L)	8	147.19	0.9	0.5196
T	7	1324.47	9.30	0.0001
L*T	7	176.56	1.24	0.2972

Tests of hypotheses using the type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	1	22.31	1.21	0.3028

Contrast	DF	SS	F Value	Pr > F
SS4 VS EP4	1	122.41	6.02	0.0173
SS8 VS EP8	1	35.43	1.74	0.1923
CSS4 VS CEP4	1	585.04	28.75	0.0001
CSS8 VS CEP8	1	38.75	1.90	0.1731
SS4 VS CSS4	1	20.97	1.03	0.3144
SS8 VS CSS8	1	4.44	0.22	0.6423
EP4 VS CEP4	1	73.00	3.59	0.0634
EP8 VS CEP8	1	3.36	0.17	0.6859

5.2.2. Length of germ tubes

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	113	2433899.38	21538.93	38.71	0.0
Error	686	381742.84	556.47		
Corrected Total	799	2815642.22			

R-Square	C.V.	Root MSE	Mean
0.8644	35.78	23.59	65.91

Source	DF	Type III SS	F Value	Pr > F
L	1	137.78	0.25	0.6189
R(L)	98	73306.44	1.34	0.0204
T	7	2351195.66	603.59	0.0
L*T	7	9259.50	2.38	0.0209

Tests of hypotheses using the type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	1	137.78	0.18	0.6687

Contrast	DF	SS	F Value	Pr > F
SS4 VS EP4	1	38088.00	68.44	0.0001
SS8 VS EP8	1	618716.88	1111.85	0.0
CSS4 VS CEP4	1	27471.68	49.37	0.0001
CSS8 VS CEP8	1	534164.48	959.90	0.0
SS4 VS CSS4	1	605.52	1.09	0.2973
SS8 VS CSS8	1	8502.08	15.28	0.0001
EP4 VS CEP4	1	2918.48	5.24	0.0223
EP8 VS CEP8	1	21882.32	39.32	0.0001

5.3. Ascospore germination of *S. sclerotiorum* with presence of extract from culture filtrate of *E. purpurascens* (Re:Fig 6.3).

T, treatments; WS, extracts for sterile wheat seed medium; EPWS, extracts from culture filtrate of *E. purpurascens*. Contl, control.
D, dilutions.

5.4.1. inhibition of percent germination

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	81976.10120	10247.01265	1303.17	0.0001
Error	45	353.84013	7.86311		
Corrected Total	53	82329.94133			

R-Square	C.V.	Root MSE	Y Mean
0.995702	3.849822	2.804124	72.8377778

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	2	37895.97918	18947.98959	2409.73	0.0001
D	3	24639.18197	8213.06066	1044.50	0.0001
T*D	3	25118.66171	8372.88724	1064.83	0.0001

T tests (LSD)

Alpha= 0.05 df= 45 MSE= 7.863114
Critical Value of T= 2.01
Least Significant Difference= 2.3057

T Grouping	Mean	N	T
A	94.7	24	WS
A			
A	94.4	6	Contl
B	45.5	24	EPWS

5.4.2. inhibition of length of germination

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	318244.3852	39780.5481	96.43	0.0001
Error	261	107674.6667	412.5466		
Corrected Total	269	425919.0519			

R-Square	C.V.	Root MSE	Mean
0.747195	33.88554	20.31124	59.9407407

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T	2	230718.0222	115359.0111	279.63	0.0001
D	3	66829.0000	22276.3333	54.00	0.0001
T*D	3	50146.8667	16715.6222	40.52	0.0001

T tests (LSD)

Alpha= 0.05 df= 261 MSE= 412.5466
Critical Value of T= 1.97
Least Significant Difference= 7.302

T Grouping	Mean	N	T
A	98.5	30	CONTL
B	80.2	120	WS
C	30.0	120	EPWS

Appendix 6

Statistical analyses for the data in section 7.

6.1. Effects of carbon source on conidial germination of *E. purpurascens*. (Re:Table 7.1)

T, Treatments (carbon compounds).
L, trials.
R, replicates.

6.1.1. percent germination (data were arcsine-transformed)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	27	2.31432223	0.08571564	7.59	0.0001
Error	44	0.49665766	0.01128767		
Corrected Total	71	2.81097989			

R-Square	C.V.	Root MSE	Mean
0.823315	15.30924	0.106243	0.69398277

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	0.00003058	0.00003058	0.00	0.9587
R(L)	4	0.02102021	0.00525505	0.47	0.7606
T	11	1.73059210	0.15732655	13.94	0.0001
L*T	11	0.56267933	0.05115267	4.53	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	0.00003058	0.00003058	0.01	0.9429

6.1.2. Length of germ tubes

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	81	253533.6000	3130.0444	5.86	0.0001
Error	638	340800.1778	534.1696		
Corrected Total	719	594333.7778			

R-Square	C.V.	Root MSE	Mean
0.426585	36.71827	23.11211	62.9444444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	1017.6889	1017.6889	1.91	0.1680
R(L)	58	36948.0889	637.0360	1.19	0.1628
T	11	165706.5778	15064.2343	28.20	0.0001
L*T	11	49861.2444	4532.8404	8.49	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	1017.688889	1017.688889	1.60	0.2113

6.2. Effects of amino acids on conidial germination of *F. purpurascens* (Re:Table 7.2)

T, Treatments (amina acids).
L, trials.
R, replicates.

