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Ulocladium atrum as an antagonist of grey mould (*Botrytis* cinerea) in grapevine

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Referent:	Prof. Dr. H.-W. Dehne
Korreferent:	Prof. Dr. G. Noga
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Abstract

PHILIP SCHOENE

ULOCLADIUM ATRUM AS AN ANTAGONIST OF GREY MOULD *(BOTRYTIS CINEREA)* IN GRAPEVINE

Plant diseases inhibiting cultivation of crops are typical of intensive agriculture and plant protection measures with frequent applications of pesticides are therefore required. Problems occur by using chemical pesticides: e.g. development of resistance to pesticides and side-effects on beneficials and the environment, which calls for new strategies of crop protection. *Botrytis cinerea* (Pers.), one of the main pathogens in grapevine, *Vitis vinifera* L., is capable of infecting leaves, stems, flowers and fruits and reduces quality and quantity of yield. The saprophytic hyphomycet *Ulocladium atrum* (Pers.) proved to be antagonistic to *B. cinerea* in some crops, reducing growth and sporulation of the pathogen by competition for nutrients and space, limiting the epidemic spread of *B. cinerea* on plants and within the crop.

The efficacy of *U. atrum* for biological control of grey mould in grapevine was assessed under controlled conditions and in field studies. The antagonist reduced the development of *B. cinerea in vitro* and on necrotic and healthy grapevine leaf material, respectively. *U. atrum* reduced spore production of the pathogen on mature grapevine berries as well as sporulation of sclerotia. Competition for nutrients and space proved to be the principal mode of action. The antagonist showed, similar to the pathogen, an ecological fitness persisting unfavourable climatic conditions under controlled conditions and in field experiments. Moreover, a low sensitivity of *U. atrum* to many pesticides enabled an integration of the antagonist into conventional crop protection measures. Direct combination with some pesticides as tank mixture resulted in a lower vitality of the antagonist and therefore reduced its efficacy. Nonetheless, alternating treatments with pesticides and the biocontrol agent are a potential strategy to enrich strategies of integrated crop protection.

Large-scale experiments on biological control of grey mould by *U. atrum* were carried out in white grapes in 1997-99 in three German vine-growing areas. Three to four applications of the antagonist reduced *B. cinerea* effectively on plant tissue during the season and on berries at harvest. Furthermore, a reduced air-load of *B. cinerea* conidia was measured before vintage in 1997. This indicates that an effect of *U. atrum* could be enhanced when the antagonist is applied over a much larger area. The potential of this biocontrol agent was shown applied alone or combined with botryticides. In all three seasons with low to moderately high disease pressure, grey mould was reduced by *U. atrum* alike chemical botryticide treatments. Nonetheless, at a high disease pressure of grey mould, an integrated approach together with chemical control and biological control measures should be envisioned. Negative effects of *U. atrum* were observed neither on plants, inflorescences and berries nor on vinification. Hence, the antagonist provides a suitable biocontrol agent for grey mould in grapevine, and it can be used in both, biological and integrated crop protection.

Kurzfassung

PHILIP SCHOENE

ULOCLADIUM ATRUM ALS ANTAGONIST DER GRAUFÄULE (*BOTRYTIS CINEREA*) AN WEINREBEN

Wie bei allen intensiv bewirtschafteten Kulturpflanzen erschweren Krankheiten auch den Anbau von Weinreben, *Vitis vinifera* L.; ein Verzicht auf Pflanzenschutz kann schon nach wenigen Jahren die Pflanzen so sehr schwächen, dass ein Totalverlust des Bestandes möglich ist. Jedoch können auch Probleme bei dem Einsatz von chemischen Pflanzenschutzmitteln auftreten, wie zum Beispiel das Auftreten von Resistenzen gegenüber diesen Pestiziden, was nach neuen Bekämpfungsstrategien verlangt. Eines der bedeutendsten Rebenpathogene ist *Botrytis cinerea* (Pers.), das vegetative und generative Pflanzenteile befällt und so zu einem quantitativen und qualitativen Verlust führt. Der saprophytisch lebende Hyphomycet *Ulocladium atrum* (Pers.) zeigte sich in einigen Kulturen als Antagonist von *B. cinerea*. Als Konkurrent um Nährstoffe und Raum verringerte *U. atrum* das Wachstum und die Sporenbildung des Pathogens und reduzierte so die epidemische Ausbreitung von *B. cinerea* auf den Pflanzen und im Bestand.

Die Effizienz von *U. atrum* gegenüber der Graufäule an Weinreben wurde unter kontrollierten Bedingungen und im Freiland untersucht. Der Antagonist reduzierte die Entwicklung von *B. cinerea in vitro* und auf nekrotischen und gesunden Blättern. *U. atrum* verminderte die Sporenproduktion des Pathogens, ausgehend von infizierten reifen Beeren oder Sklerotien. Der hauptsächliche Wirkmechanismus bei diesen Interaktionen war Nährstoffkonkurrenz. Der Antagonist zeigte sich gut an die ungünstigen Umweltbedingungen der Phyllossphäre angepasst und wies eine geringe Sensitivität gegenüber im Weinbau üblichen Pestiziden auf; in beiden Bereichen war die ökologische Fitness von *U. atrum* besser als die des Pathogens, was wichtig für eine erfolgreiche Konkurrenz ist. Eine direkte Kombination mit einigen Pflanzenschutzmitteln in einer Tankmischung führte jedoch zu einer reduzierten Vitalität des Antagonisten und dadurch bedingt zu einem Verlust an Wirksamkeit gegenüber *B. cinerea*. Alternierende Behandlungen mit Pestiziden waren aber fast immer unproblematisch und stellen eine Bereicherung des Integrierten Pflanzenschutzes dar.

Zur Wirksamkeit von *U. atrum* gegenüber der Graufäule wurden 1997-99 Freilandversuche an weißen Traubensorten in drei Weinbaugebieten in Deutschland durchgeführt. Bei getrennter Ausbringung des Antagonisten reduzierten drei bis vier Applikationen das Vorkommen von *B. cinerea* und die Graufäule ähnlich einer konventionellen Botrytizidbehandlung. Negative Auswirkungen auf die Pflanzengesundheit oder die Herstellung und Lagerfähigkeit des Weines wurden nicht beobachtet. Wegen der guten Kombinationsmöglichkeit von *U. atrum* mit Pflanzenschutzmitteln und geringeren Bekämpfungserfolgen bei einem starken Auftreten der Graufäule sollte in Jahren mit hohem Befallsdruck von *B. cinerea* ein integrierter Einsatz von *U. atrum* angestrebt werden.

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1 Introduction

The grapevine, *Vitis vinifera* L., has been cultivated over millennia for several qualities that distinguish it from other plants, notably its vigorous growth and the ability of grapes to ferment into alcohol. It is particularly for the latter property that civilisation valued the grapevine. Members of the genus *Vitis* (family *Vitaceae*) are woody, climbing creepers growing in temperate regions. Viticulture, and especially viniculture, is very labour intensive, planting, pruning, fertilisation and crop protection to produce high quality grapes are as important and expensive as a skilful winemaking. Wine, the final product of this high-value crop, repays winegrowers for their ingenious work and justifies crop protection to a high level permitting viticulture in many temperate regions world-wide.

As with all intensively cultivated crops, plant diseases impede growing grapevine, and successful viticulture is improbable without crop protection. In 1885, Pierre Marie Alexis Millardet discovered the 'Bordeaux mixture', a combination product of copper and sulphur, and he developed it as the first fungicide to be widely applied to many crops up to now. Millardet described its effect against grapevine diseases, amongst them downy mildew, powdery mildew and grey mould [SCHNEIDERHAN, 1933].

Grey mould, caused by *Botrytis* spp., is one of the first plant diseases reported: MALPIGHI [1675] described the phytopathogenic fungi *Penicillium*, *Mucor*, *Rhizopus* and *Botrytis*. MICHELI [1729] showed the importance of fungal spores, conidia of *Botrytis* spp. among others for their epidemic spread. *Botrytis* spp. was validated by PERSOON [1801] but since the genus included more than 350 species, some of them wrongly ascribed, HENNEBERT [1973] assigned 22 species to the genus *Botrytis*. These necrotrophic fungi attack a wide variety of crops and they occur as saprophytes on necrotic plant material [JARVIS, 1980]. The most common species, *Botrytis cinerea*, attacks more than 200 plant species and causes economically significant yield losses in production as well as in storage [COLEY-SMITH *et al*., 1980]; conidia of *B. cinerea* are therefore almost omnipresent [COERTZE *et al*., 2001].

Botryotinia fuckeliana (De Bary) Whetzel, teleomorph of *B. cinerea*, forms apothecia but natural occurrences have been reported only infrequently [LORBEER, 1980]. Nonetheless, molecular analysis of strains showed that there is great genetic diversity and that sexual reproduction is therefore efficient [GIRAUD *et al*., 1998]. Furthermore, heterokaryosis generates genetic variability of *B. cinerea*, and the pathogen is therefore highly adapted to environmental changes [LORBEER, 1980].

MÜLLER-THURGAU [1888] described *B. cinerea* as a pathogen of grapevine infecting leaves, stems, flowers and fruits but he also mentioned its beneficial effect causing noble rot. This symptom of *B. cinerea* appears on late-harvested grapes under favourable weather conditions and causes the grapes to dry out and shrink so that the natural sugars become highly concentrated. Examples of botrytised white wines include French *Sauternes*, German *Auslesen*, Hungarian *Tokay* and Sicilian *Muscatel*. The French *Banyuls* is a red dessert wine made from Grenache grapes and there are also some German *Auslesen* made from red wine varieties. The significance of *B. cinerea* in grapevine is therefore ambiguous; nevertheless, *B. cinerea* mostly occurs as a pathogen causing grey mould and other symptoms in grapevine, which reduces quantity and quality of yield. Without *Botrytis* control, severity of grey mould range about 20 % and can reach up to 100 % and *B. cinerea* is therefore an important risk factor in viniculture [FARETRA & POLLASTRO, 2001]. Furthermore, modification of chemical composition is a serious damage in viniculture; *B. cinerea* converts sugars to glycerol, glycolic acid and β-glucan, secretes catabolic enzymes oxidising phenolic compounds and affects colour and flavour of wine and causes a musty smell [PEARSON & GOHEEN, 1988].

Moreover, fungal toxins produced by *B. cinerea* such as botrydial have been thought to inhibit vinification but these metabolites are not stable, and the high sugar content of botrytised must hampers fermentation [ALTMAYER *et al.*, 1985]. The most aggressive isolates of *B. cinerea* produced highest concentration of this metabolite *in planta*; botrydial is therefore classified as a phytotoxin [DEIGHTON *et al.*, 2001]. Nonetheless, other toxins excreted by *B. cinerea* were found to be more stable, and their toxicity was shown *in vitro* [BESSIS *et al.*, 1992]. Even if more important toxins are produced by fungi such as *Alternaria* spp., *Penicillium* spp. or *Trichothecium roseum* [SCHWENK *et al.*, 1989], increasing public awareness of residues of antibiotics produced by *B. cinerea* in wine called for a method to determine *Botrytis*-disease rate at harvest [COLLADO *et al.*, 1996].

Depending on catabolic conditions, stability of fungal metabolites differs widely, and immunological tests were developed to give reliable results. Initially, assays via antibodies were problematic because of their specificity to transient structures; mixtures of antigens derived from all fungal stages are needed for a confident quantitative analysis [MENDGEN, 1986]. Immunological tests developed in the past [BOSSI & DEWEY, 1992; BAKER, 1994; DEWEY & COLE, 1996] were not satisfactory until an antibody that recognises a thermostable antigen present in the extracellular matrix surrounding the hyphae was used, and *B. cinerea*

can now be suitably quantified [MEYER & DEWEY, 2000]. Despite the fact that the test is based on only one antibody, this diagnostic method provides reliable results.

B. cinerea can be found in every vineyard [JARVIS, 1980; COERTZE *et al*., 2001]. The pathogen achieves this omnipresence by reason of its wide host range, distribution by airborne conidia and low sensitivity to fungicides from different chemical classes. The fungus is also known for its rapid development of resistance to various classes of botryticides with different modes of action, impeding control of grey mould in grapevine [BLAICH *et al*., 1982; DEHNE *et al*., 1990; DUBOS, 1992; LORENZ *et al*., 1994a; LEROUX, 2000; ROßLENBROICH & STÜBLER, 2000]. Indeed, the 'Fungicide Resistance Action Committee' (FRAC) reported that *B. cinerea* was the first pathogen, whose onset of fungicide resistances caused severe problems in viticulture [RUSSELL, 1993].

In recent years some new compounds active against *B. cinerea* have been developed, which was highly needed for an anti-resistance strategy against grey mould [MUCKENSTURM & DECOIN, 2000; CAPELLA, 2001]. These new compounds are also not absolutely immune from losing their efficacy against this adaptive pathogen, however. It was shown that 'ATP-Binding-Cassette'-transporters are important in fungicide resistance of *B. cinerea*; botryticidal compounds are secreted actively and thereby the fungus is protected from their toxic effects [SCHOONBEEK *et al*., 2001]. This and other mechanisms enable *B. cinerea* to develop resistance to synthetic and natural botryticides, and even a single gene of this polykaric pathogen can determine its cross-resistance to several fungicides [CHAPELAND *et al*., 1999]. In addition to problems occurring in anti-resistance management, a lack of new fungicide introductions occurs in minor crops, and the number of available botryticides is restricted in various areas, which calls for new strategies in plant protection.

All kinds of plant diseases, weeds, animal pests have on their part antagonists, enemies or natural compounds, which can affect their development. In the biological control these agents are applied to reduce their epidemics and this was already practised before 900 AD; Chinese citrus growers placed nests of ants in trees to reduce populations of foliage feeding insects [SIMMONDS *et al*., 1976]. Biocontrol agents have been generally successful and there has been no major damage to the environment, certainly nothing on the scale of recent industrial accidents [HOKKANEN *et al*., 1995]. Nonetheless, biocontrol agents are not generally harmless and adequate research must be undertaken beforehand [LYNCH, 1992]. Furthermore, they have to contend with problems occurring in production, formulation, storage and their efficacy and,

therefore, with their economical profit that makes biocontrol often less accepted on the market than chemical pesticides [REINECKE, 1990; COPPING & MENN, 2000]. However, pollution of the environment and residues of pesticides make consumers desire biologically produced food, even if it is more expensive and quality standards are sometimes not achieved. At present, over 2600 agrobiological products from some 470 companies are used for controlling animal pests, diseases and weeds, as nitrogen fixers, silage and composts/soil activators, fertilisers, products for animals, for diagnostics or traps & monitoring devices [ANONYMOUS, 2001a]. Nevertheless, none of them is used world-wide or in all crops; hence many diseases or pests cannot be controlled biologically, and currently less than 5 % of pesticides are biocontrol agents [BUTT & COPPING, 2000]. Studies in research on new biocontrol agents and their development have to be done to enlarge the spectrum of biocontrol strategies and to fulfil plans of biological or organic farming, which aimed to reduce or to restrict pesticide applications.

Biological control of plant pathogens has been developed since SANFORD [1926] described interactions of microorganisms in the phyllosphere. Nonetheless, plant pathologists were late to recognise that microorganisms other than plant pathogens were associated in the phyllosphere and that they could affect disease development [BLAKEMAN, 1985]. Pesticides, especially those with a brought spectrum of pathogens to be controlled, eliminate naturally occurring antagonists, which can be replaced by applied antagonists [NEWHOOK, 1957; MANGIAROTTI *et al*., 1987; FOKKEMA, 1990].

Products based on nearly 35 antagonistic microorganisms against more than 40 plant pathogens have been commercialised in different countries world-wide and the number of commercialised biocontrol agents used to control plant diseases is increasing [ANONYMOUS, 2001a; WHIPPS & LUMSDEN, 2001]. Some of these products are listed in Table 1.

For intensively cultivated crops such as grapevine, combining biocontrol with balanced fertilisation and pruning is the only major alternative to the use of pesticides for controlling grey mould [CARGNELLO *et al*., 1991; R'HOUMA *et al*., 1998; SMITHYMAN *et al*., 1998]. Biocontrol agents enlarge the spectrum of *Botrytis* control; however, their use in grapevine is low at present. Due to their non-eradicative effect, biological agents are often less effective than chemical botryticides, even though, they can provide a sufficient control of grey mould in viniculture, where a moderate level of *B. cinerea* is tolerable, desirable or even enhanced for making dessert wines [MÜLLER-THURGAU, 1888; GANGL & TIEFENBRUNNER, 1999].

Table 1: Fungi registered and commercially marked as biocontrol agents (revised from WHIPPS & LUMSDEN, 2001).

	antagonistic microorganism(s)	product name and source
soil-borne and root pathogens	*Coniothyrium minitans*	Contans® WG, Prophyta, Germany
	Gliocladium virens	SoilGard®, Thermo Trilogy, USA
	Trichoderma albidus	Trichoseal®, Anchor Yeast, South Africa
	T. harzianum	Supresivit®, Borregard & Reitzel, Denmark
	T. harzianum	T-22G®, BioTrek® or Root Shield®, Bio-Works Inc., USA
	T. harzianum and *T. polysporum*	BINAB-T® WP, Svenska Predator AB, Sweden
postharvest diseases	*Candida oleophila*	Aspire®, Ecogen Inc., USA
	Cryptococcus albidus	Yield*Plus*®, Anchor Yeast, South Africa
aerial pathogens	*Ampelomyces quisqualis*	AQ10®, Biofungicide, Ecogen Inc., USA
	Bacillus subtilis	Serenade®, AgraQuest Inc., USA
	B. subtilis	FZB24® WG, FZB-Biotechnik GmbH, Germany
	Peniophora giganta	Rotstop® Kemira Agro Oy, Finland
	T. harzianum	Trichodex®, Makhteshim Chemical Works Ltd, Israel
	T. harzianum and *T. polysporum*	BINAB-T® WP, Svenska Predator AB, Sweden
	T. harzianum and *T. viride*	Trichoseal®, Agrimm Technologies Ltd, New Zealand

WOOD [1951] described first the biocontrol of grey mould in viticulture; he tested bacteria, actinomycetes and fungi on their antagonistic potential against *B. cinerea* in grapevine. DUBOS *et al*. [1982] studied the interaction of *Trichoderma viride* and *B. cinerea* in grapevine, and many other studies were conducted with *Trichoderma* species in grapevine [MEZZALAMA *et al*., 1985; SHIMSHONI *et al*., 1988; ELAD, 1994; HARMAN *et al*., 1996; O`NEILL *et al*., 1996; ELAD *et al*., 1998; SESAN *et al*., 1999]. Strains of *T. harzianum* were applied alone or integrated in chemical crop protection in grapevine [SHTIENBERG & ELAD, 1997; EGGER, 2001]. Some of them are commercialised, amongst others the strain T-39 as Trichodex®

(Table 1). Nonetheless, due to the low tolerance of diseases in table grapes, *T. harzianum* provided only insufficient control of grey mould in this crop [LATORRE *et al*., 1997]. A more successful antagonist of *B. cinerea* in table grapes, the mycoparasite *Pythium periplocum*, enters the pathogen's mycelium, grows inside metabolising its contents and finally produces numerous spores [PAUL, 1999].

Yeasts such as *Aureobasidium* spp. [BISIACH *et al*., 1985] and *Candida oleophila* [LIMA *et al*., 1997], *C. guilliermondii* or *Acremonium cephalosporium* [ZAHAVI *et al*., 2000], *Pichia anomala* and *P. membranifaciens* [MASIH *et al*., 2000; MASIH & PAUL, 2002] have been used as biocontrol agents against *B. cinerea* in grapevine, especially in table grapes. A strain of *C. oleophila* was commercialised as Aspire® (Table 1). Nevertheless, application of yeasts in grapevine might be critical, because the balance of naturally occurring yeasts important for vinification can be disturbed, or the antagonistic yeasts could also grow during fermentation [LONGO *et al*., 1991]. Therefore, most of the yeasts were tested in table grapes and proved their antagonistic potential against post-harvest diseases.

The antagonistic effectiveness of bacteria against grey mould in grapevine was proved for *Bacillus subtilis* [PUSEY, 1989; PAUL *et al*., 1997], *B. brevis* [SEDDON, 2000], *Pseudomonas* species [KRÓL, 1998], *Serratia liquefaciens* [WHITEMAN & STEWART, 1998] and also for compost extracts with *Pseudomonas* spp. and spore-forming bacteria [KETTERER *et al*., 1992]. In grapevine, *B. subtilis* is used as commercial biobotryticide Serenade® (Table 1), which is the first foliar applied, *Bacillus*-based biofungicide [ESTERIO *et al*., 2000].

Biological control depends on expressing the antagonistic mechanisms antibiosis, hyperparasitism, competition and induced systemic resistance, in various ways [KOCH, 1996]. Direct interaction, antibiosis, hyperparasitism, impedes registration as a biocontrol agent, which is almost as expensive and restricted as the registration of a chemical pesticide; therefore, many antagonists are marketed as plant growth promoters or stimulants, soil conditioners or wound protectants [WHIPPS & LUMSDEN, 2001]. Nonetheless, most screenings of biocontrol agents were conducted similar to active ingredients of chemical pesticides, focussed on direct inhibition of pathogens, because these effects are most convenient and promise success. Activity of fungi or bacteria selected against *B. cinerea* in grapevine often depends on antibiotics suppressing spore germination or mycelial growth [DUBOS *et al*., 1982; KETTERER *et al*., 1992; PAUL *et al*., 1997; GUETSKY *et al*., 2001]. Nonetheless, onset of resistance against these mechanisms is possible, just as with chemical pesticides.

The other direct effect of fungi, hyperparasitism, has been described for *Trichoderma* species against *B. cinerea* in grapevine [DUBOS, 1987], and other antagonists as *Pythium periplocum* are known as mycoparasites; unfortunately, this phenomenon has not been much exploited by now [PAUL, 1999].

Competition is considered important in biocontrol of grey mould in grapevine because germination of *B. cinerea* conidia depends on the presence of nutrients [PAULITZ, 1989]. Furthermore, the saprophyte needs nutrient sources for its epidemic spread, and retardation of the epidemic is a promising strategy in biocontrol [FOKKEMA, 1990]. Competition between aggressive and non-aggressive strains of *B. cinerea* was proved to reduce disease symptoms in French bean, suggesting that the two strains are co-existing in lesion [WEEDS *et al.*, 2000]. Nonetheless, antagonists, whose effect only bases on competition, were used rarely as biocontrol agents, due to their limited success. Another non-direct biocontrol strategy, induced systemic resistance, has been proved to reduce grey mould in grapevine [ELAD, 1996; BARKA *et al.*, 2000] but its success is sometimes inappropriate and depends on the grapevine variety [KASSEMEYER & BUSAM, 1998]. Grey mould resistant varieties were bred, and pathogenesis-related proteins were found that could be induced by salicylic acid or by *B. cinerea*; furthermore, genes promoting these proteins could be integrated in the genome of other plants [RENAULT *et al.*, 1996]. Nonetheless, these methods have to be refined, and finally, new grapevine cultivars are often less accepted on the traditional market of grapevine than familiar ones.

Infection process of *B. cinerea* is well studied in grapevine, though forecasting models for grey mould in this crop are difficult to generate [BRUNELLI & CORTESI, 1990]. Models were created to estimate disease risk [ELLISON *et al.*, 1998a] based on growth stage, conidial infection, mycelial infection, injury, symptoms and cultivar susceptibility; nonetheless, problems encountered in validation of the expert system [ELLISON *et al.*, 1998b]. Another expert system based on wetness duration and temperature was developed by BROOME *et al.* [1995] to use fungicides more efficiently in the time before *véraison*. Integration of biological and chemical control of *B. cinerea* in glasshouses within one crop protection strategy is realised in the BOTMAN forecast system following these lines: when slow disease progress or none is expected no spraying is needed. When an outbreak of epidemics is expected the use of chemical fungicides is recommended, and an application of the antagonist is otherwise advisable [SHTIENBERG & ELAD, 1997].

An adequate system is also feasible for other biocontrol agents and for other crops, though it would have to be accommodated to the more complex situation in field and to the biology of the antagonist. Nevertheless, many uncertainties in *Botrytis* forecast-systems obstruct a suitable control of grey mould in grapes based on these systems [MORIONDO *et al*., 1999]. Furthermore, preventive application of biocontrol agents is recommended due to their modes of action. Manufacturers' recommendation for applications of Serenade® (Table 1) against grey mould in grapevine suggests an application similar to conventional crop protection measures at certain growth stages.

As mentioned before, pesticides with a broad spectrum of pathogens reduce the number and variety of microorganisms in the phyllosphere. There are only a few highly specific fungicides without side-effects against naturally occurring saprophytes [SCHIEFER & KAST, 2002]. A biocontrol agent must not be sensitive to pesticides used at the same time in the respective crop. Otherwise, biological control is impeded, shown for species of *Trichoderma* applied against *B. cinerea* in grapevine; although, mutant strains of these antagonists could be combined with chemical pesticides [MEZZALAMA *et al*., 1985; KAY & STEWARD, 1994].

Antagonists of grey mould were used in grapevine either to replace or to reduce chemical pesticide treatments as alternating or combined applications [DUBOS *et al*., 1982; HARMAN *et al*., 1996; SHTIENBERG & ELAD, 1997]. Nonetheless, ELAD [1997] concluded that the moderate effectiveness of biocontrol agents calls for their integration with chemical and cultural control measures. Furthermore, most antagonists affect only some pathogens, which necessitate a combination with other crop protection programmes. An exception might be the biobotryticide Serenade® that is recommended for all important diseases of grapevines, though combinations with other plant protection programmes is advised due to the above-mentioned possibility of an onset of resistance against this agent.

Application technique, especially pressure and droplet size, have to be adapted for biocontrol agents in respect of the physiology and required density of the antagonist [SUTTON & PENG, 1993; BATEMAN & CHAPPLE, 2001]. Furthermore, for an antagonist it is desirable to be used in tank mixture applied with conventional spray equipment to minimise the amount of work and therefore to reduce costs of crop protection measures [BUTT *et al*., 2001]. An application as a tank mix also means direct contact with pesticides; an antagonist has to prove both, compatibility with chemical pesticides and robustness to application techniques to guarantee a suitable survival at its target place.

Early attempts to use antagonists in the phyllosphere were often unsuccessful until it was established that environmental conditions are crucial in affecting the microorganisms [BLAKEMAN & FOKKEMA, 1982]. Therefore, most successful biocontrol agents were used against soil-borne pathogens. To find a euryoecious antagonist is still one of the main problems; little information is available in literature about fate and ecological fitness of saprophytic antagonists in the phyllosphere [KÖHL & MOLHOEK, 2001]. Antagonists must have the capacity to grow rapidly during periods favourable for growth, and they have to be able to colonise niches to affect the pathogen, especially those antagonists whose mode of action depends on competition for nutrients [ELMER & KÖHL, 1998]. This fitness has to be evaluated under controlled conditions to obtain knowledge on the interactions of microorganisms at specific conditions, and results have to be proved in the phyllosphere; otherwise, biocontrol will not be a 'realistic goal' but only a 'false hope' [ANDREWS, 1990].

Drought tolerance was a major selection criterion for screening competitive antagonists of *Botrytis* spp. on necrotic leaves [KÖHL *et al*., 1993]; strains of *U. atrum*, especially the isolate Ua 385, were most suitable candidates for competition with *Botrytis* spp. under these conditions. The saprophytic hyphomycet *U. atrum* degrades diverse organic material and forms dark pigmented, pluricellular conidia [DAVID & KELLEY, 1995]. DIEM [1971] described differences between dark pigmented and hyaline fungi, and that cell walls of dermatiaceous spores provide protection against unfavourable climatic conditions. Microorganisms with dark pigmented spores as *U. atrum* are therefore suitable as biocontrol agents in the phyllosphere exposed to UV-radiation and rapid changes of temperature and availability in nutrients and water. Furthermore, another *Ulocladium* species, *U. botrytis*, was described as an antagonist of root-diseases [REAVES & CRAWFORT, 1994] and against nematodes [UNTIVEROS, 1975].

The antagonistic potential of *U. atrum* against *Botrytis* spp. was first described by KÖHL *et al*. [1993], and an EU-project entitled 'biological control of airborne necrotrophic plant pathogens by suppression of spore production' was initiated with nine collaboration partners. The aim of this project was to evaluate the antagonistic potential of *U. atrum* against *B. cinerea* in grapevine, strawberry and tomato, and against *Septoria* spp. in wheat. The title of the project indicates the antagonistic effect of *U. atrum* on the pathogens. Epidemic development of the pathogen is affected by a reduction of sporulation, which was shown to be a successful strategy for *U. atrum* [KÖHL *et al*., 1995b]. The mode of action of *U. atrum* was described as rapid and extensive competition for necrotic tissue excluding competitors,

without other antagonistic action such as parasitism or antibiosis [KÖHL *et al*., 1997]. Correspondingly, other *U. atrum* isolates were described as competitive antagonists of *B. cinerea* [YOHALEM, 1997]. This antagonist has therefore two advantages: risk of an onset of resistance against this antagonist is low and registration costs are less due to the missing direct fungicidal effect.

In the present study the biology of *U. atrum* Ua 385 and its antagonistic potential against *B. cinerea* in grapevine was investigated under controlled conditions and in field experiments, with special regard to the integration into existing crop protection programmes. The antagonist proved its efficacy in some crops [KÖHL *et al*., 1995b], and the first prerequisite for a successful use of *U. atrum* as an antagonistic competitor for nutrients in grapevine is its capability of colonising grapevine tissue. Grapevine synthesises fungicidal compounds [PEZET & PONT, 1992], which might affect *U. atrum*, and therefore, colonisation of necrotic grapevine tissue would be inhibited. Furthermore, as a permanent presence at sufficient density is needed for the antagonist to compete successfully with *B. cinerea*, *U. atrum* had to prove its ability to survive in the phyllosphere of grapevine. Adequate knowledge on dispersal of *B. cinerea* conidia is crucial to understand its epidemic and for evaluating possibilities for an antagonistic competition. Conidial spread of *B. cinerea* and the epidemic of grey mould was therefore investigated.

The antagonistic activity of *U. atrum* against *B. cinerea* was evaluated *in vitro* and at different growth stages of grapevine under controlled conditions and in field trials and the effect of *U. atrum* on other grapevine diseases was investigated. Furthermore, a low sensitivity of *U. atrum* to pesticides is important for its integration into crop protection; its compatibility with chemical pesticides was tested *in vitro* and under field conditions. Application methods were adapted to this antagonist in respect on its biology and the necessity of dispersal. Different modes of an application of *U. atrum* were evaluated; the antagonist was used in tank mixtures with conventional spray equipment. The possibility of an integration of this antagonist into crop protection, especially a combination with the botryticide fenhexamid was evaluated in order to enhance this integrated control strategy. Timing of applications was studied to determine the best dates of *U. atrum* application. Last of all, the effect of *U. atrum* on the plant health and on vinification was checked to guarantee an uncomplicated use of *U. atrum* as a biocontrol agent in grapevine.

The limited knowledge on the ecology of biocontrol agents seems to be a major constraint on successful biocontrol of foliar diseases [FOKKEMA, 1995]. Biological control, however, is not simple; 'its successful use requires trained specialists to conduct research on natural antagonists and well-informed extension workers and farmers to implement their use' [VAN DRIESCHE & BELLOWS JR., 1996]. The overall objective of this project was to demonstrate the potential of this antagonistic fungus to be integrated into an effective integrated crop protection strategy and to simplify an application of *U. atrum* for biocontrol of grey mould in grapevine.

2 Materials and Methods

2.1 Organisms

The following organisms were used for experiments or observed in field studies:

Host plants:	*Vitis vinifera* (L.) cv. 'Müller-Thurgau', 'Riesling', 'Kerner' and 'Thompson Seedless'
Pathogenic fungi:	*Botrytis cinerea* (Pers.) ex Fr., teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel)
	Penicillium expansum (Link) ex Thom.
	Phomopsis viticola (Sacc.) Sacc.
	Plasmopara viticola (Berk. & Curt.) Berl. & de Toni
	Pseudopezicula tracheiphila (Müll.-Thurg.) Korf & Zhuang
	Uncinula necator (Schw.) Burr.
Antagonistic fungus:	*Ulocladium atrum* (Pers.)

2.2 Production of plants and plant material

Plants were grown in glasshouses at 20 to 30 °C and relative humidities between 60 and 80 %. Plants were illuminated (High Pressure Sodium bulb, SON-T Agro SGR 140, Philips Electronics N.V., The Netherlands) for 16 h per day to accomplish illumination of approx. 8000 lx. Cuttings of grapes were grown as hydroponics with 'Oasis-growing-medium' (Agrimedia, Germany) for 28 days and potted into plastic pots (Ø 10 cm) with standard soil (type T). To avoid infection by *Uncinula necator* during rooting and growing, sulphur was applied to grapevine plants once a week with a 'Kombi-Verdampfer' (Paul Hübecker GmbH & Co KG Fleur Ami, Germany). Plants were irrigated by flooding greenhouse tables twice a day and fertilised with 0.2 % 'Flori 2' (Euflor, Germany) once a week.

Plants with six leaves were used for experiments. The last four fully developed leaves were taken for experiments with detached leaves or to produce dried leaf discs. Discs (Ø 18 mm) were punched out with a cork borer, dried for four weeks between paper towels at room temperature and sterilised by γ-irradiation (γ Gamm STER B. V., The Netherlands).

Table grapes (cv. 'Thompson Seedless') were used for experiments with mature berries under controlled conditions. Berries cut at 0.5 cm of the pedicel were surface-sterilised for 10 min with 1.5 % NaOCl.

2.3 Assessment of growth stages of grapevine

Growth stages of grapevine were assessed according to the BBCH code shown in Figure 1. The system described by LANCASHIRE *et al*. [1991] for the identification of grapevine growth stages is an adaptation of a basic scale developed to cover all mono- and dicotyledonous crops.

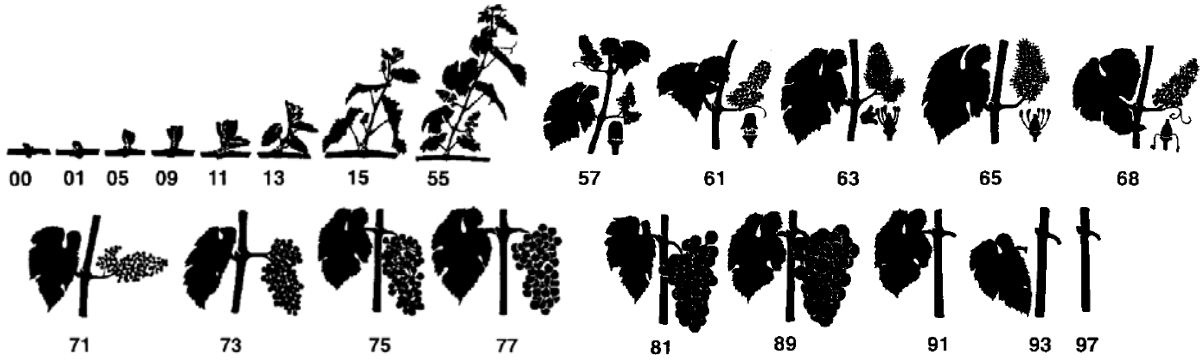


Figure 1: Phenological growth stages and BBCH-identification keys of grapevine (*Vitis vinifera* L. ssp. *vinifera*) [LORENZ *et al*., 1994b].

2.4 Cultivation of fungi

Ulocladium atrum, isolate Ua 385, was isolated from a necrotic leaf tip of a field grown onion, *Botrytis cinerea*, isolate Bc 700, from necrotic leaf tips of onion [KÖHL *et al*., 1995b]. Plant Research International (PRI, WAGENINGEN, The Netherlands) provided both organisms. Six other isolates of *B. cinerea* were isolated from grapevine berries sampled at Marienthal (Mt 1 and Mt 2), Geisenheim (Gh 1 and Gh 2) and Kirrweiler (Kw 1 and Kw 2) to assess the antagonistic potential of *U. atrum* against pathogenic isolates from vineyards.

2.4.1 Maintenance of fungi and preparation of spore suspensions

All fungi were stored in aqueous 1 % sucrose solutions at -80 °C and cultured in Petri dishes (Ø 92 mm) on oatmeal agar.

Prescription of oatmeal agar:

15 g agar
20 g oat flakes (Schmelzflocken, Kölln KGaA, Germany)

Ingredients were mixed, filled up to 1 l with H_2O_{demin} and autoclaved for 20 min at 121 °C.

The agar plates were inoculated with spore suspension (20 µl, 10^6 spores·ml^{-1}) and incubated in the dark for 14 days at 20 °C. Conidial spore suspensions were obtained by flooding cultures with sterile tap water containing 0.01 % Tween® 20 (Merck, Germany), and spores were gently removed from fungal cultures by rubbing with a spatula. Suspensions containing spores and hyphae were used unfiltered for mycelial growth rate tests. For tests on spore germination, the spore suspension of *U. atrum* was filtered through cotton gauze and washed twice with sterile tap water (0.01 % Tween® 20), conidia of *B. cinerea* were knocked off to the cover plate of the Petri dish and flooded with sterile tap water (0.01 % Tween® 20), to avoid residues of nutrients and mycelial fragments in the suspension.

To produce a higher amount of *U. atrum* spores for field experiments, the antagonist was grown on oat grain. 300 g oat kernels in 300 ml of tap water were moistened overnight inside a plastic bag. Water not absorbed was drained through a sieve and the oatmass was filled into autoclavable spawn bags (type 3LS, Sylvan, Horst, The Netherlands). The open end of the bag was closed with a cotton plug and sealed with an autoclavable tape. The spawn bags with the oat kernels were sterilised by autoclaving twice at 121 °C for 45 min with a 24 h time interval. The autoclaved oat kernels were inoculated with 5 ml of conidial spore suspension of *U. atrum* (10^6 spores·ml^{-1}) and incubated for four weeks at 20 °C. To avoid a predominant formation of mycelial growth, bags were shaken every second to third day. Two bags of kernels were transferred into cotton bags, washed two times for 5 min in 5 l tap water with 0.01 % Tween® 20 in a small washing machine (Nova MW super 2000 SR, Nova, The Netherlands). Suspensions were kept cold and applied within the next 6 h to avoid spore germination before application. The concentration of spore suspensions was determined with a counting chamber (Fuchs-Rosenthal) and adjusted with sterile tap water (0.01 % Tween® 20) to the concentration required.

2.4.2 Production of sclerotia

Six isolates of *B. cinerea* (Mt 1, Mt 2, Gh 1, Gh 2, Kw 1 and Kw 2) were used for the production of sclerotia. The isolates were grown on oatmeal agar for 28 days in daylight at room temperature, and sclerotia (Ø 2 mm) were picked with a spatula, washed with sterile water for 1 h, air dried and stored at 7 °C.

2.5 Application of fungi

As spores of both fungi stick together easily, it was necessary to use 0.01 % Tween® 20 as a wetting substance to facilitate an even dispersion of spores within the solution.

2.5.1 Application under controlled conditions

Sterilised leaf discs or mature berries were inoculated with *U. atrum* and *B. cinerea* as spore suspensions of $1 \cdot 10^6$ spores·ml^{-1}. Leaf discs were placed in Petri dishes with water agar, berries were placed in moist chambers and spore suspensions were applied with a commercial sprayer on the surface (approx. 2000 spores·cm^{-2}).

Detached leaves were inoculated with *U. atrum* as a spore suspension of $2 \cdot 10^6$ spores·ml^{-1}. *B. cinerea* was cultivated on oatmeal agar for two weeks, discs (Ø 5 mm) were punched out from the colonised agar and placed with the covered side on upper side of leaves.

2.5.2 Application of *Ulocladium atrum* under field conditions

In investigations on the optimisation of application technique, the spray pressure was varied in the range of 2 to 6 bar in 0.5 bar intervals using a Gloria knapsack sprayer with a Pin-to-cone nozzle (type 2010G, Gloria, Wadersloh, Germany). One day after application of the spore suspension on grapevine leaves, the viability of *U. atrum* was determined. Leaves were incubated for 14 h at room temperature in moist chambers and the germination rate was determined. The viability of spores in the spore suspension was 98 %. An application pressure of min. 2 bar was required for an evenly spread of the suspension. Conidia of *U. atrum* showed a high germination rate over the range of 2 to 5 bar. Above this pressure spores were affected and the germination rate was reduced (Figure 2). It was decided not to exceed a pressure of 4 bar, to have evenly spread conidia with a high survival rate.

In field experiments at Marienthal and Geisenheim *U. atrum* was applied as spore suspension with $1 \cdot 10^6$ spores·ml^{-1} (1997) and $2 \cdot 10^6$ spores·ml^{-1} (1998 and 1999) using a Gloria knapsack sprayer at 3 bar. At growth stage BBCH 03 the spore suspension was applied at a rate of 200 l·ha^{-1}. At later stages the rate was 600 l·ha^{-1}. At Kirrweiler the antagonist was sprayed with other pesticides as a tank mixture using the spray equipment for general crop protection at 4 bar with reduced application volumes. The concentration of spore suspensions was calculated for $2 \cdot 10^6$ spores·ml^{-1} at an application rate of 600 l·ha^{-1}.

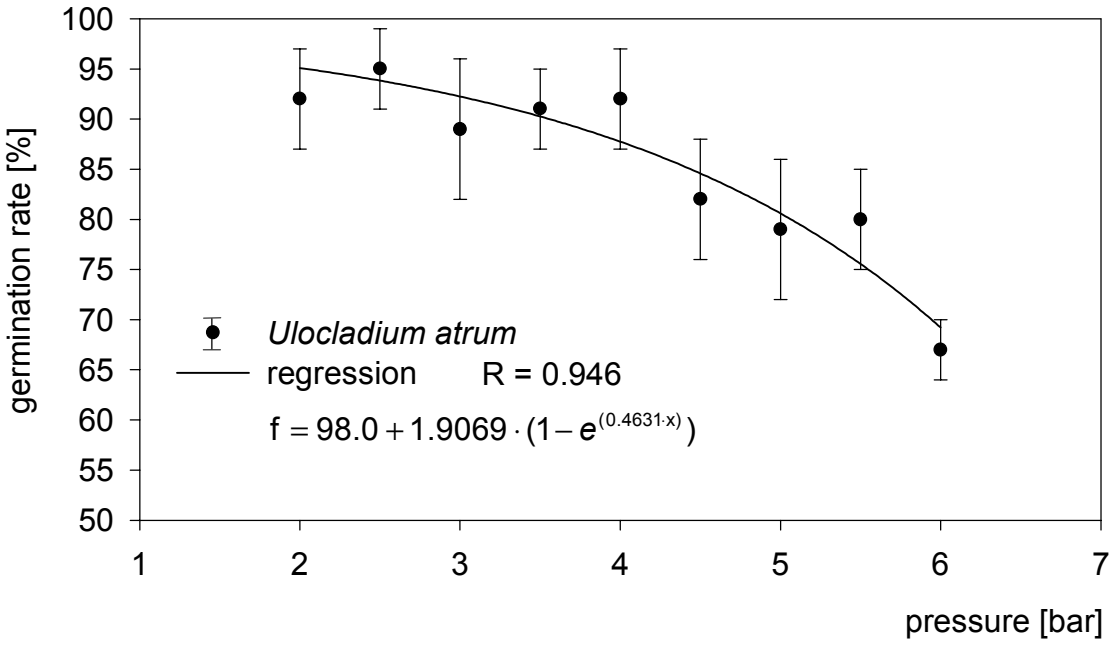


Figure 2: Effect of application pressure on the germination rate of *U. atrum* on grapevine leaves, depending on the application pressure (application of 10^6 spores·ml^{-1}, 14 h incubation at 20 °C, 99 % r.h.) showing an exponential regression from the germination rate against the pressure. Bars show the standard deviation of mean.

2.6 Evaluation of fungal growth

2.6.1 Germination rate

Germination rate was assessed in aqueous solutions, on agar or on leaf surface. Spores were incubated for 14 h at room temperature. Germination rate of 100 spores per replicate was assessed microscopically; germination was rated complete, when germ tube length was at least twice the spore diameter for *B. cinerea* or longer than the width of the *U. atrum* conidia.

2.6.2 Mycelial growth

A droplet of a suspension (15 µl, 10^6 spores·ml^{-1}) with spores and mycelium fragments was pipetted on the oatmeal agar. Petri dishes were incubated for 5 days at room temperature, and mycelial growth was determined by measuring radial growth of colonies.

2.6.3 Sporulation

The spore production was studied on sterilised grapevine leaf discs, on mature berries, and from sclerotia under controlled conditions. The number of spores produced was determined by stirring the leaves, berries or sclerotia for 10 min at 300 rpm in 10 ml tap water (0.01 % Tween® 20) and counting the spores microscopically in a counting chamber (Fuchs-Rosenthal). For low spore densities, spore suspensions were centrifuged and the volume was reduced by 90 or 99 %. The spore production of fungi was calculated per berry or sclerotia or per cm^2 of leaf tissue.

2.7 Assessment of the survival of *Ulocladium atrum*

To determine the viability of *U. atrum* on wounded and healthy grapevine leaves under controlled conditions plants were incubated in the greenhouse after application of the antagonist. Germination rate was assessed weekly by punching out leaf discs (Ø 2 cm), incubating the discs for 14 h at room temperature in moist chambers and counting the germinated spores microscopically.

During field experiments the persistence of *U. atrum* was tested on bark, inflorescences and berries. The viability of the antagonist was assessed by incubating 9 mm^2 bark samples, individual flower buds or berries on Petri dishes with water agar for 10 days, and counting the number of samples colonised with *U. atrum*. From each plot 25 samples were taken.

2.8 Assessment of the incidence of Botrytis cinerea

2.8.1 Spore trapping of airborne conidia

A Burkhard® seven-day recording volumetric spore trap (Burkhard Manufacturing Co. Ltd., Great Britain) was placed close to the vineyard at Marienthal in order to monitor the concentration of airborne conidia of *B. cinerea*. The vacuum pump was driven by 220 V to guarantee a flow rate of 10 $l \cdot min^{-1}$. The trap was kept in operation during the growing seasons in all three years. An adhesive of 50 ml Vaseline, 6 g Paraffin, 0.5 g Phenol and 0.5 ml Toluene was spread on the Melinex tape which was fixed to the recording drum. The tape was changed once a week, prepared by cutting into lengths of 48 mm (equivalent to 24 h), fixed on a glass slide and stained with 0.05 % acid Fuchsin. Concentrations of *B. cinerea* conidia per cubic metre were calculated for every hour.

Another spore sampler (Rotorod® Sampler Model 20, Sampling Technologies Inc., USA) was

used to estimate the spore concentration of *B. cinerea* in different plots. The sampler consists of a rotating arm with two fixed sampling heads, driven by a 12 V car battery. On one rod spores within 2.18 l of air were collected in 1 min. The same adhesive was used as for the Burkhard® spore trap. Rotorods® were placed within two plots per treatment and ran simultaneously. Samplers ran for 15 to 30 min and a second time directly after the first run for double the time. Samples were collected on the rods which were then analysed like the Melinex tape from the Burkhard® volumetric spore trap. The concentrations of *B. cinerea* conidia were calculated from the number of spores collected from all rods of one sampling and presented as spores per cubic metre.

2.8.2 Incidence of *Botrytis cinerea* on grapevine

Flower parts, berries and bark samples (9 mm^2) were collected to determine the incidence of *B. cinerea* on grapevine. Bark samples were taken from two one year old canes per plant. In order to assess latent infections with *B. cinerea* berries were surface-sterilised for 3 min with 1.3 % NaOCl at 20 °C, washed two times, dried for 10 min and cut in halves before incubation. The incidence of *B. cinerea* was measured by counting the samples colonised with *B. cinerea* after incubation on water agar for 10 days.

2.8.3 Assessment of *Botrytis* leaf blight

Botrytis leaf blight was assessed in detached leaves under controlled conditions. Oatmeal agar discs with cultivated *B. cinerea* were placed with the covered side on the upper side of the leaves. After 4 days of incubation at 20 °C in moist chambers, symptoms of *Botrytis* were classified on a scale of four classes: 1.) no symptoms, 2.) small circular lesion below the agar, 3.) lesion without sporulation, 4.) lesion with sporulation. Disease score data are given as percentage of each class.

The disease rate of *Botrytis* leaf blight in the field was assessed in 20 leaves per plant and ten plants per plot by counting the incidence of leaf infections. Disease data were converted to mean percent of infected plants.

2.8.4 Assessment of grey mould

For classification of grey mould intensity on grapevine berries the following scale of six classes was used: 1.) 0 %, 2.) 0-2 %, 3.) 2-10 %, 4.) 10-25 %, 5.) 25-50 % and 6.) 50-100 % in diseased berries. A total of 192 tagged bunches per treatment (four for each vine) were

assessed visually for disease severity from berry set to harvest. Disease score data were converted to mean percent berries of bunches or mean percent bunches infected by *B. cinerea* in the following formula:

$$\text{Mean disease severity on bunches} = \frac{n_2 + 6 \cdot n_3 + 17.5 \cdot n_4 + 37.5 \cdot n_5 + 75 \cdot n_6}{\sum_{6}^{i=1} n_i}$$

$$\text{Mean disease incidence on bunches} = \sum_{6}^{i=2} n_i \Big/ \sum_{6}^{i=1} n_i$$

n_i=number of bunches in class i

2.9 Assessment of other grapevine diseases

Incidence and severity of grapevine diseases like *Penicillium* rot (*Penicillium expansum*), *Phomopsis* cane or leaf spot (*Phomopsis viticola*), *Rotbrenner* (*Pseudopezicula tracheiphila*), downy mildew (*Plasmopara viticola*) or powdery mildew (*Uncinula necator*) were assessed for 10 plants per plot. For *Phomopsis* the number of cane spots was determined on two canes per plant. For *Rotbrenner*, downy and powdery mildew, the disease rate was determined in 20 leaves per plant by classifying the infected leaf area analogous to *B. cinerea* (2.8.3). In berries the disease rate of all pathogens was determined according to the same scheme as used for *B. cinerea* (2.8.4). Disease data were converted to mean percent of infected plants.

2.10 Variation of environmental conditions during incubation

The effect of temperature on the sporulation of fungi was studied at 99 % relative humidity (r.h.) and the following temperatures: 5, 10, 15, 20, 25, 30 and 35 °C. Temperatures were adjusted on ± 0.2 °C in four air-conditioned chambers run simultaneously.

In experiments on the effect of relative humidity on spore production the relative humidity above the leaf surface was adjusted within plastic boxes using a reservoir of saturated salt solutions (H_2O_{demin} >99 %, Na_2CO_3 92 %, KCl 86 %, NaCl 76 % and NaO_2 65 %) at 20 °C.

2.11 Pesticides

Pesticides were used in vineyards and for experiments under controlled conditions as formulated products listed in Table 2.

Table 2: Listing of pesticides used in the experiments. Active ingredients are listed with weight proportions and licence holders.

active ingredient(s)	product	company
Al-fosetyl + mancozeb (440+260 g/kg)	Mikal® MZ	AgrEvo
carbendazim + diethofencarb (250+250 g/kg)	Botrylon®	AgrEvo
Cu-oxychloride (756 g/kg)	Cupravit® OB 21	Bayer CropScience
cymoxanil + dithianon (100+250 g/kg)	Aktuan®	Cyanamid Agrar
cyprodinil + fludioxonil (375+250 g/kg)	Switch®	Novartis Agro
dichlofluanid (500 g/kg)	Euparen®	Bayer CropScience
dimethomorph (150 g/l)	Forum®	Cyanamid Agrar
dithianon (750 g/l)	Delan® SC 750	Cyanamid Agrar
fenarimol (120 g/l)	Rubigan® SC	Dow AgroScience
fenhexamid (510 g/kg)	Teldor®	Bayer CropScience
fluquiconazole (250 g/kg)	Castellan®	AgrEvo
folpet + metalaxyl-M (400+50 g/kg)	Ridomil® Gold Combi	Novartis Agro
iprodione (500 g/kg)	Rovral®	Rhône-Poulenc agro
kresoxim-methyl (500 g/kg)	Discus®	BASF
mancozeb (750 g/kg)	Dithane® Ultra WG	AgrEvo
metiram (800 g/kg)	Polyram® Combi	BASF
parathion-methyl (405 g/kg)	ME 605® -Spritzpulver	Bayer CropScience
penconazole (100 g/kg)	Topas®	Novartis Agro
propineb (705 g/kg)	Antracol® WG	Bayer CropScience
pyrifenox (200 g/l)	Dorado®	Novartis Agro
pyrimethanil (400 g/l)	Scala®	AgrEvo
spiroxamine (499 g/l)	Prosper®	Bayer CropScience
sulphur (800 g/kg)	Netzschwefel 80 WP	Stähler Agrochemie
tebuconazole + tolylfluanid (100 + 400 g/kg)	Folicur® EM	Bayer CropScience
tolylfluanid (505 g/kg)	Euparen® M WG	Bayer CropScience
triadimenol (52 g/kg)	Bayfidan® spezial WG	Bayer CropScience
vinclozolin (500 g/kg)	Ronilan® WG	BASF

2.11.1 Application of pesticides under controlled conditions

Pesticides were diluted with sterile H_2O_{demin} to the concentration required. For testing the effect of pesticides on spore germination, spores of *U. atrum* and *B. cinerea* were exposed to an aqueous 2 % sucrose solution with diluted pesticides on glass slides. For tests on mycelial growth, pesticides (1.5 ml) were added to 150 ml liquid oatmeal agar at approx. 50 °C. 12 ml of the medium was poured into each Petri dish, and after cooling off, plates were inoculated with the fungi.

Sterilised leaf discs, mature berries, detached leaves or plants for experiments under controlled conditions were sprayed with pesticides to the point of dripping and dried for 2 h at room temperature before the inoculation with *U. atrum* or *B. cinerea*.

2.11.2 Crop protection under field conditions

In field experiments the respective associate carried out general crop protection such as application of fertilisers and pesticides and pruning, according to local standard regulations. At Marienthal and Geisenheim botryticides were applied with a Gloria knapsack sprayer (type 2010G) similar to the application of the antagonist. At Kirrweiler, all the pesticides were sprayed as a tank mixture with or without *U. atrum* at 4 bar at reduced standard application volumes.