6.2.1 percent germination (data were arcsine transformed)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	5.40030939	0.13171486	10.13	0.0001
Error	66	0.85798894	0.01299983		
Corrected Total	107	6.25829833			

R-Square	C.V.	Root MSE	Mean
0.862904	21.32768	0.114017	0.53459544

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	2	0.02158878	0.01079439	0.83	0.4404
R(L)	6	0.02143018	0.00357170	0.27	0.9469
T	11	4.28350324	0.38940939	29.95	0.0001
L*T	22	1.07378720	0.04880851	3.75	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	2	0.02158878	0.01079439	3.02	0.1236

6.2.2. Length of germ tubes

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	81	71463.71557	882.26809	3.99	0.0001
Error	629	139099.43492	221.14378		
Corrected Total	710	210563.15049			

R-Square	C.V.	Root MSE	Mean
0.339393	34.02372	14.87090	43.7074543

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	145.03896	145.03896	0.66	0.4183
R(L)	58	10536.83175	181.66951	0.82	0.8240
T	11	49479.30780	4498.11889	20.34	0.0001
L*T	11	11854.27821	1077.66166	4.87	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	145.0389647	145.0389647	0.80	0.3753

6.3. Effects of carbon source and amino acids on conidial germination of *E. purpurascens* (Re: Table 7.3)

T, treatments.
L, trials.
R, replicates.

6.3.1. Percent germination (data were arcsine transformed)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	37	7.05957286	0.19079927	28.93	0.0001
Error	64	0.42213073	0.00659579		
Corrected Total	101	7.48170359			

R-Square	C.V.	Root MSE	Mean
0.943578	12.05571	0.081214	0.67365995

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	5.76118193	5.76118193	873.46	0.0001
R(L)	4	0.00723625	0.00180906	0.27	0.8935
T	16	0.97207209	0.06075451	9.21	0.0001
L*T	16	0.31908259	0.01994266	3.02	0.0009

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
L	1	5.76118193	5.76118193	3184.62	0.0001

6.3.2. Length of germ tubes

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	51	115613.60	2266.93	16	0.0
Error	958	135767.69	141.72		
Corrected Total	1009	251381.29			

R-Square	C.V.	Root MSE	Mean
0.46	30.29	11.9	39.3

Source	DF	Type III SS	F Value	Pr > F
L	1	41173.70	290.53	0.0001
R(L)	18	1922.98	0.75	0.7557
T	16	55589.83	24.52	0.0001
L*T	16	15802.36	6.97	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	1	41173.70	385.41	0.0001

6.4. Growth and sporulation of *E. purpurascens* and its inhibition of *S. sclerotiorum* on agar media. (Re: Table 7.4)

T, treatments.
L, trials.
R, replicates.

6.4.1 Radial growth

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	43	164.77	3.83	15.02	0.0001
Error	108	27.54	0.255		
Corrected Total	151	192.31			

R-Square	C.V.	Root MSE	Mean
0.8567	15.50	0.5050	3.06

Source	DF	Type III SS	F Value	Pr > F
L	1	4.05	15.87	0.0001
R(L)	6	22.47	14.68	0.0001
T	21	101.31	18.92	0.0001
L*T	15	20.35	5.32	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	1	4.047	1.08	0.3386

6.4.2. Percent inhibition (Data were arcsine-transformed)

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	73	4.1199	0.056	4.26	0.0001
Error	185	2.4494	0.013		
Corrected Total	258	6.5694			

R-Square	C.V.	Root MSE	Mean
0.6271	15.96	0.1151	0.72

Source	DF	Type III SS	F Value	Pr > F
L	2	0.8231	31.08	0.0001
R(L)	9	0.1613	1.35	0.2121
T	21	1.3044	4.69	0.0001
L*T	41	1.8217	3.36	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	2	0.8231	22.96	0.0003

6.4.3. Inhibition zone

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	73	805.21	11.03	14.81	0.0001
Error	186	138.53	0.74		
Corrected Total	259	943.74			

R-Square	C.V.	Root MSE	Mean
0.8532	42.22	0.8630	2.04

Source	DF	Type III SS	F Value	Pr > F
L	2	3.3946	2.28	0.1052
R(L)	9	7.6577	1.14	0.3350
T	21	339.0669	21.68	0.0001
L*T	41	455.4804	14.92	0.0001

Tests of Hypotheses using the Type III MS for R(L) as an error term

Source	DF	Type III SS	F Value	Pr > F
L	2	3.3945	1.99	0.1918

6.5. Ascospore germination in present of extracts of filtrates of cultures from *E. purpurascens* grown in CSB, ME and PDB.

(Re: Fig. 7.1, Data were percentage of inhibition).

T, treatments: CSB, ME and PDB are extracts from related sterile media.
EPCSB, EPME and EPPDB are extracts from *E. purpurasecns* cultures in CSB, ME and PDB.