2.12 Determination of the sensitivity of fungi to pesticides

2.12.1 Assessment of EC_{50} values *in vitro*

The EC_{50} value, which is defined as the concentration of an active ingredient causing 50 % inhibition, was assessed for commercial products relative to the control (without active ingredient). The fungi were tested for the following concentrations of the active ingredient: 0, 0.01, 0.1, 1, 10 and 100 ppm. For compound products, the total of the active ingredients was used for the calculation of the EC_{50} value. The commercial products were adjusted in sterile H_2O_{demin} to the concentration needed. The effect of pesticides on the germination of fungi was assessed in aqueous sucrose solution; the effect of pesticides on mycelial growth was determined on oatmeal agar containing diluted pesticides. The EC_{50} value was determined with a sigmoidal regression (5 parameters) from the reduction of mycelial growth against the log-transformed concentrations. An example is shown in Figure 3.

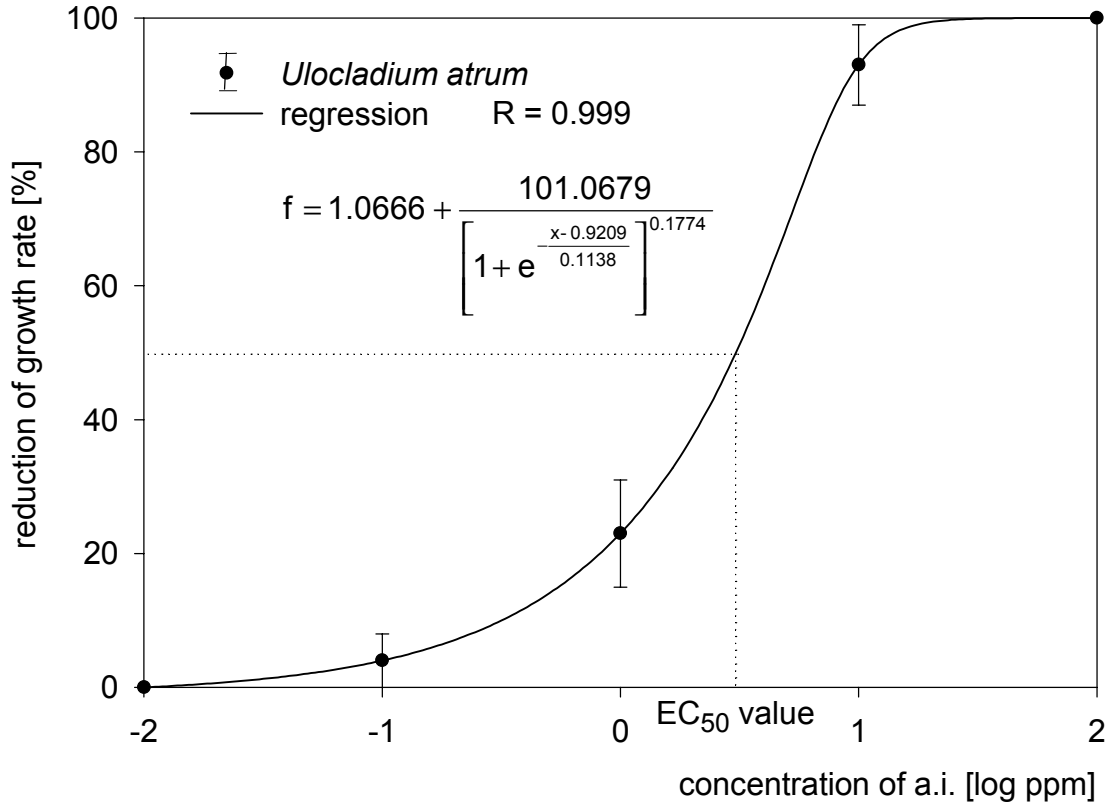


Figure 3: Determination of the EC_{50} value of iprodione for the mycelial growth of *Ulocladium atrum* with the sigmoidal regression from the relative reduction of growth against the log-transformed concentrations. Bars show the standard deviation of mean.

2.12.2 Assessment of the sensitivity of *Ulocladium atrum* under field conditions

In field experiments the survival rate of *U. atrum* on grapevine differentially treated with pesticides was determined on bark, inflorescences and berries. For the evaluation of a long term effect of pesticides on the antagonist *U. atrum* was sprayed 2 days before, simultaneously and 2, 4, 7, 14 and 21 days after application of pesticides on the leaves of grapevine plants at Bonn. Pesticides were applied at the highest concentration approved in 400 l/ha. Incidence of rainfall was negligible during the experiments. For each treatment 10 leaf discs (Ø 2 cm) were punched out 7 days after the application of *U. atrum* and incubated for 14 h at room temperature and 99 r.h.. Samples were stained with Blankophor. 100 spores per leaf disc were assessed microscopically to determine germination rate and mycelial growth of *U. atrum* using transmitted light and fluorescence technique. Reduction in both parameters was calculated based on the growth of *U. atrum* on plants without pesticides.

2.13 Design of the experimental analysis of interactions between fungi

2.13.1 Assessment of the effect of *Ulocladium atrum* on spore germination of *Botrytis cinerea in vitro*

For testing the antagonistic effect of *U. atrum* on spore germination of *B. cinerea*, spore suspensions of both fungi were inoculated together or individually and incubated for 14 h at room temperature in aqueous 0, 0.001, 0.01, 0.1, 1 and 2 % sucrose solutions on glass slides. Germination rate was assessed microscopically. Each treatment consisted of 10 replicates.

2.13.2 Assessment of the effect of *Ulocladium atrum* on mycelial growth of *Botrytis cinerea in vitro*

The effect of the antagonist on the mycelial growth of *B. cinerea* was studied within an interval between application of *U. atrum* and of *B. cinerea* on oatmeal agar. *U. atrum* was inoculated 0, 1, 2 and 3 days before *B. cinerea* and *vice versa*. Agar plates were incubated in daylight at 20 °C. Spore production of fungi was assessed 10 days after the first inoculation. Each treatment consisted of 10 replicates.

2.13.3 Determination of interactions between *Ulocladium atrum* and *Botrytis cinerea* on sterilised leaf discs

Sporulation of the fungi was studied on dried and γ-irradiated grapevine leaf discs under controlled conditions. Each treatment consisted of 10 replicates.

2.13.3.1 Assessment of the effect of environmental factors on spore production

Fungi were inoculated individually or in combination to assess the antagonistic potential at different environmental conditions. Spore production of the fungi was assessed in two days' intervals 2 to 16 days after inoculation. The effect of temperature on the kinetics of sporulation was studied at 99 % r.h. and at the following temperatures: 5, 10, 15, 20, 25, 30 and 35 °C. In experiments on the effect of atmospheric humidity on spore production moisture above the leaf surface was adjusted to 99 %, 92 %, 86 %, 76 % and 65 % r.h..

2.13.3.2 Evaluation of the effect of staggered inoculations on sporulation of *Ulocladium atrum* and *Botrytis cinerea*

The effect of an interval between application of *U. atrum* and *B. cinerea* was studied on dried and γ-irradiated grapevine leaf discs. *U. atrum* was inoculated 0, 0.5, 1, 2, 4, 7 and 10 days

before *B. cinerea* and *vice versa* and water agar plates were incubated at 20 °C and 99 % r.h.. Spore production of fungi was assessed 24 days after the first inoculation to have at least 14 days of inoculation for each fungus.

2.13.4 Determination of interactions between *Ulocladium atrum* and *Botrytis cinerea* on healthy leaves

The effect of *U. atrum* on the germination of *B. cinerea* and the severity of *Botrytis* leaf blight was studied on detached grapevine leaves under controlled conditions. Each treatment consisted of 20 replicates.

Testing germination fungi were applied as spore suspensions in sucrose solutions of 0, 0.001, 0.01, 0.1, 1 and 2 %; when applied with a time interval, the sucrose was administered with *U. atrum*. The rate of germination was determined 14 h after incubation at 99 % r.h..

In tests on the effect of *U. atrum* on the severity of *Botrytis* leaf blight, *U. atrum* was applied as a spore suspension and *B. cinerea* was inoculated with colonised agar discs. Fungi were inoculated separately, in combination or with a time-interval of 7 h between the applications of *U. atrum* and *B. cinerea*, in order to determine the antagonistic effect of *U. atrum*. The disease assessment was carried out 4 days after incubation in moist chambers (20 °C) by rating symptoms in four classes (2.8.3).

2.13.5 Assessment of the effect of *Ulocladium atrum* on the infection of inflorescences

Flowering grapevine plants cv. 'Müller-Thurgau' with at least ten leaves were placed in a greenhouse. Clusters were sprayed with *U. atrum* at growth stage BBCH 61 similar to field experiments. The inflorescences were inoculated with spore suspension of *B. cinerea* at growth stage BBCH 61 or BBCH 65 (fallen flowerhoods). Plants were kept for 18 h at 99 % r.h. and following that under normal glasshouse conditions. The infection of *B. cinerea* was assessed 14 days after inoculation of *B. cinerea* by determining the latent infection rate of berries. Each treatment had five plants with 50 observed inflorescences within two clusters.

2.13.6 Assessment of the effect of *Ulocladium atrum* on the spore production of *Botrytis cinerea* on berries

Sporulation of fungi was studied on surface sterilised, mature berries cv. 'Thompson Seedless', wounded with a needle roller. *U. atrum* was applied directly after injury, *B. cinerea*

was inoculated simultaneously or within a time interval of 2 days after wounding. Samples were incubated in moist chambers at 20 °C and 99 % r.h.. For one batch spores were washed off after 24 h to act as a reference for spore production. Spore production of *B. cinerea* was determined 7 days after inoculation of *B. cinerea* by counting the number of spores per berry, minus the number of spores detected after 24 h of incubation. Each treatment consisted of 30 replicates.

2.13.7 Assessment of the effect of *Ulocladium atrum* on spore formation of sclerotia

Six isolates of *B. cinerea* (Mt 1, Mt 2, Gh 1, Gh 2, Kw 1 and Kw 2) were used in tests with *U. atrum* to evaluate the effect of *U. atrum* on the spore production of sclerotia. Dried sclerotia were watered on water agar for 4 h and *U. atrum* was applied as a spore suspension at 50 µl per sclerotia. The fungi were incubated 40 days at 5 °C, 30 days at 10 °C and 20 days at 20 °C and 99 % r.h. and conidial spore production of the sclerotia was assessed. Each treatment had 20 replicates.

2.14 Field experiments

Field experiments were conducted at four locations in Germany: Marienthal (1997-1999, Ahr valley), Geisenheim (1998-1999, Rhinegau), Kirrweiler (1999, Palatinate) and Bonn (1998-1999, Rhine).

2.14.1 Sites and experimental layout

The **Ahr valley** is Germanys northernmost red wine area in a narrow valley with dry and warm climatic conditions. Rainfall in this area is 600-650 mm per year. The trial was located in a commercial vineyard of the ‘Staatliche Weinbaudomäne Marienthal‘ at the Stiftsberg, a plane field in a valley between two hills. Grapevine of the variety ‘Müller-Thurgau’ was used, planted in 1986 and cane pruned on a single curtain two-arm trellis system. Plot size was about 65 m^2 comprising three rows with 12 plants per row. The middle row was used for the reference; these vines were taken for sample collection, disease and yield assessment.

In **1997** conventional and integrated fungicide spray programmes were tested with *U. atrum*, to investigate the antagonistic potential of *U. atrum* against different grapevine pathogens with special respect on *B. cinerea*. Plots were placed in two blocks, one receiving fungicide treatments including botryticides and the other one an integrated spray programme without

botryticides (Figure 4). *U. atrum* was applied as a suspension of $1 \cdot 10^6$ spores·ml^{-1} at the end of bud swelling (growth stage BBCH 03), at the beginning of flowering (BBCH 61), when the majority of berries were touching (BBCH 78), and at the beginning of ripening (BBCH 81). Seven different treatments were carried out with six replications: a conventional treatment with and without four applications of *U. atrum* (BBCH 03, 61, 78 and 81), an integrated fungicide treatment without botryticides, with and without the antagonist, applied two (BBCH 61 and 81), three (BBCH 03, 61 and 81), or four times (BBCH 03, 61, 78 and 81), and one treatment was conducted as an integrated fungicide treatment with botryticides (Table 3).

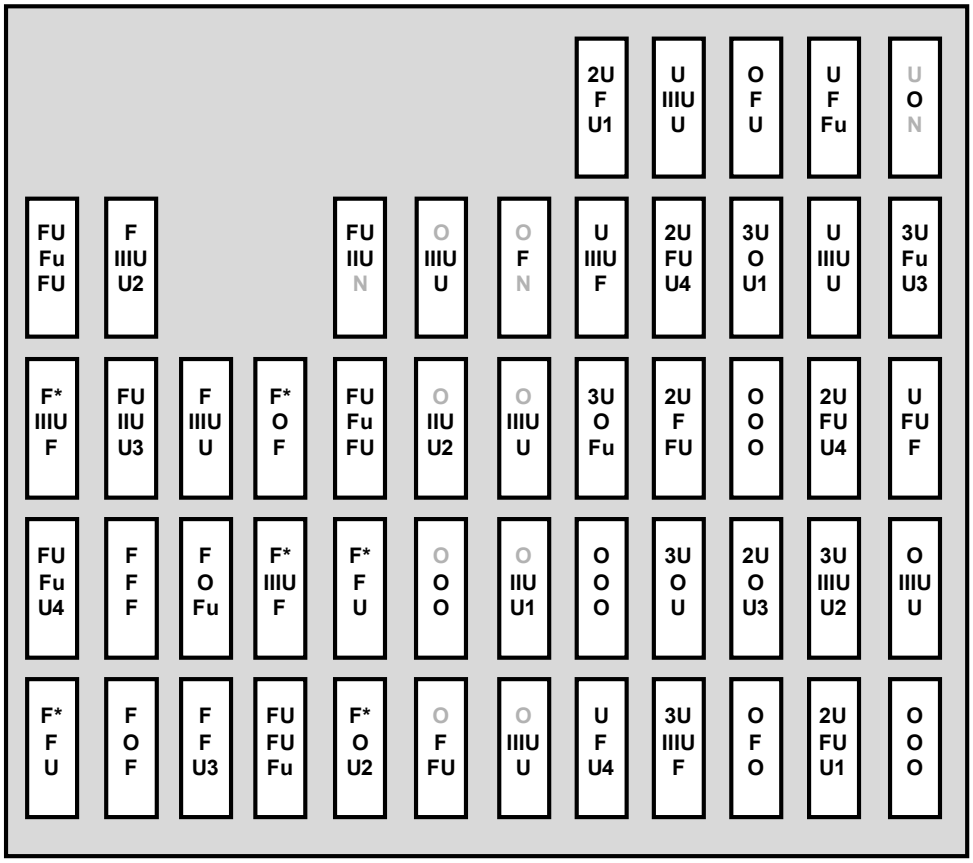
In **1998**, the total field was generally treated with pesticides without botryticides. Additionally, some plots were treated three times with botryticides (BBCH 61: pyrimethanil, BBCH 78: carbendazim & diethofencarb and BBCH 81: cyprodinil & fludioxonil) to accomplish a conventional crop protection. Due to long lasting presence of *U. atrum* on the bark in 1997 and a late touching of berries followed by a fast ripening, the last application of *U. atrum* (BBCH 81) was omitted in 1998. To guarantee a high density of spores on the plant, the antagonist was applied at double the concentration as in 1997. Suspensions of $2 \cdot 10^6$ spores·ml^{-1} were applied at the end of bud swelling (growth stage BBCH 03), at the beginning of flowering (BBCH 61), and once the majority of berries were touching (BBCH 78). Six different treatments were carried out with four replications (Table 3): a conventional treatment with and without three applications of *U. atrum*, a fungicide treatment without botryticides, with and without the antagonist, applied two (BBCH 03 and 78) or all three times. One treatment was conducted with a combination of two applications of *U. atrum* (BBCH 03 and 78) and one application of the botryticide fenhexamid at BBCH 61 to investigate a complementary effect of biological and chemical crop protection. To study the long-term effect of the *Botrytis* control on the epidemic of *B. cinerea*, the remaining plots were treated conventionally with and without botryticides, or with *U. atrum*, so that there were four plots of all combinations of these three treatments over the two years.

In **1999** the total field was generally treated with pesticides without botryticides, and, to accomplish a conventional crop protection, some plots were treated twice with fenhexamid (BBCH 61 and 81). Due to the results of the previous years, the application at BBCH 81 was resumed in the spray programme, to ensure a high population density of *U. atrum* on the grapevine at *véraison*. *U. atrum* was applied four times at an application rate of $2 \cdot 10^6$ spores·ml^{-1}: at the end of bud swelling (BBCH 03), before flowering (BBCH 55), at the

end of flowering (BBCH 69), and at the beginning of ripening (BBCH 81). Ten different treatments with four replications were carried out to prove the antagonistic effect of *U. atrum* and the combinatory effect with fungicides of *U. atrum*, and to determine the effect of one-time applications (Table 3).

Table 3: Design of the field experiments at Marienthal in 1997-1999.

year	pesticide treatment	abbrev.	number (and growth stages) of applications of *U. atrum*
1997	conventional fungicide treatment	F	0
	conventional fungicide treatment	FU	4 (BBCH 03, 61, 78 and 81)
	integrated fungicide treatment with botryticides	F*	0
	integrated fungicide treatment without botryticides	O	0
	integrated fungicide treatment without botryticides	U	4 (BBCH 03, 61, 78 and 81)
	integrated fungicide treatment without botryticides	3U	3 (BBCH 03, 61 and 81)
	integrated fungicide treatment without botryticides	2U	2 (BBCH 61 and 81)
1998	conventional fungicide treatment	F	0
	conventional fungicide treatment	FU	3 (BBCH 03, 61 and 78)
	conventional fungicide treatment	Fu	2 (BBCH 03 and 78)
	conventional fungicide treatment without botryticides	O	0
	conventional fungicide treatment without botryticides	IIU	2 (BBCH 03 and 78)
	conventional fungicide treatment without botryticides	IIIU	3 (BBCH 03, 61 and 78)
1999	conventional fungicide treatment	F	0
	conventional fungicide treatment	FU	4 (BBCH 03, 55, 69 and 81)
	conventional fungicide treatment	Fu	2 (BBCH 03 and 69)
	conventional fungicide treatment without botryticides	O	0
	conventional fungicide treatment without botryticides	U	4 (BBCH 03, 55, 69 and 81)
	conventional fungicide treatment without botryticides	U1	1 (BBCH 03)
	conventional fungicide treatment without botryticides	U2	1 (BBCH 55)
	conventional fungicide treatment without botryticides	U3	1 (BBCH 69)
	conventional fungicide treatment without botryticides	U4	1 (BBCH 81)



F	conventional fungicide treatment	2U	O with 2x *U. atrum* (appl. 2 & 4)
F*	integrated fungicide treatment	3U	O with 3x *U. atrum* (appl. 1, 2 & 4)
O	reduced/ integrated fungicide treatment (without botryticides)	IIU	O with 2x *U. atrum* (appl. 1 & 3)
		IIIU	O with 3x *U. atrum* (appl. 1, 2 & 3)
FU	F with max. *U. atrum*	U1	O with 1x *U. atrum* (appl. 1)
Fu	F with 2x *U. atrum* (appl. 1 & 3)	U2	O with 1x *U. atrum* (appl. 2)
U	O with 4x *U. atrum* (appl. 1, 2, 3 & 4)	U3	O with 1x *U. atrum* (appl. 3)
N	not used plot	U4	O with 1x *U. atrum* (appl. 4)

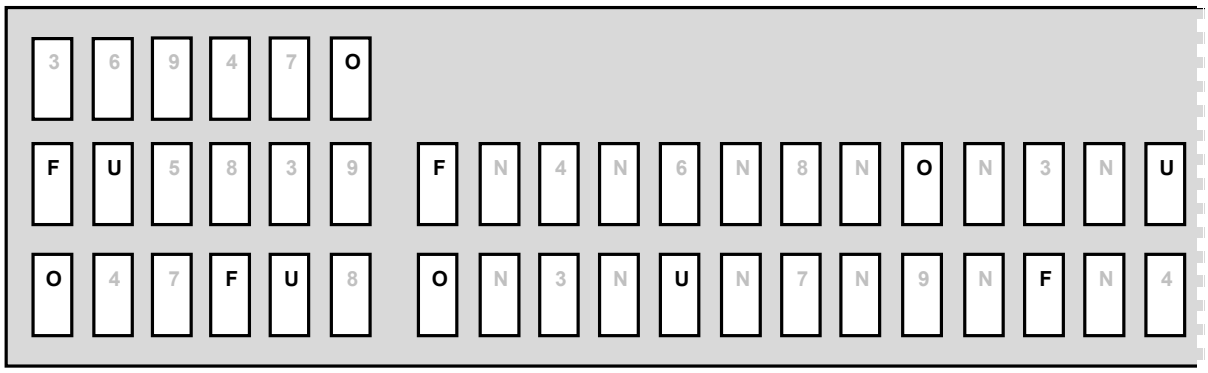
Figure 4: Experimental layout of the field trials 1997-1999 at Marienthal, Stiftsberg. Each rectangle corresponds to a plot of 3 x 12 vines cv. 'Müller-Thurgau'. The upper lettering relates to 1997, the middle to 1998 and the lowest to 1999. Plots with grey letters have not been assessed in that year.

Geisenheim is located in a plane white wine growing area of the Rhinegau with moderate rainfall (700-780 mm per year). Experiments with the grapevine variety 'Riesling' were conducted together with tests of the 'Fachhochschule-Geisenheim'. Plot size was about 31 m^2 comprising one row with 12 plants per row; all these vines were used for sample collection and disease and yield assessment. A standard fungicide treatment without botryticides was used in all plots. Due to the other cultivar and local conditions, the effect of *U. atrum* was applied at growth stages BBCH 55, 68, 79 and 81 (Table 4).

Table 4: Design of the field experiments at Geisenheim in 1998 and 1999.

year	pesticide treatment	abbrev.	number (and growth stages) of applications of *U. atrum*
1998	conventional fungicide treatment	F	0
	conventional fungicide treatment without botryticides	O	0
	conventional fungicide treatment without botryticides	U	4 (BBCH 55, 68, 79 and 81)
1999	conventional fungicide treatment	F	0
	conventional fungicide treatment without botryticides	O	0
	conventional fungicide treatment without botryticides	U	4 (BBCH 55, 68, 79 and 81)
	O with fenhexamid (BBCH 68 and 81)	T	0
	O with fenhexamid (BBCH 68 and 81)	UT	2 (BBCH 55 and 79)

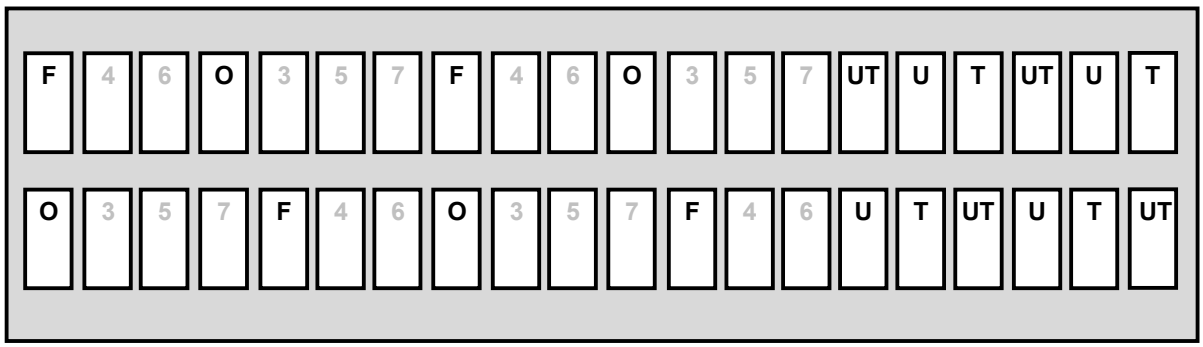
In 1998 grapevine *cv*. 'Riesling' was used in a commercial vineyard 'Ruck', planted in 1978, and cane pruned on a single curtain two-arm *Pendelbogen* system. The effect of *U. atrum* on the epidemic development of grey mould was investigated using three different treatments with four replications in a randomised block design (Figure 5). *U. atrum* was applied at a rate of $2 \cdot 10^6$ spores·ml^{-1}. Times of application were before flowering (BBCH 55), when 80 % of flowerhoods were fallen (BBCH 68), when the majority of berries were touching (BBCH 79), and at the beginning of ripening (BBCH 81).



F: conventional fungicide treatment
O: conventional fungicide treatment without botryticides
U: conventional fungicide treatment without botryticides with 4x *U. atrum*

Figure 5: Experimental layout of the field trials at Geisenheim 'Ruck' in 1998. Each rectangle corresponds to a plot of 12 vines cv. 'Riesling'. Plots with grey letters have not been assessed for this experiment.

In 1999 the grapevine variety ‘Riesling’ was used in a commercial vineyard ‘Fuchsberg’, planted in 1992, and cane pruned on a single curtain two-arm *Pendelbogen* system. The field was divided in two blocks; one block with the conventional fungicide treatment with and without botryticides was conducted together with tests of the ‘Fachhochschule-Geisenheim’ as a randomised block design. The other three treatments were placed next to the first one in another block (Figure 6). Additionally to the experiment in 1998 the effect of combination of fenhexamid and *U. atrum* was assessed in two treatments.



F: **conventional fungicide treatment**
O: **conventional fungicide treatment without botryticides**
T: **conventional fungicide treatment without botryticides with 2 x fenhexamid**
UT: **conventional fungicide treatment without botryticides with 2 x fenhexamid and 2 x *U. atrum***
U: **conventional fungicide treatment without botryticides with 4x *U. atrum***

Figure 6: Experimental layout of the field trials at Geisenheim ‘Fuchsberg’ in 1999. Each rectangle corresponds to a plot of 12 vines cv. ‘Riesling’. Plots with grey letters have not been assessed for this experiment.

Kirrweiler, close to ‘Neustadt an der Weinstraße’, is located in a plane white wine growing area of the Palatine with moderate rainfall (580 mm per year) and high average temperatures. Experiments were carried out in co-operation with BAYER VITAL according to the BAYER guidelines for screening tests. Grapevine of the variety ‘Kerner’ was used, cane pruned on a single curtain two-arm *Pendelbogen* system. The plot size was 12 or 20 plants in one row of which the 10 plants in the centre were used for sample collection and disease and yield assessment. Four treatments were conducted with four replications, located side by side. In control plots, a treatment without any use of fungicides was conducted, in the other three treatments; a standard pesticide programme without botryticides was used. One treatment consisted of *Botrytis* control with fenhexamid. Times of application of fenhexamid and

U. atrum were at inflorescences swelling (BBCH 55), at 80 % of flowerhoods fallen (BBCH 68), at majority of berries touching (BBCH 79), and at the beginning of ripening (BBCH 81). The effect of *U. atrum* was investigated singularly or in combination with fenhexamid.

Field experiments in **Bonn** were conducted in the experimental fields of the University of Bonn with four rows of 30 vines to evaluate the long-term effect of botryticides on the antagonist and to investigate the effect of *U. atrum* against *Uncinula necator*, the fungus causing powdery mildew of grapevine. This experimental field was chosen because of the absence of grapevine diseases other than powdery mildew. Experiment in 1998/99 included treatments without pesticides, with five applications of *U. atrum* at BBCH 00, 11, 15, 51 and 97, and a fungicide programme against powdery mildew with sulphur. The trial was designed as a randomised block design, consisting of ten plants in one row. Each treatment consisted of four replications.

2.14.2 Measurement of meteorological data

At Marienthal meteorological data were recorded at the edge of the trial area. Within the bunch canopy at the height of 1 and 2 m, respectively, dead leaf wetness sensors (PRI, Wageningen, The Netherlands) were positioned. These sensors evaluate the water content of dead plant material as an ohmmeter using a voltage of 15 V over 8 mm of dead grapevine leaf tissue. The sensitive area of measurement is 800 mV (dry sensor) to 2000 mV (wet sensor). Air temperature, wind speed and direction were measured 1 m above the plants and all data were stored with a Opus II-data logger (G. Lufft Mess- und Regeltechnik GmbH, Germany). Measurements were performed at 10 min intervals and data were calculated for hourly values. Data were transferred to a laptop at the vineyard and evaluated with Opus-SmartGraph version 1.16 (G. Lufft Mess- und Regeltechnik GmbH, Germany). Rainfall was measured daily at the ‘Staatliche Domäne Marienthal’.

2.14.3 Yield assessment

Yield assessments were possible for the field trails at Marienthal and Kirrweiler where vintage was by hand picking. At Marienthal only grapes fulfilling the health requirement of a standard vintage were harvested. Weight, acid and sugar content were assessed per plot. At Kirrweiler all grapes were harvested per treatment, mixed for further fermentation, and acid and sugar content were assessed.

2.14.4 Vinous fermentation and assessment of wine ripening

Four different treatments were taken for the vinification at Marienthal and Kirrweiler: a conventional treatment with and without applications of *U. atrum*, and a fungicide treatment without botryticides, with and without the antagonist. For further fermentation grapes were mixed per treatment, must was pressed, enriched with sugar and fermentation agents and filled into glass vessels for standard fermentation. At Kirrweiler the decrease in weight was observed during the fermentation by weighing the vessels once a day. Wine was filled in 0.7 l bottles with screw caps after vinification. Acid and sugar contents were assessed before and after fermentation. The gustatory quality of flavour and odour was determined in examinations according to BBA guidelines for sensory tests [ENGLERT *et al.*, 1988]. The acid content was checked every year to assess the stability of wines.

2.15 Microscopic examinations

Microscopic studies, tests on the germination rate and counting the number of spores on Melinex® tapes and/or Rotorod®-sticks were conducted with a Leitz DMRP photo microscope (Leitz, Germany) with light and fluorescence optic. Photos were taken with a camera system (Leica Wild MPS, Germany) or a digital camera Hitachi HV-C20*A* driven with the image-processing programme Discus 32 (Hilgers, Germany).

Spore germination in aqueous solutions was assessed with a 100-fold magnification without staining. *B. cinerea* spores on the Melinex tape were stained with 0.05 % acid Fuchsin for 24 h. Subsequently, tapes were gently washed twice with H_2O_{demin} and spores were counted using a 100-fold magnification of a microscope. Fungal structures on leaves were stained with 0.05 % Blankophor for 10 min, gently washed two times with H_2O_{demin} and examined with a fluorescence microscope at different magnifications (used filter combination: excitation filter: BP 340-380, dichromatic mirror: FT 400 and suppression filter LP 430). A combination of light- and fluorescence- techniques was used for better orientation on the leaf surface.

2.16 Statistical analyses

Experiments under controlled condition have been conducted two times. The statistical analyses were performed using SigmaStat 2.2 (SPSS Inc., USA). Means, variances and significant differences were worked out. Regressions were fitted and graphed with SigmaPlot 4.0 (SPSS Inc., USA). A multifactorial analysis of variances (ANOVA) was

performed on data. With no homogeneity of variances data were transformed for statistical analyses but the means of the original data are shown in the tables and graphs. Significant differences between means were determined by Tukey-test ($p \leq 0.05$). With two treatments, significant differences between means were determined with the *t*-test ($\alpha = 0.05$). The Kruskal-Wallis analysis on ranks ($p \leq 0.05$), or, in case of two different treatments, the Mann-Whitney rank sum test ($p \leq 0.05$) were performed for data, which were not normally distributed or without homogeneous variances. Standard deviations of means, standard deviations of frequencies or, for not normal distributed data, quartiles and maximal quartiles (max*QA*), respectively, were calculated. To determine significant differences of progressions, data of samples, taken at one point of time, were transformed to ranks, and differences between the progressions of a treatment and the control were determined according to the Mann-Whitney rank sum test.

3 Results

The saprophytic fungus *U. atrum* (Ua 385) was isolated from necrotic onion leaves and is known to colonise dead material under certain climatic conditions. It has shown an antagonistic potential against *Botrytis* spp. in different crops. It is desirable for biocontrol agents, to be effective in as many host-pathogen relationships as possible. This study was designed to extend the information about *U. atrum*: its antagonistic potential against *B. cinerea*, its sensitivity to pesticides, and to evaluate the possible applications of *U. atrum* in grapevine.

3.1 The development of *Ulocladium atrum* on grapevine

The survival of an antagonist at the target place is important for its success, especially, if its effect is based on competition, as it supposed for *U. atrum*. In this chapter, results are presented showing the ability of *U. atrum* to survive and to hibernate on bark of grapevine. Additionally, a potential effect of the antagonist on the plant has been tested.

3.1.1 Survival of *Ulocladium atrum* spores on grapevine

A sufficient density of spores is necessary for *U. atrum* to be able to compete with *B. cinerea*. Therefore, a high survival rate after application is essential for the success of *U. atrum* as a biocontrol agent and influences the frequency of required applications. The viability of *U. atrum* was tested on different parts of grapevine throughout the season.

To test the presence of *U. atrum* on the grapevine plant throughout the growth period, samples of bark from one-year-old canes were taken. The sample-size of bark (9 mm^2) was chosen to have at least one colony forming unit of *U. atrum* per sample immediately after application, and to be suitable to show an early decline of this value within the following weeks. Despite of different climatic conditions or the lower spore concentration of *U. atrum*, used in 1997, there was a similar decrease of *U. atrum* on the bark in the seasons 1997 to 1999 (Figure 7). After application of the antagonist, the percentage of bark samples with *U. atrum* decreased within one week to 70-80 %. After four weeks, the antagonist could be re-isolated from 30-50 % of the bark segments. In plots without applications of *U. atrum*, the antagonist was found rarely on the bark, and at a density lower than 2 %.

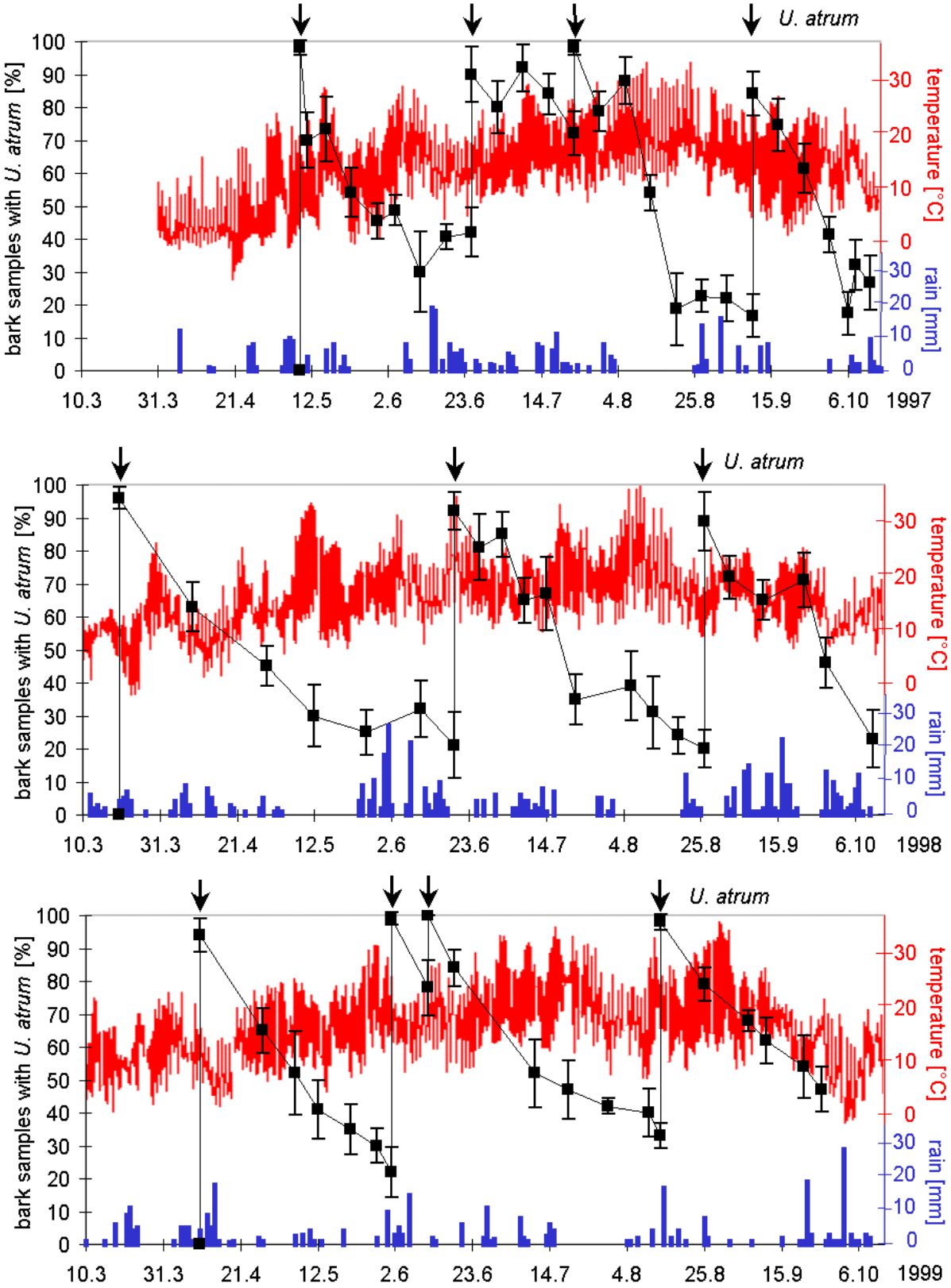


Figure 7: Survival of *Ulocladium atrum* on the bark of grapevine after application (↓) and the meteorological data of temperature and rainfall at Marienthal, 1997-1999. Bars show the standard deviation of mean.

After application, *U. atrum* could also be re-isolated from inflorescences and berries of grapevine. After incubation in a moist chamber, the incidence of *U. atrum* was assessed either microscopically (Figure 8, II), or macroscopically (Figure 8, I, III-V). The antagonist could rarely be found growing on wounded or withering berries in the vineyard (Figure 8, VI).

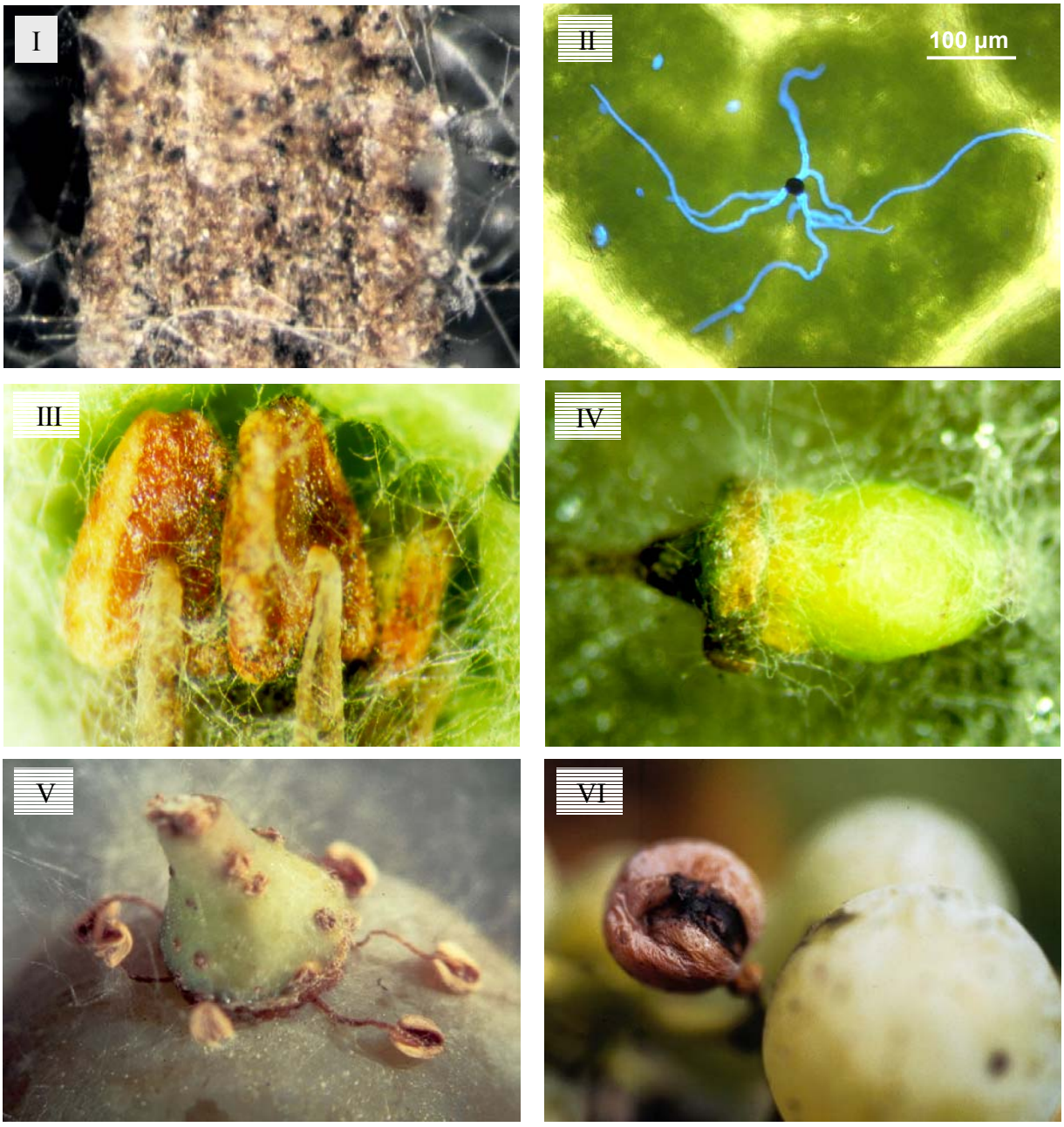


Figure 8: Growth of *Ulocladium atrum* on the bark (I), on the leaf surface (II, fluorescence microscopy), on inflorescence (III), on young berries (IV), at the basis of mature berries (V) and on necrotic berries (VI). Samples were taken after application of *Ulocladium atrum* at Marienthal. Samples I-V were incubated in a moist chamber.

After an early application (BBCH 03) of *U. atrum* there was only a low population density on the inflorescences, while an application at BBCH 55 resulted in the presence of *U. atrum* throughout the flowering period (Table 5). For all treatments later than BBCH 03, *U. atrum* was able to survive on more than 75 % of the flowers or berries until vintage. In plots without the application of *U. atrum* the antagonist was found rarely on inflorescences, and at densities, lower than 2 %. There was no significant effect of the application of botryticides on the incidence of *U. atrum* on the inflorescences or berries at Marienthal in 1999.

In experiments on latent *Botrytis* infection, berries have been surface sterilised for 3 min with 1.3 % NaOCl before incubation in moist chambers. *U. atrum* could not be re-isolated from these surface sterilised berries.

Table 5: Effect of application time and frequency on the incidence of *Ulocladium atrum* on inflorescences and berries of grapevine at Marienthal in 1999.

growth stage [BBCH]	conventional fungicide treatment without botryticides						conventional fungicide treatment	
	1x *U. atrum* (03)	1x *U. atrum* (55)	1x *U. atrum* (69)	1x *U. atrum* (81)	4x *U. atrum* (03, 55, 69, 81)	2x U. atrum (03, 81)	4x *U. atrum* (03, 55, 69, 81)	2x *U. atrum* (03, 81)
15	10 ±7	0 ±0	0 ±0	0 ±0	12 ±10	7 ±7*	5 ±9*	9 ±7*
55	11 ±9	100 ±0	0 ±0	0 ±0	99 ±3*	12 ±6	100 ±0	6 ±6*
61	7 ±6*	88 ±9	0 ±0	0 ±0	92 ±8	13 ±11	90 ±6	8 ±6*
67	9 ±6	91 ±7*	0 ±0	0 ±0	86 ±9	10 ±6*	91 ±9*	11 ±8*
71	5 ±6*	94 ±10*	98 ±3*	0 ±0	100 ±0	98 ±4*	99 ±3*	100 ±0
75	10 ±7	87 ±9	94 ±6*	0 ±0	97 ±4*	95 ±7*	90 ±10*	93 ±7*
81	8 ±7*	91 ±6	86 ±11	100 ±0	100 ±0	82 ±10	99 ±3*	85 ±6
89	7 ±7*	92 ±12*	76 ±13	94 ±7*	96 ±7*	91 ±9*	100 ±0	83 ±11

Raised data show standard deviation of mean;
*data not normally distributed, raised data show max*QA*

3.1.2 Hibernation of *Ulocladium atrum* in the vineyard

The ability of *U. atrum* to hibernate on grapevine bark was examined to investigate the possibility of an antagonistic long-term effect of *U. atrum* on *B. cinerea*.

The survival of the antagonist was determined by re-isolation of *U. atrum* from the bark of the stem, because, due to the training system the one year old canes were pruned. In the season after application less than 10 % of the samples were colonised with *U. atrum*, which is equal to a density of *U. atrum* smaller than one colony forming unit per cm^2 (Figure 9). In the second year after application, observations could only be made in one plot at Marienthal; *U. atrum* was found in less than 2 % of samples on the stem of the grapevines, and *U. atrum* could not be isolated from samples of the one year old bark, from inflorescences, or from berries.

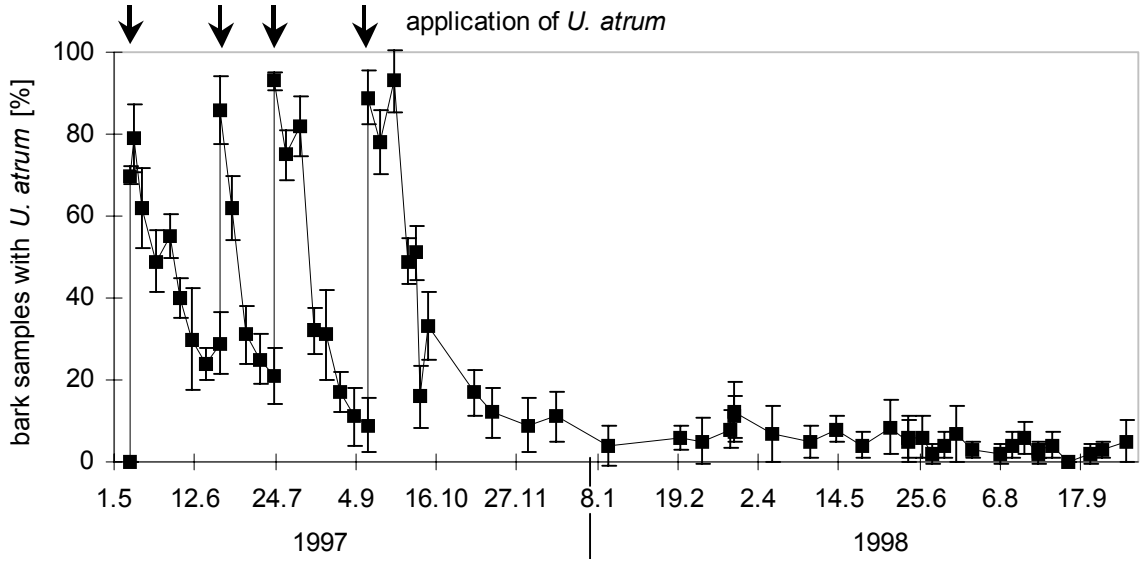


Figure 9: Survival of *Ulocladium atrum* on the bark of grapevine in plots with conventional fungicide treatment after four applications (↓) in 1997 (Marienthal, 1997-1998). Bars show the standard deviation of the mean.

3.1.3 Effect of *Ulocladium atrum* on grapevine plants

Some species within the genus *Ulocladium* are described to be phytopathogenic fungi. For the use of *U. atrum* as a biopesticide it had to be proved that the antagonistic fungus does not affect the crop.

To determine the endophytic growth of the antagonist the presence of *U. atrum* was assessed after surface sterilisation of plant material sprayed with the antagonist and incubated for at least one week. *U. atrum* was able to colonise the surface of leaves and berries, but after surface sterilisation, *U. atrum* was not growing from inside the plant material. Neither in experiments under controlled conditions nor under field conditions *U. atrum* caused any lesion on healthy leaves, inflorescences, berries, or canes of grapevines.

The decay of grapevines was documented at Marienthal at the end of the seasons 1997 to 1999 to study the effect of *U. atrum* on the vitality of grapevine plants. Observations were made in plots, where the antagonist had been used for up to three years. Due to the age of vines, the natural decay of grapevines was high in this vineyard. The occurrence of withered plants was not distributed uniformly, and new-planted grapevines changed the presupposition that there were high standard deviation of the means and no significant differences between the treatments. In the field experiment, the antagonistic fungus did not affect the decay of old grapevines over the three seasons (Table 6). Also, the vitality of new-planted grapevines was not affected by *U. atrum*.

Table 6: Effect of *Ulocladium atrum* on the decay of grapevines per plot (36 plants) at Marienthal in 1997-1999. Raised data show standard deviation of mean.

		fungicide treatment without botryticides				conventional fungicides	
year	n	without *U. atrum*	one year *U. atrum*	two years *U. atrum*	three years *U. atrum*	without *U. atrum*	with *U. atrum*
1997	6	2.0 $^{\pm 1.3}$	2.0 $^{\pm 1.7}$	-	-	3.8 $^{\pm 3.1}$	1.8 $^{\pm 1.3}$
1998	4	2.8 $^{\pm 0.5}$	1.5 $^{\pm 1.3}$	1.5 $^{\pm 1.3}$	-	2.0 $^{\pm 1.4}$	1.8 $^{\pm 1.0}$
1999	4	5.3 $^{\pm 1.0}$	4.3 $^{\pm 2.1}$	3.0 $^{\pm 1.4}$	2.5 $^{\pm 0.7}$*	4.0 $^{\pm 2.0}$**	3.5 $^{\pm 3.1}$

decay was assessed in only two*, or three plots**, respectively

3.2 Effect of pesticides on the viability of *Ulocladium atrum*

For an integrated use of the antagonist within general crop protection, the possibility of an integrated use of chemical and biological control agents is a prerequisite. The sensitivity of *U. atrum* to fungicides used in grapevine, and to an insecticide, known for side-effects on fungi, was tested *in vitro* and under field conditions using commercial products.

3.2.1 In vitro sensitivity of *Ulocladium atrum*

The tests on the *in vitro* sensitivity of *U. atrum* were carried out to provide a survey of pesticides, which may be applied in combination with the antagonist under field conditions. All pesticides used in the field experiments were checked for potential side-effects on the antagonist. The fungi were tested for the following concentrations of the active ingredient: 0, 0.01, 0.1, 1, 10 and 100 ppm. For compound products, the total of the active ingredients was used for the calculation of EC_{50} values. The EC_{50} value was determined with a sigmoidal regression from the reduction of mycelial growth against the log-transformed concentrations. The sensitivity of *B. cinerea* against pesticides was tested to evaluate the biological activity of botryticides against the target organism in relation to *U. atrum*, and to determine the side-effect of the other pesticides on the non target organisms *B. cinerea* and *U. atrum*. The approved basic concentration of active ingredients (a.i.) was calculated from the basic dosage rate at 1600 l/ha and represents the reference concentration the fungi are receiving in contact under field conditions.

U. atrum showed low sensitivity against most of the fungicides used in grapevine in studies under controlled conditions (Table 7). The inorganic fungicides copper and sulphur, and also the dithiocarbamate propineb, the combination of the dithiocarbamate mancozeb with the organophosphate fosetyl-Al, the morpholine dimethomorph, the phenylsulfamide dichlofluanid, and the hydroxyanilide fenhexamid did not reduce the growth of *U. atrum* by more than 50 %. For germination of *U. atrum*, most EC_{50} values of the fungicides and of the insecticide parathion-methyl were above 50 ppm, but mycelial growth was affected by some of these pesticides at lower dosages: the combination of the anilinopyrimidine cyprodinil and the phenylpyrrole fludioxonil, the dicarboximide iprodione, the triazole penconazole and the pyridine pyrifenox. The EC_{50} values of the dithiocarbamates mancozeb and metiram and the phenylsulfamide dichlofluanid were lower for germination than for the mycelial growth of *U. atrum*. Only the quinone dithianon and the anilinopyrimidine pyrimethanil had an effect on spore germination and mycelial growth of the antagonist at concentrations lower than 10 ppm.

The sensitivity of *B. cinerea* against the botryticides cyprodinil and fludioxonil, dichlofluanid, fenhexamid, iprodione, pyrimethanil and tebuconazole was high, but many other fungicides and also the insecticide parathion-methyl affected the germination and mycelial growth of the isolate Bc 700 under controlled conditions.

Table 7: Effect of pesticides on germination of *Ulocladium atrum* and *Botrytis cinerea* in aqueous solution and on mycelial growth on oatmeal agar (20°C, 99 % r.h.).

active ingredient(s)	[g/l]*	EC_{50} – value [ppm]			
		U. atrum Ua 385		*B. cinerea* Bc 700	
		germination	mycelial growth	germination	mycelial growth
Al-fosetyl + mancozeb	1.75	>50	>50	>50	15
carbendazim + diethofencarb	0.63	>50	20	>50	6
Cu-oxychloride	3.78	>50	>50	>50	>50
cymoxanil + dithianon	0.44	10	1	0.6	7
cyprodinil + fludioxonil	0.38	>50	3	0.6	0.4
dichlofluanid	1.00	41	>50	0.7	0.9
dimethomorph	0.18	>50	>50	>50	>50
dithianon	0.56	4	0.5	4	11
fenarimol	0.02	>50	12	>50	3
fenhexamid	0.82	>50	>50	0.4	0.1
fluquiconazole	0.05	>50	15	>50	0.3
folpet + metalaxyl-M	0.68	>50	11	2	0.7
iprodione	0.38	>50	3	9	0.2
mancozeb	1.50	23	>50	36	>50
metiram	1.60	33	>50	42	>50
parathion-methyl	0.20	>50	28	5	7
penconazole	0.02	>50	9	32	4
propineb	1.41	>50	>50	>50	47
pyrifenox	0.04	>50	6	6	0.2
pyrimethanil	0.50	9	0.9	>50	0.1
spiroxamine	0.03	>50	12	>50	13
sulphur	4.80	>50	>50	>50	>50
tebuconazole + tolylfluanid	1.25	>50	13	2	4
tolylfluanid	1.26	44	12	1	3
triadimenol	0.03	>50	11	0.6	13

*approved basic concentration of a.i. for using the pesticide in the vineyard

3.2.2 Possible combinations of *Ulocladium atrum* with botryticides

The combination of *U. atrum* with botryticides can only be utilised, when the pesticides do not affect the antagonistic fungus. In the following field experiment, *U. atrum* was tested for its compatibility with botryticides, applied simultaneously or with a time interval between applications. Botryticides were applied at the highest approved concentration in 400 l/ha. Samples were incubated, stained with Blankophor and examined under a microscope, using a combination of light and fluorescence techniques (Figure 10). The reductions in germination and growth rate were calculated relative to the water control. The period a botryticide had to be applied prior to the antagonist without affecting the mycelial growth of *U. atrum* by more than 50 % is marked in Table 8.