CON, Dilutions: 1, 0.5; 2, 0.25; 3, 0.1; 4, 0.05.
R, replicates.
LO, Trials.

6.5.2 Percent germination (Data were sorted by CON).

General Linear Models Procedure

CON=1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	97786.48709	7522.03747	270.23	0.0001
Error	28	779.40784	27.83599		
Corrected Total	41	98565.89493			

R-Square	C.V.	Root MSE	Mean
0.992093	9.557417	5.275983	55.2030176

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	170.19914	170.19914	6.11	0.0197
T	6	96489.36204	16081.56034	577.73	0.0001
LO*T	6	1126.92591	187.82098	6.75	0.0002

T tests (LSD)

Alpha= 0.05 df= 28 MSE= 27.83599
Critical Value of T= 2.05
Least Significant Difference= 6.2396

CON=2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	83418.61682	6416.81668	630.99	0.0001
Error	28	284.74235	10.16937		
Corrected Total	41	83703.35917			

R-Square	C.V.	Root MSE	Mean
0.996598	10.57085	3.188945	30.1673595

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	15.80475	15.80475	1.55	0.2229
T	6	82957.51043	13826.25174	1359.60	0.0001
LO*T	6	445.30164	74.21694	7.30	0.0001

T tests (LSD): Alpha= 0.05 df= 28 MSE= 10.16937
Critical Value of T= 2.05 Least Significant Difference= 3.7714

CON=3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	74848.16864	5757.55143	99.94	0.0001
Error	28	1613.12865	57.61174		
Corrected Total	41	76461.29729			

R-Square	C.V.	Root MSE	Mean
0.978903	27.12393	7.590240	27.9835490

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	181.12716	181.12716	3.14	0.0871
T	6	73821.47408	12303.57901	213.56	0.0001
LO*T	6	845.56740	140.92790	2.45	0.0499

T tests (LSD): Alpha= 0.05 df= 28 MSE= 57.61174
Critical Value of T= 2.05 Least Significant Difference= 8.9766

CON=4

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	44374.60582	3413.43122	266.89	0.0001
Error	28	358.10911	12.78961		
Corrected Total	41	44732.71493			

R-Square	C.V.	Root MSE	YP Mean
0.991994	22.37961	3.576257	15.9799776

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	6.72217	6.72217	0.53	0.4745
T	6	44099.36807	7349.89468	574.68	0.0001
LO*T	6	268.51558	44.75260	3.50	0.0104

T tests (LSD): Alpha= 0.05 df= 28 MSE= 12.78961
Critical Value of T= 2.0 Least Significant Difference= 4.2295

6.5.2 Length of germ tube (data were sorted by CON).

General Linear Models Procedure

CON=1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	80995.24387	6230.40337	315.90	0.0001
Error	28	552.23957	19.72284		
Corrected Total	41	81547.48344			

R-Square	C.V.	Root MSE	Mean
0.993228	7.62	4.441041	58.27

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	153.02083	153.02083	7.76	0.0095
T	6	76170.01495	12695.00249	643.67	0.0001
LO*T	6	4672.20810	778.70135	39.48	0.0001

T tests (LSD) Alpha= 0.05 df= 28 MSE= 19.7228
Critical Value of T= 2.05 Least Significant Difference= 5.2522

CON=2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	70819.14586	5447.62660	190.80	0.0001
Error	28	799.42857	28.55102		
Corrected Total	41	71618.57442			

R-Square	C.V.	Root MSE	Mean
0.988838	13.67	5.343315	39.08

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	70.69410	70.69410	2.48	0.1268
T	6	68318.51337	11386.41890	398.81	0.0001
LO*T	6	2429.93839	404.98973	14.18	0.0001

T tests (LSD): Alpha= 0.05 df= 28 MSE= 28.55102
Critical Value of T= 2.05 Least Significant Difference= 6.3193

CON=3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	62969.38098	4843.79854	47.36	0.0001
Error	28	2863.66067	102.27360		
Corrected Total	41	65833.04165			

R-Square	C.V.	Root MSE	Mean
0.956501	28.41	10.11304	35.58

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	375.07462	375.07462	3.67	0.0658
T	6	58884.20452	9814.03409	95.96	0.0001
LO*T	6	3710.10184	618.35031	6.05	0.0004

T tests (LSD): Alpha= 0.05 df= 28 MSE= 102.27
Critical Value of T= 2.05 Least Significant Difference= 11.96

CON=4

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	30316.68561	2332.05274	32.20	0.0001
Error	28	2027.65833	72.41637		
Corrected Total	41	32344.34395			

R-Square	C.V.	Root MSE	Mean
0.937310	30.96	8.509781	27.48

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LO	1	267.72188	267.72188	3.70	0.0647
T	6	27135.77725	4522.62954	62.45	0.0001
LO*T	6	2913.18648	485.53108	6.70	0.0002

T tests (LSD): Alpha= 0.05 df= 28 MSE= 72.41637
Critical Value of T= 2.05 Least Significant Difference= 10.064