Most of the botryticides showed similar effects in the field as in the *in vitro* test on agar: mycelial growth of the antagonist was more affected than germination (Table 8). Applied simultaneously and at the highest approved concentration, *U. atrum* was affected by almost all botryticides, except fenhexamid, but after a period of maximal 14 days all botryticides had no more than 50 % effect on mycelial growth of applied spores. Pyrimethanil had a long-lasting effect on both, spore germination and mycelial growth. Viability testing of *U. atrum* 21 days after application of the botryticides showed no significant differences between all treatments; the antagonist was able to germinate and grow on the surface without any affect of the botryticides (Table 8).

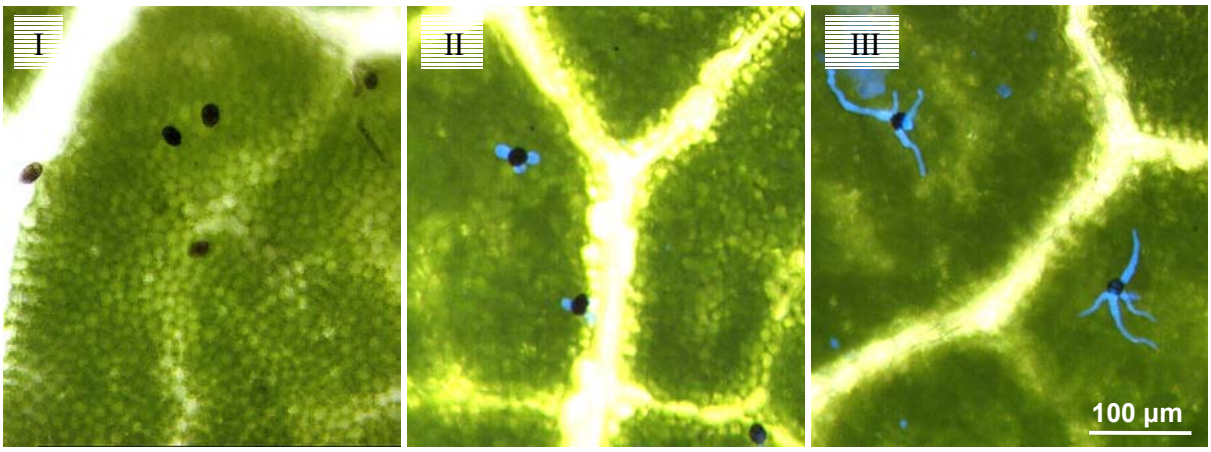


Figure 10: Effect of botryticides on germination and mycelial growth of *Ulocladium atrum* on grapevine leaf surface, applied at once with the antagonist (I: pyrimethanil, II: iprodione and III: fenhexamid, at highest approved concentration). Staining with Blankophor, combination of light and fluorescence microscopy.

Table 8: Effect of a time-interval between the application of *Ulocladium atrum* and botryticides on germination and mycelial growth of the antagonist on grapevine leaves (assessment 7 d after application of the antagonist and incubation for 14 h at 20 °C and 99 % r.h., germination on untreated leaves: 91-96 %, mycelial growth 107-115 µm).

active ingredient(s)	[g/l]	**g**ermination and **m**ycelial growth relative to untreated [%] time interval *n* between botryticide and *U. atrum* [d]											
		-2		*0*		*2*		*4*		*7*		*14*	
		g	m	g	m	g	m	g	m	g	m	g	m
carbendazim + diethofencarb	2.5	97	9*	95	10*	99	10*	100	56*	98	92	99	89
cyprodinil + fludioxonil	1.5	40*	10*	32*	9*	27*	12*	44*	61	67	98	79	101
dichlofluanid	4.0	0*	-	0*	-	10*	32*	7*	47*	21*	87	83	100
fenhexamid	3.3	100	98	97	96	101	102	99	102	101	97	100	102
iprodione	1.5	93	10*	82	9*	89	29*	98	32*	102	29*	98	89
pyrimethanil	2.0	0*	-	0*	-	0*	-	20*	12*	34*	11*	77	75
tebuconazole + tolylfluanid	5.0	8*	10*	0*	-	64*	26*	87	52*	93	89	101	97
tolylfluanid	5.1	72	10*	63*	9*	98	67*	101	98	101	95	102	98
vinclozolin	2.0	89	28*	92	32*	95	72	103	71*	98	92	100	94

required time period between applications of antagonist and botryticide for not affecting mycelial growth by more than 50 %. * Means are significant different to the control (*t*-test, α = 0.05).

3.2.3 Effect of pesticides on *Ulocladium atrum* under field conditions

In addition to the investigations on the effect of pesticides on the antagonist, the survival of *U. atrum* on grapevine bark was monitored during the field experiments to determine the effect of the applied pesticides on the antagonist under practical conditions. The effects of two different spray programmes on *U. atrum* were assessed under field conditions at Marienthal in 1997 (Figure 11). Experiments with *U. atrum* were conducted in plots with conventional fungicide treatment and an integrated fungicide treatment without the use of botryticides.

U. atrum was present on almost all bark samples in plots of both treatments directly after its application. The percentage of bark samples with *U. atrum* decreased after application I, III and IV of the antagonist; four weeks after application, the antagonist could be re-isolated from only 20-40 % of bark segments. After application II the incidence of *U. atrum* was almost constant in plots with an integrated fungicide programme; there was no application of

fungicides during this time. In conventionally treated plots, the antagonist was decimated after the use of pyrifenox and the combination of cymoxanil and dithianon, the spore density was reduced by 70 % within three weeks. This reduction was significantly different from the progression in integrated fungicide treated plots (Tukey-test, $p \leq 0.05$), while the other progressions after the applications did not differ significantly (Tukey-test, $p \leq 0.05$). There was no effect of the additional applications of botryticides carbendazim with diethofencarb or cyprodinil with fludioxonil on the antagonist.

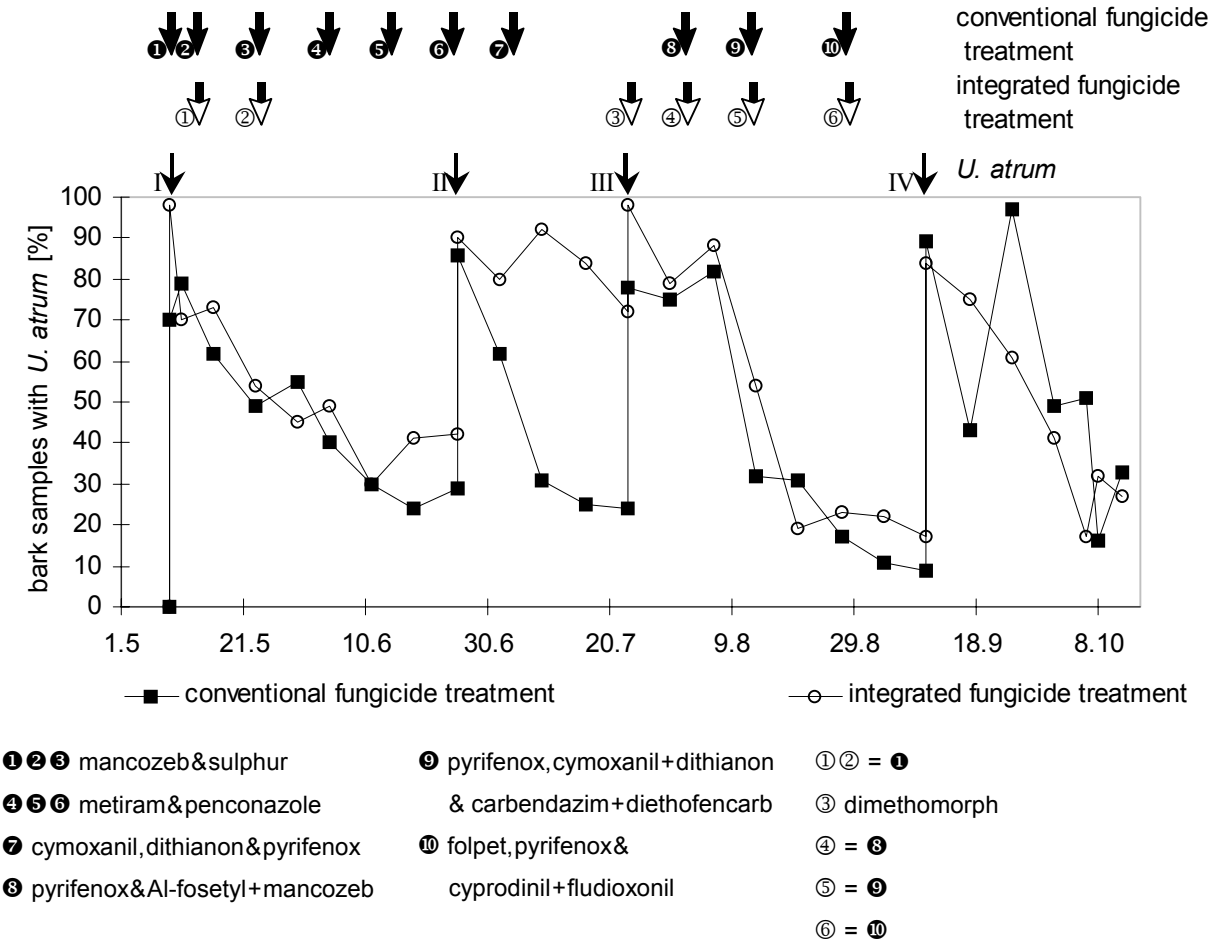


Figure 11: The effect of integrated and conventional fungicide treatments on the viability of *Ulocladium atrum* on the grapevine bark (Marienthal, 1997).

3.3 Epidemics of *Botrytis cinerea* in the vineyard

The epidemic development of pathogens depends on the primary inoculum and the subsequent spread in the vineyard, which depends on dispersal of conidia and on the spread via mycelial growth. Symptoms of the pathogen were observed on different parts of grapevine, such as leaves, tendrils, inflorescences and canes and most frequently on mature berries (Figure 12).

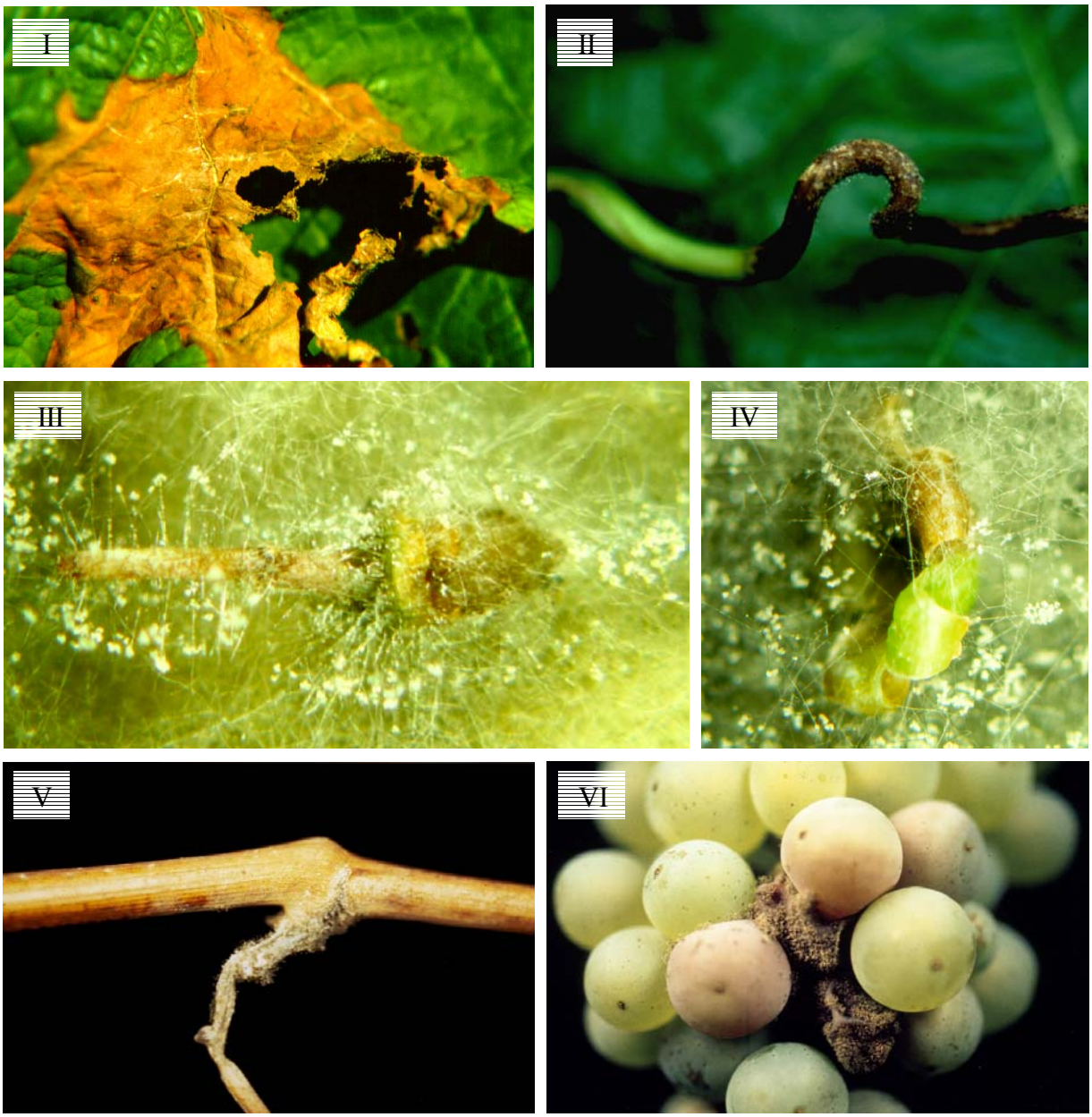


Figure 12: Symptoms of *Botrytis cinerea* on parts of grapevine: leaves (I), tendrils (II), inflorescences (III), flowerhoods (IV), canes (V) and mature berries (VI). Flower parts (III & IV) were incubated to intensify sporulation.

The conidial spread of *B. cinerea* and the epidemic development of grey mould were investigated at Marienthal. The density of *B. cinerea* spores in the air was determined using a Burkhard® seven-day recording volumetric spore trap. The trap ran throughout the growing seasons close to the vineyard at Marienthal, and the air load of *B. cinerea* spores was calculated per cubic metre air. Additionally, data of air temperature and water content of necrotic leaves are shown in Figure 13, Figure 14 and Figure 16. The sensitive range of leaf wetness sensor is between 800 mV for dry conditions and 2000 mV for wet leaf surface.

The severe winter of **1997** and late periods of frost at the end of May affected the crop, reducing plant vigour, and resulted in a low number of leaves and bunches per plant; in addition the incidence of *B. cinerea* was delayed, conidia of *B. cinerea* were trapped for the first time on June 7 (Figure 13). A cold period of long humidity in June and drought conditions in July and most of the time until mid September were not conducive to an epidemic spread of *B. cinerea* on leaves, flowers and berries. During the dry period in July, incidence of spores was low until late August, when the number of spores exceeded 100 spores/m³ for the first time. At vintage in early October, the highest spore density - 180 spores/m³ - was measured. Late in the season, however, the number of spores declined on days with heavy rain. In conclusion, 1997 can be classified as a year of low *B. cinerea* incidence.

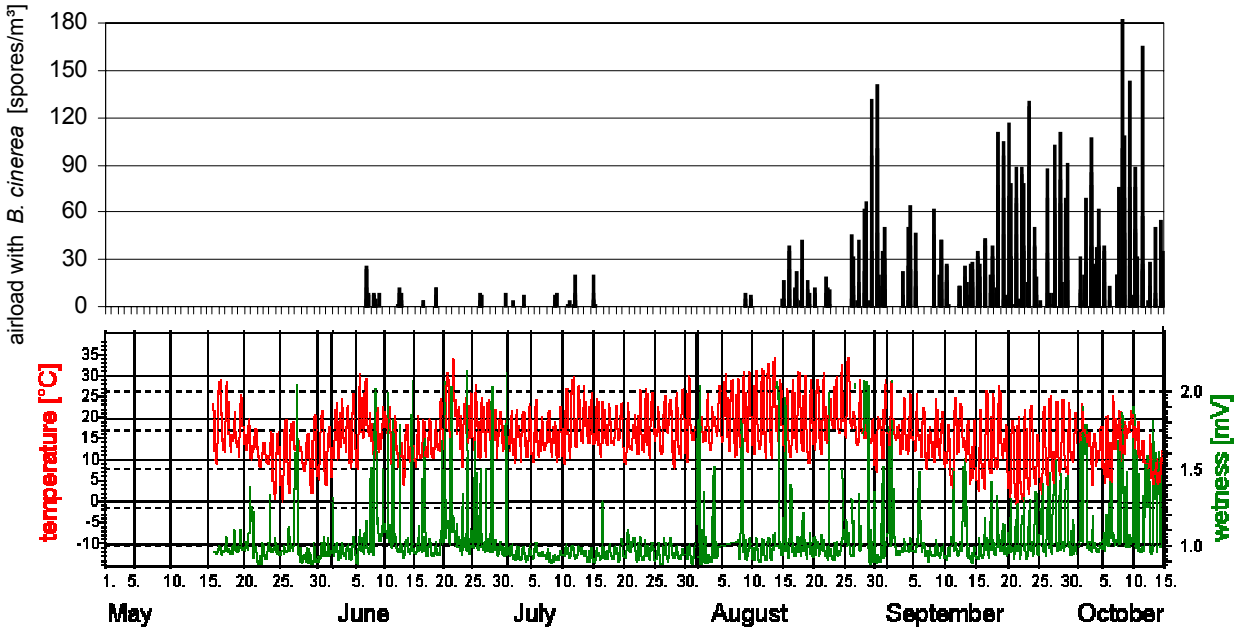


Figure 13: Air load of *Botrytis cinerea* spores per cubic metre, measured within one hour and data of air temperature and moisture of necrotic leaves (Marienthal, 1997).

The moderate winter of **1998** and an early start of the growing season resulted in a better than average crop development. *B. cinerea* did occur early in mid May, and there were spores on a low level for almost every day until July. A long cold, humid period mixed with showers of hail and wet conditions in July and August promoted the development of *B. cinerea* on leaves, flowers and grapes, though trapping of *B. cinerea* spore were infrequent, and the air load was on a level lower than 30 spores/m³. In early September, the number of spores increased, and a long period of high spore density in the air occurred. The air load with conidia reached its maximum of about 180 spores/m³ in mid October, the time of vintage in this region. Late maturation of grapes, caused by the cold weather and the massive incidence of *B. cinerea*, caused a harvest of the grapevine berries at short notice.

To show a more detailed time curve of the air load of *B. cinerea* conidia at Marienthal, and the correlation to the meteorological measurements, the spore catches of June 1998 are shown with the data of air temperature and moisture of necrotic leaves exemplarily in Figure 15. Measurements of the air load with *B. cinerea* spores showed a peak of spores at noon rising from early morning and lasting until 3 pm. After a longer period of rainfall (June 1-2 and 23-26), an increased air load of *B. cinerea* spores could be measured. The second period of spores release might have been of special importance for the epidemic, because flowering started on June 25 (Figure 15).

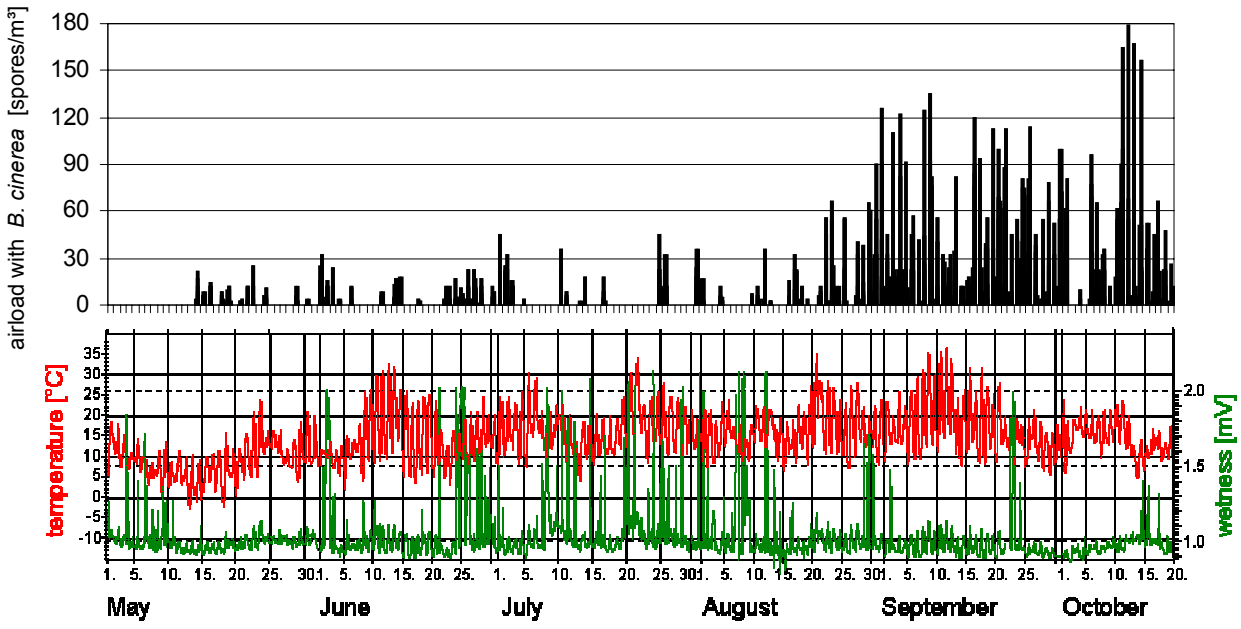


Figure 14: Air load of *Botrytis cinerea* spores per cubic metre, measured within one hour, and data of air temperature and moisture of necrotic leaves (Marienthal, 1998).

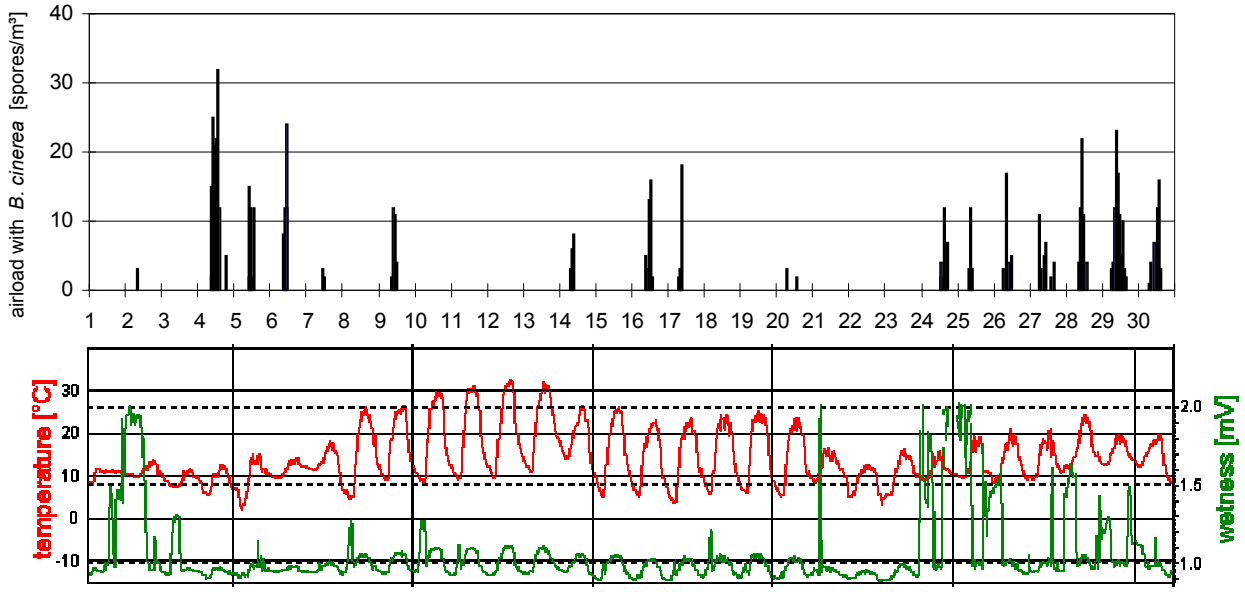


Figure 15: Air load of *Botrytis cinerea* spores per cubic metre, measured within one hour, and data of air temperature and moisture of necrotic leaves at Marienthal in June 1998.

The season in **1999** started two weeks earlier than in 1998. This resulted in a good crop development, in a higher number of grapes per plant, and also in the promotion of the incidence of *B. cinerea*. The pathogen did occur as early as the first days of April, and spores were trapped on a low level for almost the whole season until August. A long cold, humid period mixed with showers in July and until mid August were also conducive to the development of grey mould on grapes. By early August the number of spores already exceeded 100 spores/m³, and increased until rainfalls in mid September, when the spore density reached its maximum of about 250 spores/m³. Especially the cold period in September and the humid period late in the season was very conducive to sour rot of berries as well as sporulation of *B. cinerea* in dry periods. Therefore, almost none of the infected berries showed symptoms of *pourriture noble*. The harvest was put to an earlier date because of the early maturing of grapes. The maturing was caused by warm and dry weather conditions in the summer resulting too in a massive incidence of *B. cinerea* late in the late season (Figure 16).

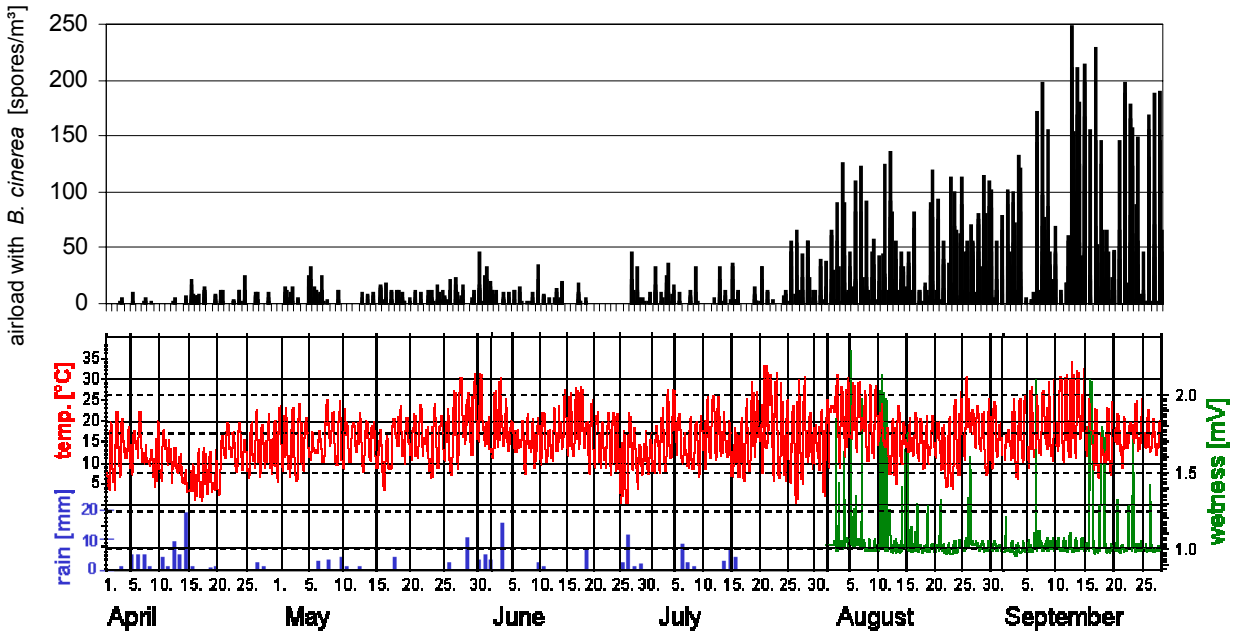


Figure 16: Air load of *Botrytis cinerea* spores per cubic metre, measured within one hour, and data of air temperature, rain and moisture of necrotic leaves at Marienthal, 1999 (due to data loss, data of rain are given instead of moisture until August 1).

3.4 Interactions between *Ulocladium atrum* and *Botrytis cinerea* under controlled conditions

The growth of microorganisms depends on the ecological circumstances: the environmental conditions and the interactions with other microorganisms. The experiments described below were conducted to assess the interactions between *U. atrum* and *B. cinerea* under controlled conditions. The antagonistic potential of *U. atrum* was investigated in aqueous solutions on sterilised and healthy grapevine leaves and on berries. The effect of *U. atrum* on the spore production of sclerotia was determined.

3.4.1 *In vitro* interactions between fungi during spore germination

The rate of germination in aqueous sucrose solutions was investigated to examine the effect of *U. atrum* on the first step of *B. cinerea* to colonise a new habitat, the germination of conidia. With all sucrose concentrations examined, there was no significant reduction of the germination rate of *B. cinerea* by *U. atrum* and *vice versa*. *B. cinerea* needed a sucrose concentration of at least 0.01 %, while *U. atrum* was able to germinate without external nutrients (Figure 17).

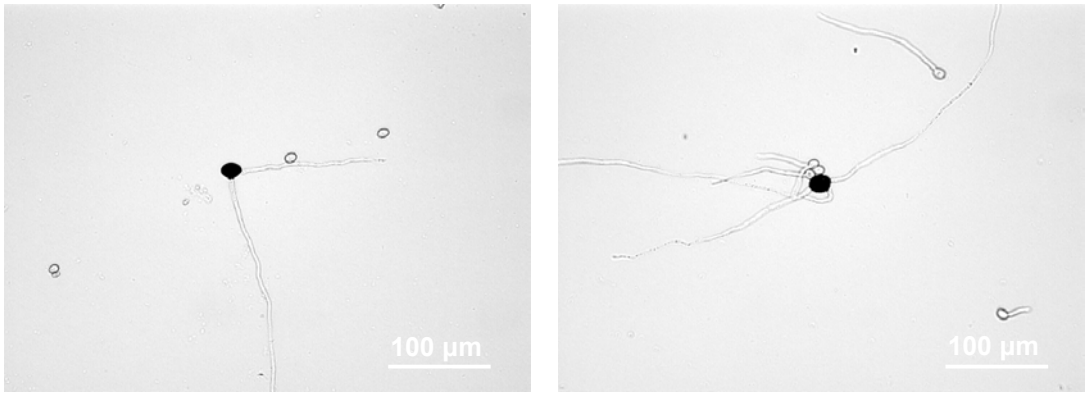


Figure 17: Effect of different concentrations of sucrose (left 0 %, right 2 % sucrose) on the germination rate of *Botrytis cinerea* and *Ulocladium atrum* in aqueous solutions after an incubation for 14 h at 20 °C.

3.4.2 *In vitro* interactions between fungi during mycelial growth

The effect of an interval between the applications of *U. atrum* and *B. cinerea*, respectively, was studied on oatmeal agar at 20 °C. Fungi were inoculated simultaneously or with a time interval of 1, 2 and 3 days before and after each other. Inoculated three days prior to the other fungus, *U. atrum* and *B. cinerea* were able to colonise almost the whole Petri dish, while a simultaneous inoculation resulted in an equipartition of both fungi (Figure 18).

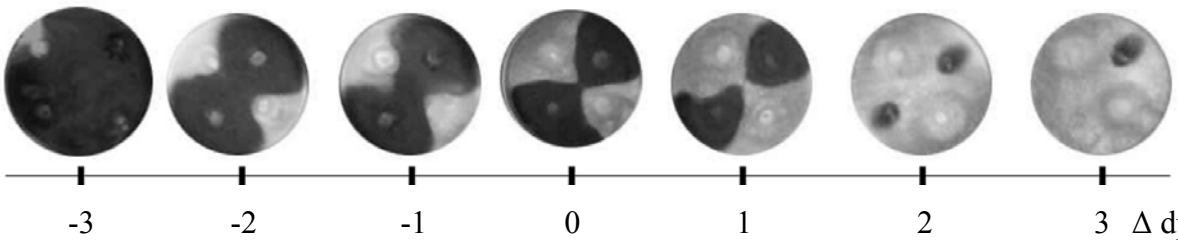


Figure 18: Mycelial growth of *Botrytis cinerea* (■) and *Ulocladium atrum* (■) on oatmeal agar in relation to the time interval between the application (Δ dpi) of *Botrytis cinerea* and of *Ulocladium atrum* (incubation for 10 d at 20 °C).

3.4.3 Effect of *Ulocladium atrum* on *Botrytis cinerea* on sterilised leaves

The major nutrient resource of *B. cinerea* is dead plant material. The pathogen is able to colonise necrotic tissue and to start an epidemic from this source by forming mycelium and conidia. Therefore, the antagonistic potential of *U. atrum* against *B. cinerea* was determined on sterilised grapevine leaves, in order to avoid interference with a background mycoflora. The effect of environmental parameters on sporulation of *U. atrum* and *B. cinerea* was studied on leaf tissue under various temperature conditions and different relative humidities.

3.4.3.1 Kinetics of sporulation on necrotic grapevine leaf tissue

Kinetics of sporulation of *B. cinerea* and *U. atrum* on necrotic grapevine leaf discs were studied at 20 °C and 99 % r.h. in two days intervals after inoculation. Fungi were applied singularly, or in co-inoculation to determine the antagonistic potential of *U. atrum*.

Spore production of both fungi reached a maximum 14 days after inoculation (Figure 19). In the first 8 days spore production of *U. atrum* and *B. cinerea* was at a same level. For the next 4 days, the sporulation rate of *B. cinerea* increased twice as fast as the sporulation rate of *U. atrum*. After simultaneous inoculation spore production of the pathogen was reduced by about 50 % compared to the individual inoculation. Also, sporulation of the antagonist was lower compared to the separate growth. Microscopic and macroscopic illustrations of the leaf surfaces are shown in Figure 20.

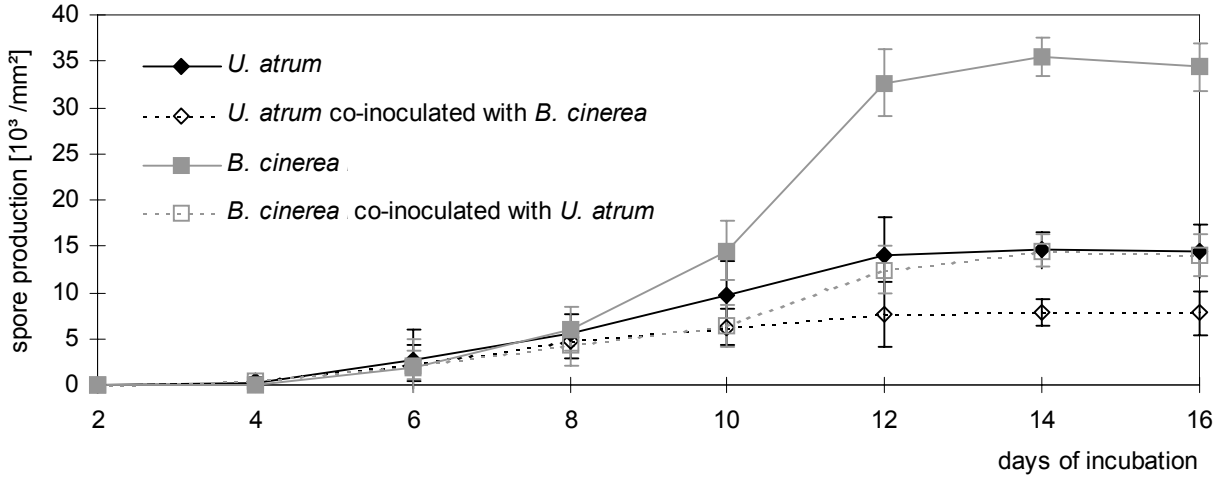


Figure 19: Sporulation of *Botrytis cinerea* and *Ulocladium atrum* on necrotic grapevine leaves, inoculated singularly or in combination with the other fungus. Incubation at 20 °C and 99 % r.h.; bars show the standard deviation of means.

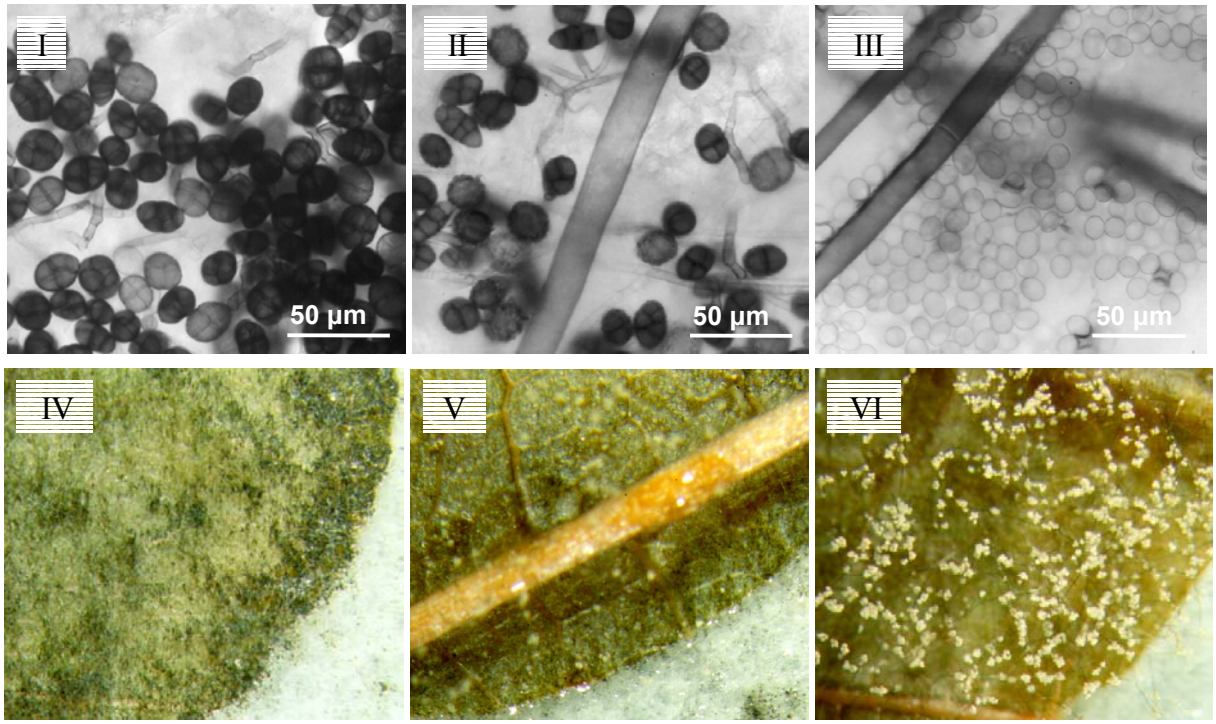


Figure 20: Sporulation of *Ulocladium atrum* (left) and *Botrytis cinerea* (right) inoculated singularly or in combination with other fungus (centre) on necrotic grapevine leaves. Incubation for 14 d at 20 °C and 99 % r.h.. Microscopic (I-III) and macroscopic (IV-VI) photographs of the colonised leaf surface.

3.4.3.2 Influence of environmental conditions on spore production

Environmental conditions are as important as nutrients for the growth and sporulation of microorganisms. The effect of environmental parameters on the sporulation of *U. atrum* and *B. cinerea* was studied on γ–irradiated grapevine leaf discs at different relative humidities and various temperatures.

Tests on the spore production of *U. atrum* and *B. cinerea* under different relative humidities were conducted on sterilised leaves. The sporulation was determined after 14 days of incubation at 20 °C.

Maximum number of spores was produced at 99 % relative humidity, whilst sporulation of *B. cinerea* depended on relative humidity higher than 90 %, the formation of spores by *U. atrum* was already possible at relative humidities above 76 % r.h. (Table 9).

Table 9: Effect of relative humidity on the spore production of *Ulocladium atrum* and *Botrytis cinerea* on sterilised grapevine leaves. Leaves were incubated for 14 days at 20 °C (raised data show the standard deviation of means).

fungus	spore production per leaf area [10^3 spores / mm^2] depending on relative humidity				
	65 %	76 %	86 %	92 %	99 %
U. atrum	$0.0^{\pm 0.0}$	$0.7^{\pm 0.6}$	$4.2^{\pm 2.2}$	$7.2^{\pm 3.0}$	$14.5^{\pm 2.0}$
B. cinerea	$0.0^{\pm 0.0}$	$0.0^{\pm 0.0}$	$0.0^{\pm 0.0}$	$1.2^{\pm 1.1}$	$35.5^{\pm 2.1}$

Tests on the sporulation of *B. cinerea* and *U. atrum* on necrotic grapevine leaf discs under various temperature conditions were conducted at 99 % r.h. and showed different optimum temperatures for the two fungi. Spore production of *U. atrum* was highest at 25 °C whereas the optimum for *B. cinerea* was 20 °C (Figure 21). *U. atrum* produced spores under a similar temperature range as *B. cinerea*, but *U. atrum* was able to produce spores even at 5 °C. There was no sporulation of both fungi at 35 °C.

Differences between the temperature optima were emphasised in co-inoculation experiments; the antagonistic effect of *U. atrum* was best at its optimum temperature, sporulation of *B. cinerea* was 28 % of the sporulation without the antagonist. Above the temperature range of 10-30 °C, *U. atrum* reduced the spore formation of *B. cinerea* by 40-72 % (Figure 21).

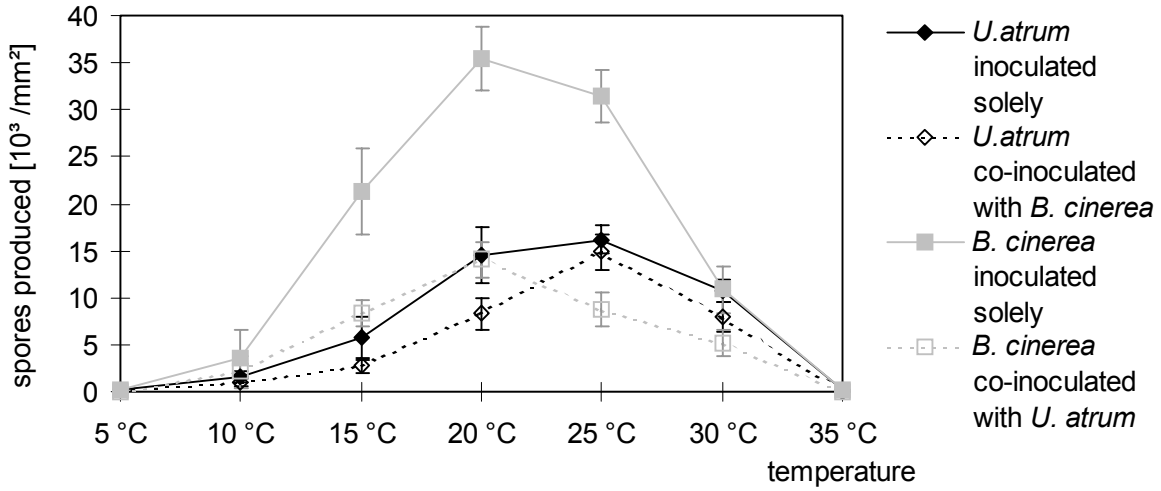


Figure 21: Effect of temperature on the sporulation of *Botrytis cinerea* and *Ulocladium atrum* on necrotic grapevine leaves. Inoculation solely or in combination with the other fungus for 14 days at 99% r.h.; bars show standard deviation of means.

3.4.3.3 Impact of different time intervals between inoculations

To determine the effect of the time interval between leaf colonisation of the pathogen and the antagonist, respectively, the antagonist was inoculated 0.5, 1, 2, 4, 7 and 10 days before *B. cinerea* and *vice versa*.

Pre-inoculation of *U. atrum* for more than one day led to a reduction of the pathogen's spore production by more than 70 %, compared to the inoculation of *B. cinerea* alone. When the antagonist became well-established on the leaves four to ten days prior to the application of *B. cinerea* spore production of the pathogen was reduced by more than 90 % (Figure 22). *B. cinerea* also reduced the spore production of the antagonist, although there was still an occurrence of 10 % of sporulation, compared to the sole inoculation of *U. atrum* when the pathogen was applied ten days before the antagonist. This experiment demonstrates that an early presence of *U. atrum* is essential for its antagonistic success.

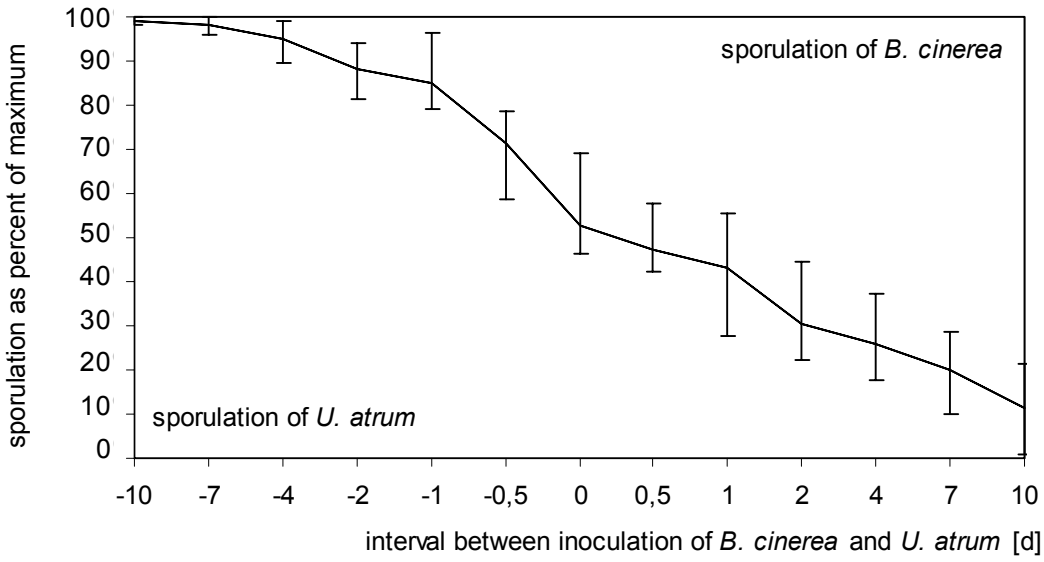


Figure 22: Effect of the interval between the inoculations of *Ulocladium atrum* and *Botrytis cinerea*, respectively, on the spore production of both fungi on sterilised grapevine leaves (24 d after inoculation of the first applied fungus, incubation at 20 °C, 99 % r.h.). The upper bars give the standard deviations of means of the sporulation of *Botrytis cinerea*; the lower ones give those for *Ulocladium atrum*.

3.4.4 Effect of *Ulocladium atrum* on *Botrytis cinerea* on healthy leaves

Disease symptoms of *B. cinerea* do occur on grapevine leaves as leaf blight in the vineyard. Under certain circumstances the pathogen is able to infect leaves thus producing necrosis and starting an epidemic spread using this source. The antagonistic potential of *U. atrum* in this niche was determined under controlled conditions on detached grapevine leaves.

3.4.4.1 Effect of *Ulocladium atrum* on spore germination of *Botrytis cinerea*

In addition to the *in vitro*-tests in aqueous sucrose solutions, the germination rate of *B. cinerea* and *U. atrum* was determined on healthy grapevine leaves. Fungi were inoculated individually or co-inoculated to examine the effect of *U. atrum* on the germination of *B. cinerea* conidia in the phyllosphere. Moreover, the effect of sucrose on the germination of fungi was tested as an additional external nutrient source.

Inoculated simultaneously with the pathogen *U. atrum* had no significant effect on the germination rate of *B. cinerea*, regardless of the concentration of added sucrose (Figure 23). Similar to the tests with aqueous sucrose solutions *B. cinerea* required a sucrose concentration of at least 0.01 %, whilst *U. atrum* also germinated without additional nutrients. *U. atrum* was capable to suppress the sporulation of *B. cinerea* by 97 % in the treatment with 0.01 % sucrose, when the antagonist was inoculated 7 h before the pathogen. At other sucrose concentrations the pre-inoculation of *U. atrum* gave no significant effect.

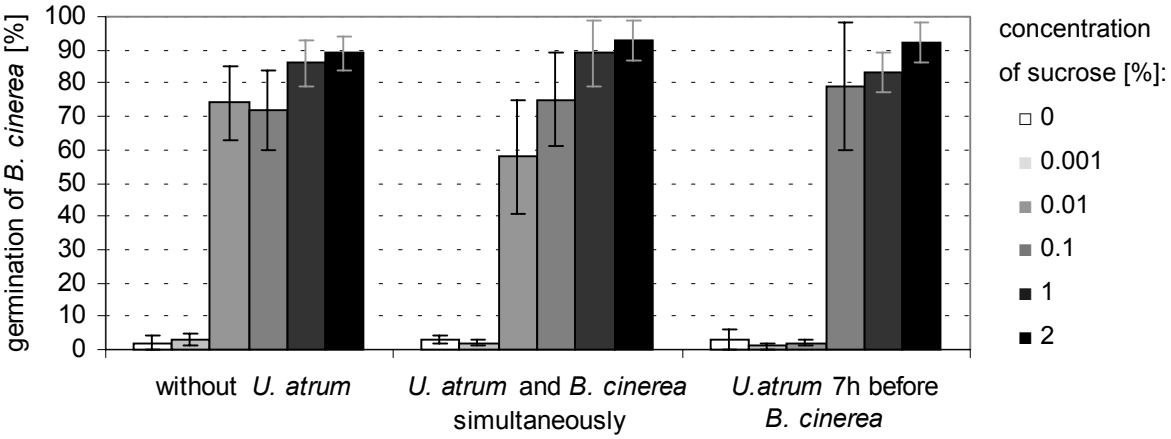


Figure 23: Effect of different sucrose concentrations and the presence of *Ulocladium atrum* on the germination rate of *Botrytis cinerea* on grapevine leaves, incubation for 14 h at 20 °C. Bars show the standard deviation of means.

3.4.4.2 Effect of *Ulocladium atrum* on expansion of lesion caused by *Botrytis cinerea*

Botrytis leaf blight does not cause severe crop protection problems in grapevine, however it is an additional source for conidia, which can lead to an infection of inflorescences or grapes later in the season. The effect of *U. atrum* on the development of *Botrytis* leaf blight was investigated on detached leaves after 4 days of incubation at 20 °C and 99 % r.h.. Symptoms due to *B. cinerea* were rated from I (no symptoms) to VI (lesion with sporulation).

There was a reduction of the incidence and the severity of *Botrytis* leaf blight by *U. atrum* on detached grapevine leaves (Figure 24 and Figure 25).

Figure 24: Effect of *Ulocladium atrum* (right) on the development of *Botrytis* leaf blight on detached leaves of grapevine, compared to water control (left) after an incubation of 4 d at 20 °C in a moist chamber.

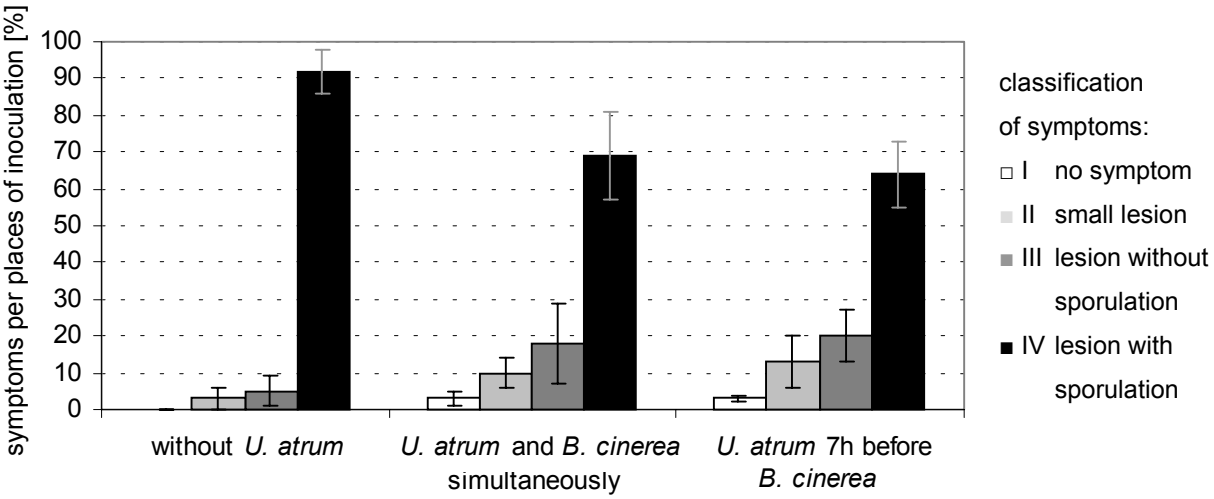


Figure 25: Effect of *Ulocladium atrum* on the development of *Botrytis* leaf blight on detached grapevine leaves; incubation for 4 d at 20 °C in a moist chamber. Bars show the standard deviation of frequencies.

The effect of *U. atrum* on the development of *Botrytis* leaf blight on detached grapevine leaves was a reduction in the percentage of sporulating lesions, and in some cases a suppression of infection (Figure 25). Due to the reduction of sporulation there were more symptoms in the class II (small circular lesion) and class III (lesion without sporulation), respectively. Staggered inoculations of *U. atrum* and *B. cinerea* did not increase the antagonistic effect significantly

3.4.5 Effect of *Ulocladium atrum* on the infection of inflorescences by *Botrytis cinerea*

Grapevine inflorescences are susceptible to infections by *B. cinerea*. An early infection of flowers can cause a loss of infected berries or even of total clusters, while grey mould, due to a latent infection, occurs at later growth stages. In this experiments the antagonistic potential of *U. atrum* was assessed at the stages of flowering (BBCH 61-69) with grapes, cv. 'Müller-Thurgau'. Clusters were sprayed with *U. atrum* at growth stage BBCH 61 and *B. cinerea* was inoculated at BBCH 61 or BBCH 65.

Berries inoculated at BBCH 61 'trickled through' without any difference between the treatments. There was an effect of *U. atrum* on latent infection of berries inoculated at BBCH 65: measured at the end of flowering (BBCH 69), the antagonist reduced latent infection significantly by 22 % ($p \leq 0.05$, Mann-Whitney rank sum test).

3.4.6 Effect of *Ulocladium atrum* on sporulation of *Botrytis cinerea* on berries

Another possibility of *B. cinerea* infection in grapevine is the transmission from berry to berry. Starting at *véraison*, grapes can be infected directly by conidia forming germtubes, or by mycelium penetrating the epidermis or growing into wounds. Therefore, the amount of spores produced on a mature grapevine berry is important for the development of grey mould in the bunch and also in the environment of the infected bunch.

Wounded berries were taken for tests to determine the effect of *U. atrum* on the sporulation of *B. cinerea* and to ensure the infection rate of 100 % of berries. The antagonist was applied directly after injury. Sporulation of *B. cinerea* was measured after 7 days of incubation in moist chambers at 20 °C. To assess the possibility of a spontaneous recovery of berries inoculation with *B. cinerea* was performed directly and two days after injury.

When inoculated simultaneously spore production of *B. cinerea* was reduced by *U. atrum* by about 30 %, compared to treatment without the antagonist. A time lag of two days between

injury and inoculation of *B. cinerea* reduced sporulation of the pathogen by 64 % and by 74 % in the presents of *U. atrum*, respectively (Figure 26). Whilst sporulation of *B. cinerea* was reduced significantly by *U. atrum* after simultaneous inoculation, the antagonist was not capable of enhancing the effect of the time lag between injury and inoculation of *B. cinerea* significantly (*t*-test, $\alpha = 0.05$ %).

There was no effect of *U. atrum* on the incidence of berry infection, but the reduction in sporulation was macroscopically visible; no pathological symptoms occurred in berries without inoculation of *B. cinerea* even on berries treated with the antagonist (Figure 27).

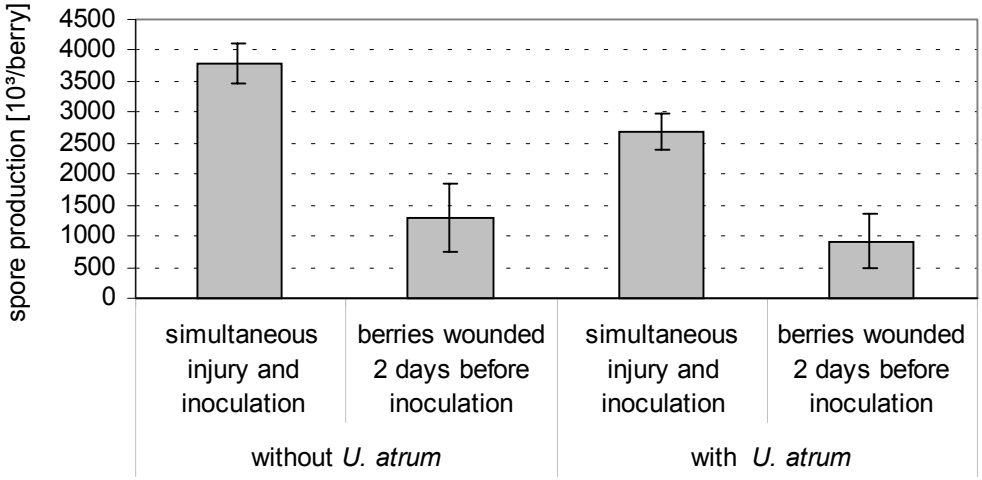


Figure 26: Effect of *Ulocladium atrum* and of the time delay before inoculation with *Botrytis cinerea* on the sporulation of the pathogen on grapevine berries. *Ulocladium atrum* was applied directly after injury, *Botrytis cinerea* was inoculated directly or 2 d after injury. Incubation for 7 d at 20°C and 99 % r.h.. Bars show the standard deviation of means.

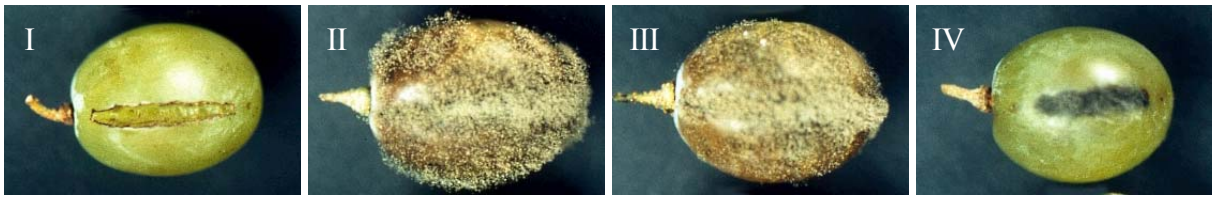


Figure 27: Sporulation of *Botrytis cinerea* with (III) and without *Ulocladium atrum* (II) on wounded grapevine berries, a berry without fungi (I) and inoculated with the antagonist (IV). Inoculation directly after injury, incubation 7 d, 20°C, 99 % r.h..

3.4.7 Effect of *Ulocladium atrum* on conidia production of sclerotia

Sclerotia of *B. cinerea* occur as hibernating organs on plant debris and on poorly hardened canes in vineyards. They may be myceliogenic or sporogenic and are a source for inoculum in springtime. Six sclerotia producing isolates were used in tests with *U. atrum* to evaluate its effect on spore formation of this form of *B. cinerea*. Berries inoculated with fungi were incubated for 40 days at 5 °C, 30 days at 10 °C or 20 days at 20 °C, and following incubation, mycelial growth of fungi was assessed macroscopically and spore production of *B. cinerea* was determined.

After incubation sclerotia were overgrown by the inoculated antagonist, although conidia were produced from all sclerotia. For each isolate there was a not significant ($p \leq 0.05$, Mann-Whitney rank sum-test) effect of *U. atrum* on spore production of sclerotia. The antagonistic fungus reduced the average mean of sporulation at a certain temperature of all isolates significantly ($p \leq 0.05$, Mann-Whitney rank sum-test, Figure 28).

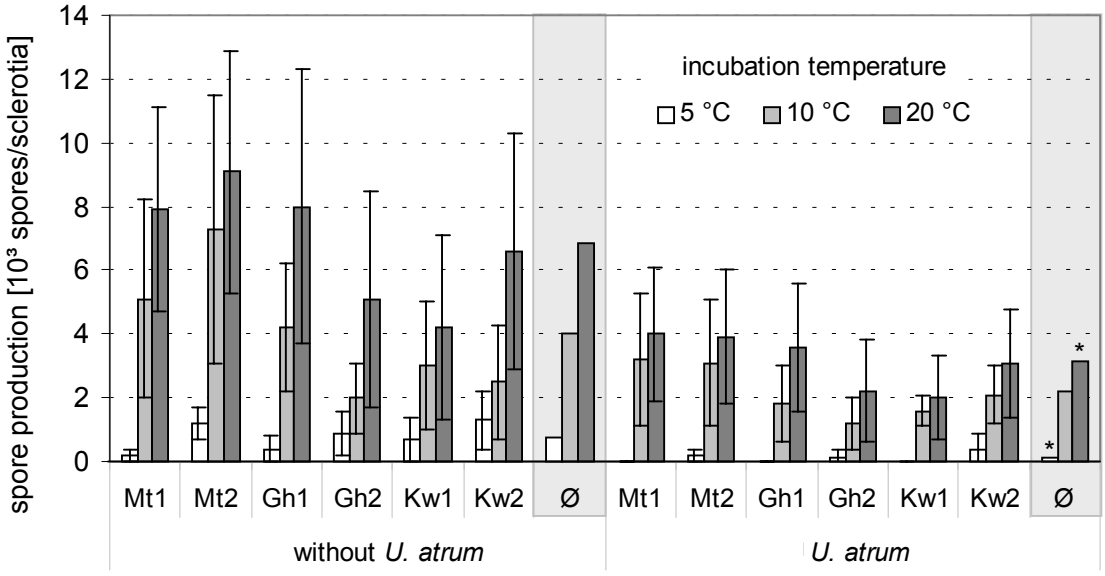


Figure 28: Effect of *U. atrum* on spore production of sclerotia of six *Botrytis cinerea* isolates (Mt 1, Mt 2, Gh 1, Gh 2, Kw 1 and Kw 2) at different incubation temperatures. Bars show the standard deviation of means, *Average mean of sporulation of all isolates at one temperature differs significantly from the untreated control ($p \leq 0.05$, Mann-Whitney rank sum-test).

3.5 Effect of *Ulocladium atrum* on grapevine diseases under field conditions

The efficacy of a biological control agent is often limited to a small ecological niche to prove its antagonistic potential. The experiments under controlled conditions showed that within a wide range of temperature and relative humidity *U. atrum* is able to grow on necrotic grapevine tissue, and thus proved to be an euryoecious antagonist of *B. cinerea*. Most of the tested pesticides too did not affect the antagonist up to a tolerable concentration. The antagonistic effect of *U. atrum* on grey mould and other grapevine diseases was assessed in three field experiments in commercial vineyards and one field trial at Bonn University. The potential of *U. atrum* as a biocontrol agent, applied alone, mixed with or in alternation with pesticides was evaluated.

3.5.1 Effect of *Ulocladium atrum* on other grapevine diseases than grey mould

It is desirable for a biological agent to control more than one pathogen of a crop. *U. atrum* proved to be antagonistic against *B. cinerea in vitro* and was tested against grey mould in grapevine under field conditions. The effect of *U. atrum* on the incidence of disease symptoms caused by *Penicillium expansum*, *Phomopsis viticola*, *Plasmopara viticola*, *Pseudopezicula tracheiphila* and *Uncinula necator* was determined in the field experiment with integrated fungicide use at Marienthal in 1997.

In this experiment, effects of *U. atrum* on other grapevine diseases than grey mould could be detected in plots with integrated fungicide treatment. In plots with conventional fungicide treatment the rate of disease incidence was too low to determine a difference between treatments with or without the antagonist. Based on visual assessments, a reduction in dead arm disease development caused by *P. viticola* was assessed on June 25 (BBCH 61); the infection on new canes was reduced significantly (*t*-test, $\alpha = 0.05$ %) by *U. atrum* (Figure 29). Due to the fungicide treatment severity of grapevine leaf diseases was low at this time.

On August 8 (BBCH 81, Figure 30) and at harvest there was no significant effect of *U. atrum* on the severity of berry diseases caused by *P. expansum*, *P. tracheiphila* (Mann-Whitney rank sum test, $p \leq 0.05$), *P. viticola* or *U. necator* (*t*-test, $\alpha = 0.05$ %). The incidence of split berries (*U. necator*) and leather berries (*P. viticola*) was reduced by conventional fungicide treatment and was significantly for the incidence of leather berries (*t*-test, $\alpha = 0.05$ %). There was also no significant effect (*t*-test, $\alpha = 0.05$ %) of the treatment with *U. atrum* on the incidence of grapevine diseases at this and at later growth stages.

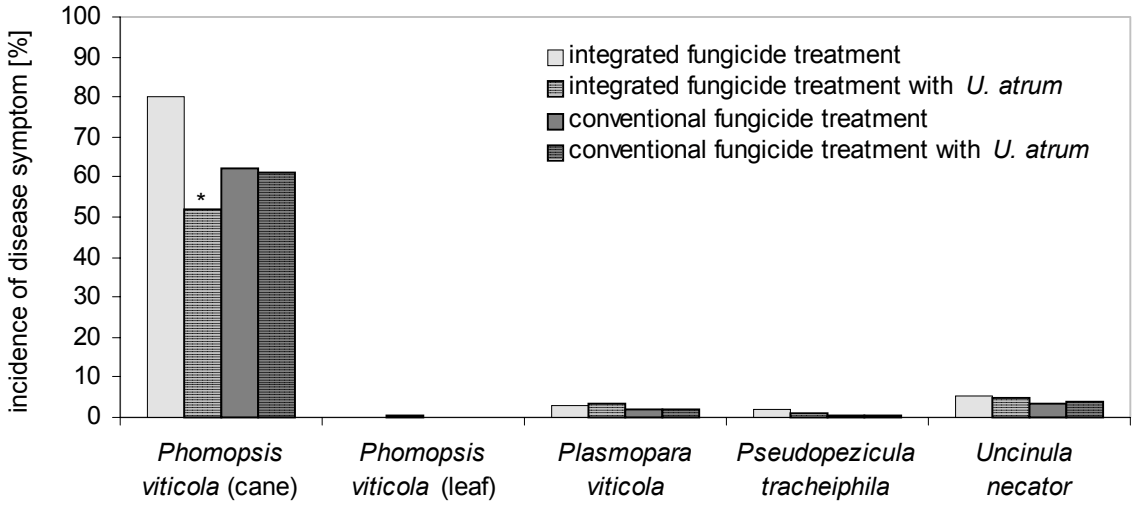


Figure 29: Effect of *Ulocladium atrum* and fungicide treatment on the incidence of symptoms of grapevine diseases (Marienthal, June 25 1997). *Average mean differs significantly from mean of the integrated fungicide treatment (*t*-test, $\alpha = 0.05$ %).

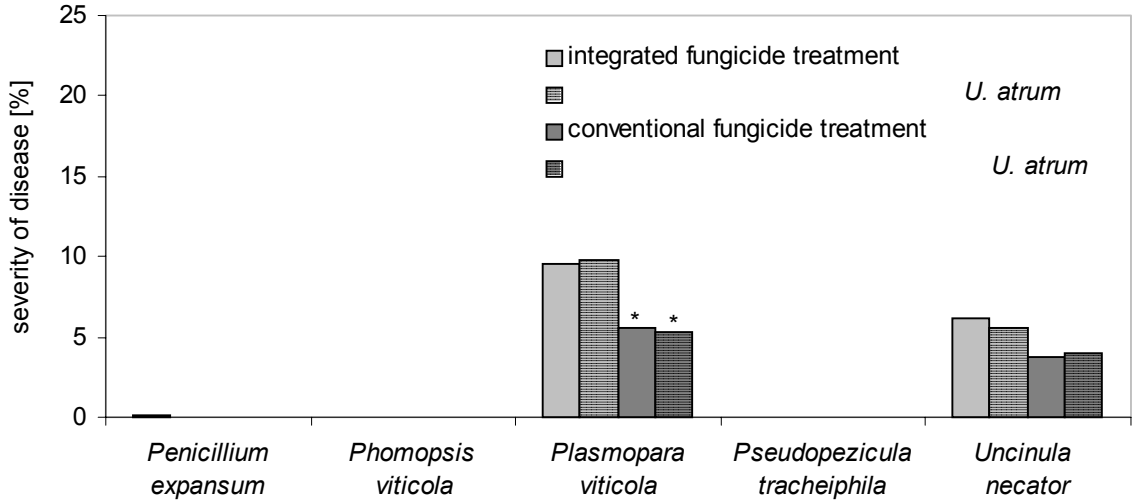


Figure 30: Effect of *Ulocladium atrum* and fungicide treatment on the severity of symptoms of grapevine berry diseases (Marienthal, August 8 1997). *Average mean differs significantly from the mean of the integrated fungicide treatment (*t*-test, $\alpha = 0.05$ %).

In 1998 a field experiment with grapevine cv. 'Müller-Thurgau' was carried out in Bonn to assess the antagonistic activity of *U. atrum* against *Uncinula necator* causing powdery mildew on grapevine. The experimental field was chosen because of the absence of other grapevine diseases than powdery mildew.

The epidemic build-up was fast in plots not sprayed with fungicides and at growth stage BBCH 68 the incidence of bunches and leaves infected with *U. necator* was 100 % in plots without treatments for *U. necator*, and the experiment had to be stopped (Figure 31). The whole experimental field had to be treated twice with fenarimol to guarantee a new healthy shoot, and therefore the survival of the grapevine plants. The experiment was continued in 1999 focussing particularly on the effect of *U. atrum* on hibernation of *U. necator*. The following season the onset of first infection occurred simultaneously in plots with and without treatments of *U. atrum* (Figure 31). At growth stage BBCH 63 the incidence of *U. necator* infected bunches and leaves was 100 % in plots without fungicide treatment, and the experiment had to be discontinued. An effect of *U. atrum* against powdery mildew was neither observed on the epidemic spread during the growing season, nor on hibernation and the outbreak in the following season.

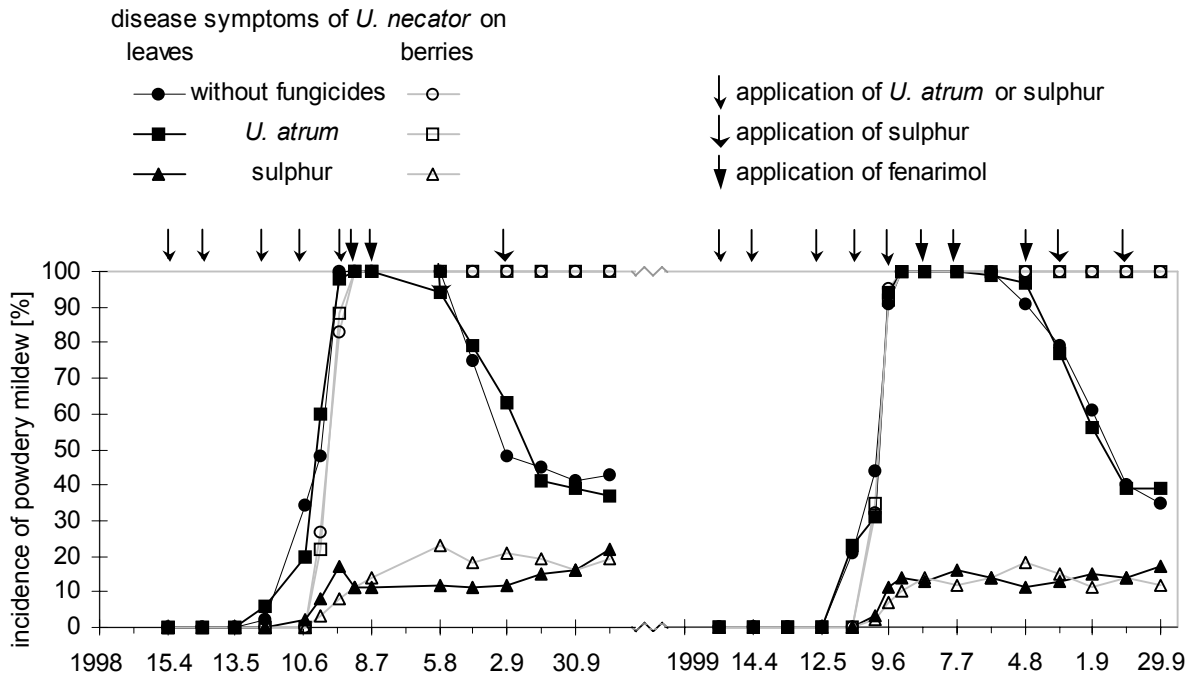


Figure 31: Effect of *Ulocladium atrum* and fungicide treatment on the incidence of powdery mildew, *Uncinula necator* (Bonn, 1998-1999).

3.5.2 Influence of *Ulocladium atrum* on the epidemics of grey mould

The epidemic spread of *B. cinerea* on grapevine begins with the hibernation on the stem, canes and other suitable material in the surrounding area, and continues with mycelial growth and sporulation on necrotic material. Consequently the initial infections of leaves, shoots and inflorescences take place, followed by latent stages of the pathogen in berries and conclude with an outbreak of grey mould on mature berries. The effect of *U. atrum* on the epidemic of grey mould was determined at all these stages.

3.5.2.1 Effect of *Ulocladium atrum* on the incidence of *Botrytis cinerea* on the bark of grapevine

Grapevine bark represents not only an ecological niche for hibernation of *B. cinerea* it also functions due to its surface structure as a spore trap. The presence of *B. cinerea* on grapevine bark was recorded at Marienthal about every two weeks by investigating 9 mm^2 bark sections. In 1997 the epidemic development of *B. cinerea* on the grapevine bark was characterised by three different periods, each of them lasted about seven weeks: 1.) May 9 to June 25, 2.) July 2 to August 20 and 3.) August 27 to October 10 (Figure 32). During the first period the incidence of the pathogen increased slowly. After June 25 the population of *B. cinerea* increased progressively; there was no significant difference between treatment with *U. atrum* and conventional fungicide treatment ($p \leq 0.05$, Mann-Whitney rank sum-test).

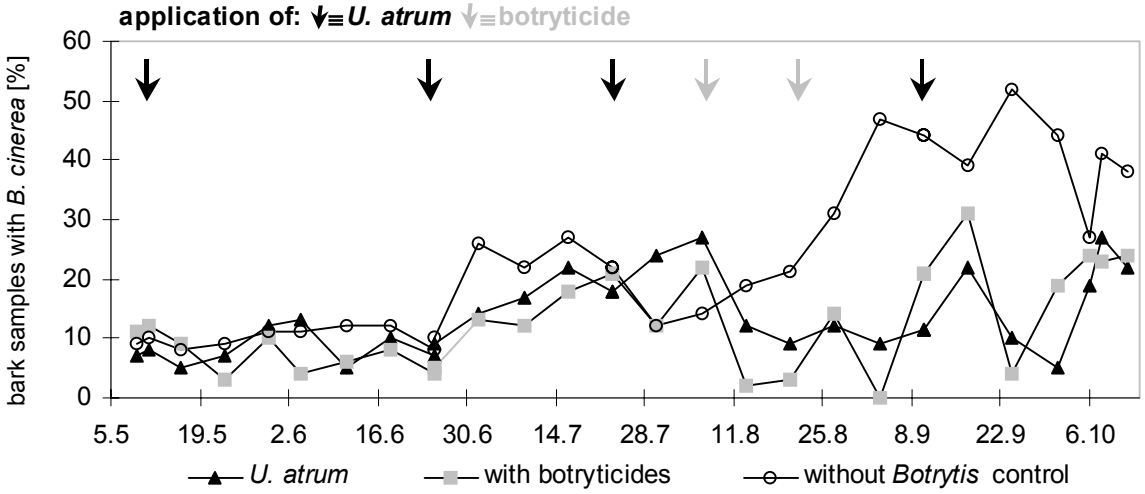


Figure 32: Effect of botryticides (August 7: carbendazim & diethofencarb, and August 22: pyrimethanil) and *Ulocladium atrum* on the epidemic build-up of *Botrytis cinerea* on grapevine bark ('Müller-Thurgau', Marienthal, 1997).

With increasing disease pressure during the third period the botryticides and the antagonist reduced the incidence of *B. cinerea* on the grapevine bark. Statistically regarded, treatments had no influence on the population of *B. cinerea* for every single date of sampling, but there was a significant ($p \leq 0.05$, Mann-Whitney rank sum-test) reduction in the incidence of *B. cinerea* for a period of six weeks before harvest.

The effect of *U. atrum* on the incidence of *B. cinerea* on the bark was determined during and after the winter season. With a low overall recurrence of *B. cinerea* in the period between growth stages BBCH 00 and 61 the average reduction of the pathogen's incidence on the grapevine bark due to *U. atrum* was 34 % (*U. atrum* /without botryticides, Figure 33); for this period, the reduction was significant ($p \leq 0.05$, Mann-Whitney rank sum-test). In plots treated with botryticides the incidence of *B. cinerea* was reduced by 13 %, which was not significant ($p \leq 0.05$, Mann-Whitney rank sum-test, with/without botryticides, Figure 33).

The incidence of *B. cinerea* on the bark increased during the growing season, and the epidemic build-up was reduced directly after the application of botryticides by up to 84 % (with/without botryticides, Figure 33). This effect lasted three to five weeks and was significant for this period ($p \leq 0.05$, Mann-Whitney rank sum-test). The effect of *U. atrum* was continuous, but on a lower level (22 %), with a significant reduction ($p \leq 0.05$, Mann-Whitney rank sum-test) of the incidence of *B. cinerea* for the entire growing period.

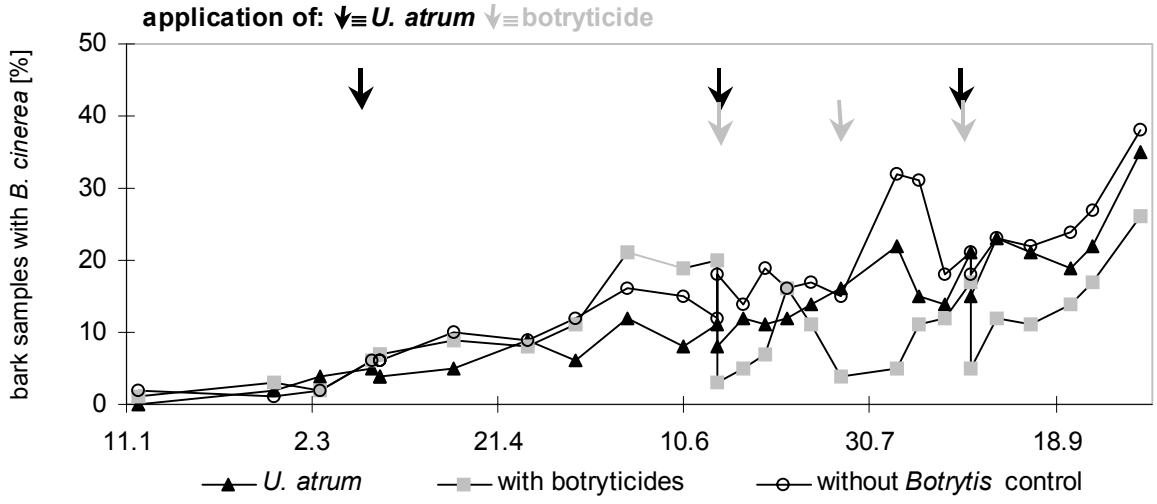


Figure 33: Effect of botryticides (June 19: pyrimethanil, July 22: carbendazim & diethofencarb and August 26: cyprodinil & fludioxonil) and *Ulocladium atrum*, applied in 1997 and 1998, on hibernation and epidemic build-up of *Botrytis cinerea* on grapevine bark ('Müller-Thurgau', Marienthal, 1998).

In 1999 the occurrence of *B. cinerea* on grapevine bark increased by about 2 % per month in the control plots at Marienthal. The epidemic development of *B. cinerea* on the bark during and after the winter season showed again a reductive effect of *U. atrum* on the pathogen. The incidence of *B. cinerea* between growth stage BBCH 00 and 61 was significantly reduced by 35 % due to applications of *U. atrum* in 1998 ($p \leq 0.05$, Mann-Whitney rank sum-test, *U. atrum*/without botryticides, Figure 34), whereas the incidence of *B. cinerea* in plots treated with botryticides was not reduced (with botryticides/without *B. cinerea* control in 1998). There was a reduction of more than 20 % in treatments with *U. atrum* or botryticides during the growth period; *U. atrum* reduced the development of *B. cinerea* especially in the first part of the vegetation period, however, the botryticides showed a strong effect for a limited time after their application.

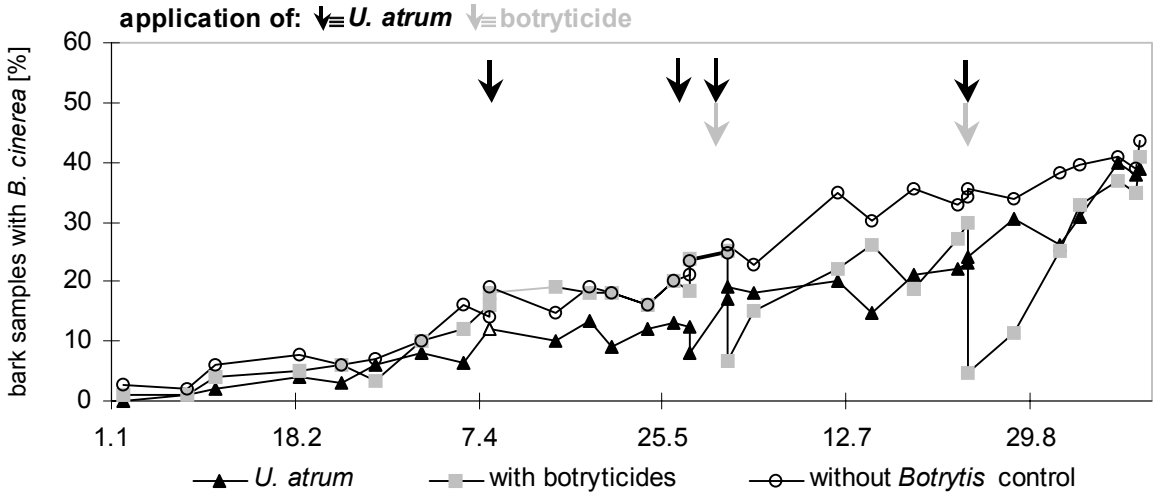


Figure 34: Effect of the botryticide fenhexamid or *Ulocladium atrum*, applied in 1998 and 1999, on hibernation and on the epidemic build-up of *Botrytis cinerea* on grapevine bark ('Müller-Thurgau', Marienthal, 1999).

3.5.2.2 Effect of *Ulocladium atrum* on the incidence of *Botrytis cinerea* on flower parts and on the infection of berries

The *Botrytis*-infections of grapes resulting in grey mould take place during flowering or at later stages. It was necessary thus to determine the incidence of *B. cinerea* from flowering to berry stages by counting the number of plant samples colonised by *B. cinerea*. Berries were surface-sterilised before incubation in order to assess the rate of latent infection.

In plots without *Botrytis* control about 40 % of the flowers and berries were infected by *B. cinerea* before harvest. The incidence of flower infection was reduced by *U. atrum* as well as by botryticides up to 40 %, whilst the latent infection rate of berries was reduced by more than 50 % (Table 10). This effect lasted for the period until vintage. The low infection rates at BBCH 81 in plots with conventional fungicide treatment were caused by application of botryticides directly before taking the samples and showed the direct effect of chemical plant protectants. This strong fungicidal effect of the botryticide at the beginning of ripening (BBCH 81) could not be detected one month later at the time of vintage (BBCH 89). In plots untreated against *B. cinerea* more than 50 % of clusters of plants were infected by grey mould at harvest time and with an average disease severity of 16 %. Both, *U. atrum* and the botryticide reduced the epidemic development of grey mould on grapevine berries. Applications of the antagonist resulted in a significantly higher reduction of the incidence of grey mould than botryticides (Tukey-test, $p \leq 0.05$), whilst the *U. atrum* and the botryticide treatment resulted in a comparable reduction of disease severity (Table 10).

Table 10: Effect of *Ulocladium atrum* and botryticides on the incidence of *Botrytis cinerea* on flower parts, latent infection of berries, as well as on the severity/incidence of grey mould at BBCH 89, vintage (Marienthal, 1999). Means within one row with different letters are significantly different (Tukey-test, $p \leq 0.05$).

growth stage [BBCH]	organ of grapevine	percentage of flowers or berries infected by *B. cinerea*			
		reduced fungicide treatment		conventional fungicide treatment	
		without *U. atrum*	4x *U. atrum*	without *U. atrum*	4x *U. atrum*
63	flowers	44 ^{a}	27 ^{b}	18 ^{b}	20 ^{b}
67	flowers	45 ^{a}	31 ^{b}	30 ^{b}	31 ^{b}
71	berries	38 ^{a}	19 ^{b}	18 ^{b}	19 ^{b}
75	berries	41 ^{a}	18 ^{b}	16 ^{b}	18 ^{b}
81	berries	45 ^{a}	21 ^{b}	5 ^{c} *	2 ^{c} *
89	berries	16 ^{A} / 54 ^{a}	8 ^{B} / 29 ^{c}	9 ^{B} / 37 ^{b}	7 ^{B} / 32 ^{bc}

* samples were taken directly after application of the botryticide

3.5.2.3 Effect of *Ulocladium atrum* on the incidence of airborne conidia of *Botrytis cinerea*

Airborne conidia of *B. cinerea* are spread by wind all over the vineyard and the density of spores at one position depends on the kind of inoculum source and its distance. Spore traps (Rotorods®) were used to assess the concentration of airborne conidia of *B. cinerea* within different plots. Rotorods® were placed within two plots of four treatments and ran simultaneously. The concentrations of *B. cinerea* conidia were calculated from the collected spores of one rod and calculated as spores per cubic metre of air.

Results of sampling spores of *B. cinerea* in different plots between September 17 and October 12 demonstrate a significant reduction (Mann-Whitney rank sum test, $p \leq 0.05$) of spores after four applications of *U. atrum* and the conventional fungicide treatment (Figure 35). Two or three applications of *U. atrum* were less effective, showing air loads similar to those of treatments without *Botrytis* control. The conventional fungicide programme, however, showed the highest efficacy especially until early October. At the end of the season the number of spores in the air also in these plots increased considerably. An additional application of *U. atrum* on fungicide treated plants provided no supplementary effect on the air load with conidia of *B. cinerea*.

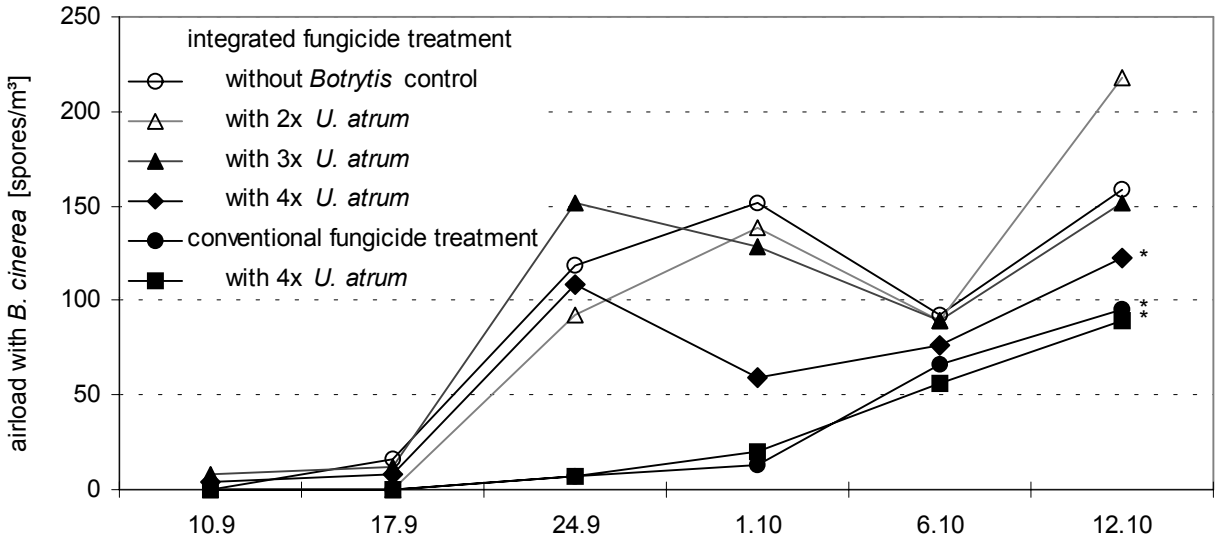


Figure 35: Effect of different treatments on the air load of *Botrytis cinerea* spores (Rotorod® samplings, Marienthal, 1997). * Progression of means is significantly different from the progression of means of the treatment without *Botrytis* control (Mann-Whitney rank sum test, $p \leq 0.05$)

Due to unfavourable weather conditions in 1998 and 1999 it was not possible to sample airborne spores of *B. cinerea* with Rotorods® in the different plots between September and October. Frequent rainfalls and wind speeds over 2 $m \cdot s^{-1}$ spread *B. cinerea* spores all over the plots. Though there were visible differences in the severity of grey mould within the treatments, no differences in the air load of *B. cinerea* were detected between the treatments.

3.5.3 Impact of timing of application on the efficacy of *Ulocladium atrum*

For the competition of *U. atrum* with *B. cinerea* it is essential that the density of viable spores is sufficient at the target place. Therefore, timing of application is important, because a thinning and a loss of vitality of the spores reduces the density of the antagonist at its target place. The effect of timing the use of *U. atrum* was evaluated within single spray application programmes at growth stages BBCH 3, 55, 69 and 81 at Marienthal in 1999.

In contrast to the application of the botryticide, the pathogen could not be reduced significantly (Tukey-test, $p \leq 0.05$) by single applications of *U. atrum* (Table 11). Only trends indicating an effect showed, but it could not be proved significantly in this experiment.

Table 11: Effect of the application time of *Ulocladium atrum* on the incidence of *Botrytis cinerea* on flower parts, on latent infection of berries, as well as on severity/incidence of grey mould at vintage (Marienthal, 1999). Means within one row with different letters are significantly different (Tukey-test, $p \leq 0.05$).

organ	growth stage [BBCH]	percentage of flowers or berries infected by *B. cinerea*					
		no *Botrytis* control	1x *U. atrum* [03]	1x *U. atrum* [55]	1x *U. atrum* [69]	1x *U. atrum* [81]	with botryticide
flowers	63	44 ^{a}	39 ^{ab}	42 ^{a}	39 ^{a}	35 ^{ab}	18 ^{b}
flowers	67	45 ^{a}	49 ^{a}	36 ^{ab}	46 ^{a}	46 ^{a}	30 ^{b}
berries	71	38 ^{a}	36 ^{ab}	31 ^{ab}	34 ^{ab}	39 ^{a}	18 ^{b}
berries	75	41 ^{a}	31 ^{ab}	22 ^{ab}	20 ^{ab}	34 ^{ab}	16 ^{b}
berries	81	45 ^{a}	46 ^{a}	32 ^{ab}	35 ^{ab}	42 ^{a}	5* ^{c}
berries	89	16 ^{a}/54 ^{A}	15 ^{ab}/50 ^{AB}	12 ^{ab}/41 ^{AB}	14 ^{ab}/38 ^{AB}	12 ^{ab}/40 ^{AB}	9 ^{b}/37 ^{B}

* samples were taken directly after application of the botryticide

3.5.4 Impact of the number of applications on the efficacy of *Ulocladium atrum*

The number of applications of *U. atrum* required to achieve a suitable control of *B. cinerea* is important for economic success. Therefore, three different spray programmes with *U. atrum* were carried out at Marienthal in 1997 to determine the impact of the number of applications on the efficacy of *U. atrum* against grey mould. Experiments were conducted in plots with integrated fungicide treatment without botryticides. The antagonist was applied two (BBCH 61and 81), three (BBCH 03, 61 and 81), or four times (BBCH 03, 61, 78 and 81).

A relatively low incidence of *B. cinerea* in 1997 caused a low level of grey mould and resulted in only small differences between treatments. The percentage of latent infection of berries with *B. cinerea* was reduced only by the botryticides, whilst the infection of berries was also significantly reduced by four or three applications of *U. atrum* (Figure 36). Due to the low infection rate and the high deviations in the results, treatment with only two applications of *U. atrum* caused a disease reduction not significantly different from those with fungicides and three or four applications of *U. atrum*.

The relation between the incidence of latent infected berries and berries with symptoms of grey mould at harvest ranged from about 10 % to 25 %. This relationship was also found in other seasons and at other locations.

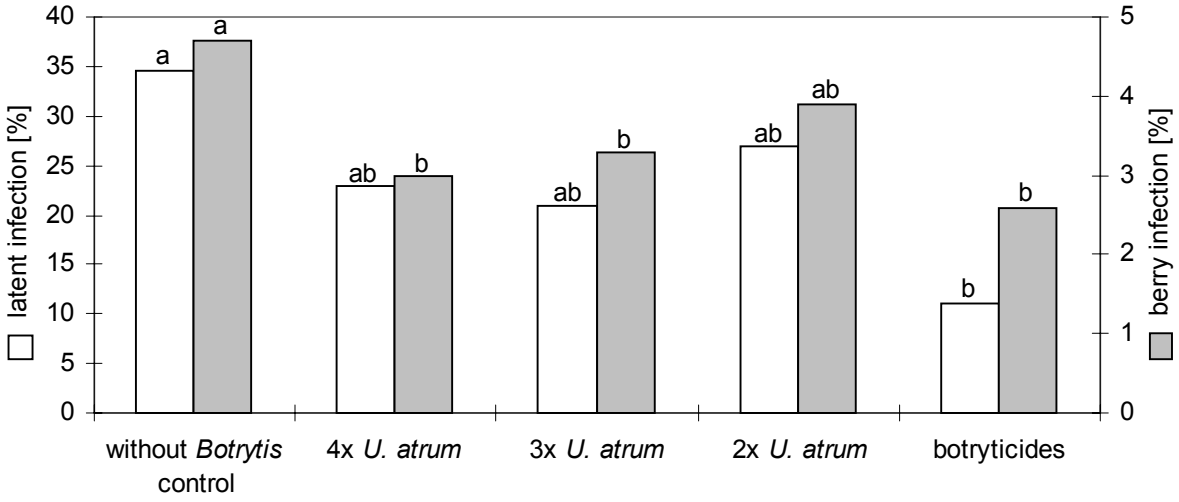


Figure 36: Effect of the number of applications on the efficacy of *Ulocladium atrum* on latent and berry infections by *Botrytis cinerea* (Marienthal, 1997). Means with different letters are significantly different (Tukey-test, $p \leq 0.05$).

3.5.5 Integration of *Ulocladium atrum* with existing crop protection programmes

A combination of *U. atrum* with pesticides is necessary to complete the plant protection against all grapevine pathogens. For the control of grey mould the antagonist can also be utilised to replace some chemical treatments using this bioagent, and to broaden the spectrum of modes of action for better disease and anti-resistance management. *U. atrum* showed a low *in vitro*-sensitivity against many fungicides used in grapevine and integrated into existing spray programmes. The antagonist survived on grapevine bark and clusters for a long time.

Combined with a reduced fungicide treatment without botryticides *U. atrum* affected the epidemic development of grey mould at Marienthal and Geisenheim in all seasons. The effect was not significantly different from the effect of botryticides (Tukey-test, $p \leq 0.05$). At Kirrweiler where the antagonist was applied in tank mixtures with pesticides, the antagonistic effect was not significantly different from the treatment without pesticides (Table 12).

In plots treated with of *U. atrum* and botryticides the potential of synergistic effects was investigated. There was no significantly synergistic effect of two or four applications of the antagonist in combination with botryticides.

Table 12: Effect of *Ulocladium atrum* and fungicides on the severity of grey mould at harvest time. Means within one row with different letters are significantly different (Tukey-test, $p \leq 0.05$).

		percentage of flowers or berries infected by *B. cinerea*				
		reduced fungicide treatment		conventional fungicide treatment		
year	location	without *U. atrum*	4x *U. atrum*	without *U. atrum*	2x *U. atrum*	4x *U. atrum*
1997	Marienthal	5 [a]	2 [b]	2 [b]	-	2 [b]
1998	Marienthal	21 [a]	7 [b*]	7 [b]	6 [b]	6 [b*]
	Geisenheim	19 [a]	8 [b*]	8 [b]	-	-
1999	Marienthal	16 [a]	8 [b]	9 [b]	8 [b]	7 [b]
	Geisenheim	21 [a]	6 [b]	9 [b]	8 [b]	5 [b]
	Kirrweiler	26 [a**]	21 [ab]	9 [c]	-	12 [bc]

* *Ulocladium atrum* was applied three times

** without any use of any pesticides

3.5.6 Long-term effect of *Ulocladium atrum* on *Botrytis cinerea*

A sustained phytosanitary effect is important for crop protection in perennial crops. A satisfactory disease control in one year retards the disease development in the following season. This objective can be achieved by chemical control and especially by biological control organism, which can be established and will persist at its target place. The long-term effects of crop protection programmes against grey mould were determined at Marienthal in 1998. Plots were treated without *Botrytis* control, with *U. atrum* or with botryticides in 1997 and in 1998, so that there were all possible nine combinations of treatments in 1998.

Within one treatment conducted in 1998 differences in infection rates between three treatments in 1997 were not significant (columns in Table 13). Independently from the treatment in the previous season, both, *U. atrum* and botryticides resulted in a significant reduction of flower infection in 1998 (rows in Table 13).

The long-term effect of *U. atrum* and botryticide treatment on the incidence of infected berries was more pronounced than the effect on the reduction in flower infection. Only within the treatment with *U. atrum* conducted in 1997, differences in infection rates of berries for treatment without *Botrytis* control and for treatment with *U. atrum* in 1998 were significant (columns in Table 14). Similar to flower infections, both treatments with *U. atrum* and with botryticides resulted in a significant reduction of berry infection independently from the treatment in the previous season (rows in Table 14).

Table 13: Long-term effect of *Ulocladium atrum* and botryticides on the percentage of flower infections by *Botrytis cinerea* (Marienthal, June 25, 1998). Means within one row with different letters show a significant difference, means within one column are not significantly different (Tukey-test, $p \leq 0.05$).

treatment in 1998 / treatment in 1997	percentage of flowers infected by *B. cinerea*		
	without *Botrytis* control	with 3x *U. atrum*	with botryticides
without *Botrytis* control	19 [a]	11 [b]	9 [b]
with 4x *U. atrum*	16 [a]	9 [b]	8 [b]
with botryticides	17 [a]	10 [b]	10 [b]

Table 14: Long-term effect of *Ulocladium atrum* and botryticides on the severity of grey mould (Marienthal, October 12, 1998). Means within one row with different small letters are significantly different; means within one column with upper case letters are significantly different (Tukey-test, $p \leq 0.05$).

treatment in 1997 \ treatment in 1998	percentage of berries infected by *B. cinerea*		
	without *Botrytis* control	with 3x *U. atrum*	with botryticides
without *Botrytis* control	22 $^{a\,A}$	9 $^{b\,A}$	10 $^{b\,A}$
with 4x *U. atrum*	18 $^{a\,B}$	5 $^{b\,B}$	8 $^{b\,A}$
with botryticides	21 $^{a\,AB}$	7 $^{b\,AB}$	7 $^{b\,A}$

3.5.7 Effect of *Ulocladium atrum* on yield quantity and quality

The yield quantity is important for the economic calculation of the benefit of crop protection. *B. cinerea* can destroy the whole yield by causing sour rot. In white wine cultivars *B. cinerea* causing noble rot can be tolerated up to a certain level and it is sometimes even enhanced, especially for dessert wines or in Riesling cultivars not only for the wine classification of *Auslesen*. The yield parameters acid and sugar content were assessed at all locations, while the weight was determined only at Marienthal.

Due to late frost periods in 1997 fruit setting was low and the average grape yield of 670 kg/ha was just one tenth the yield harvested in previous years. None of the applications increased yield parameters of sugar or acid content significantly. The quantity of grapes harvested was significantly higher in plots treated with *U. atrum* or botryticides; furthermore, higher yield of plots with conventional fungicide treatment resulted from the control of downy mildew, which can be highly destructive (Table 15). The harvest of grapevine berries in 1998 was affected by the late maturing of grapes. The number of grapes per plant was normal and the sugar content was low due to a high water content of berries. The humid period late in the season was very conducive towards sour rot of berries. Therefore, almost none of the infected berries showed symptoms of noble rot and could not be harvested. Again, there were no significant differences in yield parameters except for the quantity between treatments with *U. atrum* and the control without botryticides. At Geisenheim harvest was late in this year, and the development of grey mould could be utilised for a high sugar content of the must.

Table 15: Effect of *Ulocladium atrum* and fungicides on yield quality (**sugar** content [°Oe], **acid** content [pH]) and quantity (**m** [t/ha]). *Means within one row are significantly different (*t*-test, $\alpha = 0.05$).

		reduced fungicide treatment						conventional fungicide treatment					
		without *U. atrum*			with *U. atrum*			without *U. atrum*			with *U. atrum*		
year	location	sugar	acid	m	sugar	acid	m	sugar	acid	m	sugar	acid	m
1997	Marienthal	81	3.4	0.4	82	3.3	0.6*	82	3.4	0.9*	83	3.3	0.8*
1998	Marienthal	61	3.3	7.1	60	3.2	7.8*	63	3.1	8.1*	67*	3.2	7.9*
	Geisenheim	89	3.0	-	87	2.9	-	92	2.9	-	-	-	-
1999	Marienthal	66	3.3	10.0	65	3.2	11.5*	66	3.2	11.2*	68	3.3	11.9*
	Geisenheim	87	3.0	-	91	3.0	-	90	2.9	-	-	-	-
	Kirrweiler	90	2.9	-	88	3.2	-	85	2.9	-	93*	3.0	-

In 1999 maturing of grapes was accelerated by warm and dry weather conditions in summer and also by the massive incidence of grey mould. At Marienthal and at Kirrweiler, grapes were harvested early in order to prevent berries from sour rot, and also from a high loss in acid content. Harvest was late at Geisenheim, and the development of *B. cinerea* as noble rot could be utilised for a high sugar content of the must. Except for the yield quantity, there was no significant difference in the yield parameters between the treatment without *Botrytis* control and the treatment with *U. atrum* and/or the conventional fungicide treatment.

3.5.8 Effect of *Ulocladium atrum* on vinous fermentation and ripening of wine

As pesticides might affect the vinous fermentation or the ripening of wine it was necessary to check the influence of *U. atrum* on vinification. Four treatments were taken for standard vinification at Marienthal: reduced fungicide treatment with or without *U. atrum* and conventional treatment with or without *U. atrum*. At Kirrweiler, wine was made from grapes of all four different treated plots, and weight loss was assessed during fermentation to determine a possible effect of treatments on the activity of the yeasts during the progression of fermentation. There were no measurable differences in the fermentation between the treatments (Figure 37).

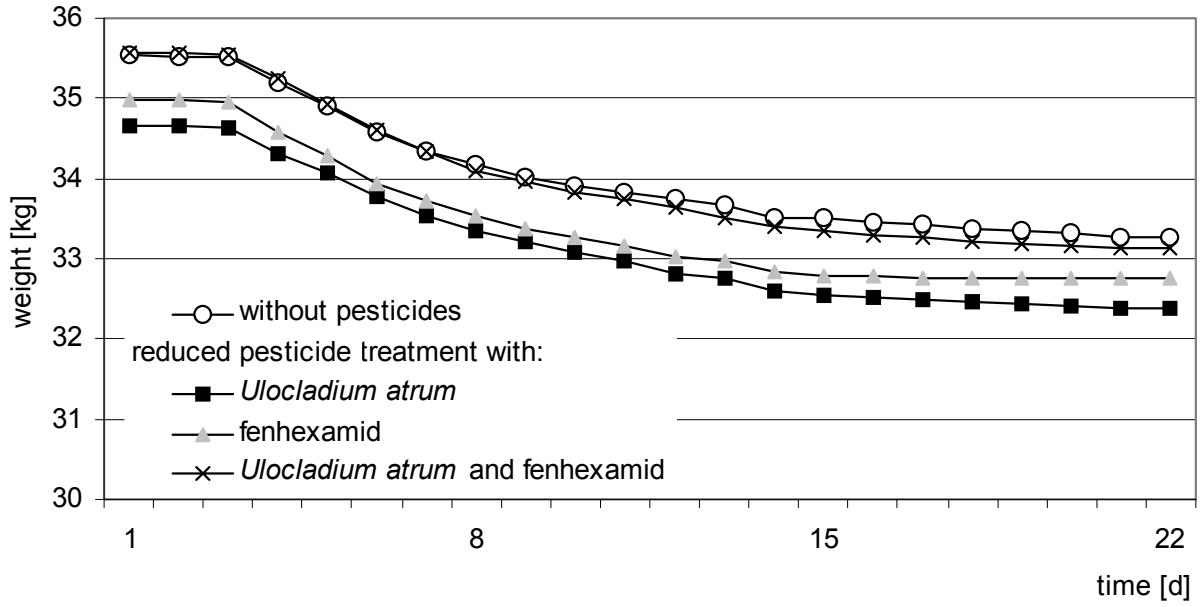


Figure 37: Effect of *Ulocladium atrum* and fenhexamid applied four times during the season on the decrease in weight during the process of vinification (Kirrweiler, 1999).

The acid and sugar content were assessed before and after vinification, and the acid content was checked annually to assess stability of the wines as well as gustatory quality.

There were neither differences of the gustatory quality of wines from Marienthal, nor any differences between the treatments in changing the acid or sugar content. There were also no changes in the acid content observed during ripening. A negative effect of *U. atrum* on vinification and ripening of wine was not observed, which completes the prerequisites of a safe use of *U. atrum* as a biocontrol agent in grapevine.

4 Discussion

The ecological balance of microorganisms in the phyllosphere is important for plant health; however, chemical pesticides with a broad spectrum of pathogens reduce variety and number of saprophytes, which might be beneficial to plants by acting as antagonists to plant pathogens [NEWHOOK, 1957; FOKKEMA, 1990]. Competitive antagonistic microorganisms are capable of colonising these vacated niches; nevertheless, limited knowledge of the ecology of antagonists seems to be a major constraint in successful biocontrol; one of the main problems is still to have a euryoecious antagonist whose population does not fade out too fast [FOKKEMA, 1995; WHIPPS & LUMSDEN, 2001]. Despite an increasing public awareness of the importance of biological control, uncertain economic profitability of biocontrol agents and their relatively narrow spectrum still limit their acceptance by growers and general public, and less than 5 % of pesticides are biocontrol agents by now [BUTT & COPPING, 2000].

The aim of this project was to study the antagonistic potential of *Ulocladium atrum* against *Botrytis cinerea* in grapevine and its integration into existing crop protection programmes. Investigations on pesticide sensitivity of the antagonist were performed for pesticides used in conventional control of grapevine pathogens. Furthermore, interactions of both fungi were studied under controlled conditions on necrotic and healthy grapevine leaves and on mature berries. The antagonistic effect of *U. atrum* against grey mould was evaluated in field experiments with different grapevine varieties at three locations. Emphasis was given to application dates and to the compatibility of the fungus with conventional crop protection in order to integrate *U. atrum* into other control strategies. Side-effects of the antagonist on other grapevine diseases were determined to evaluate the antagonistic potential of *U. atrum*. The effect of *U. atrum* on plant health and vinification were studied to ensure an application safe for the environment. This knowledge can help to simplify a successful application of *U. atrum* in grapevine and to optimise a strategy of controlling grey mould with this antagonist.

B. cinerea was described by MÜLLER-THURGAU [1888] as a pathogen in grapevine, and he also mentioned the noble rot, the beneficial effect of *B. cinerea*. The pathogen is able to infect leaves, stems, flowers and fruits and causes yield losses in the production phase as well as in storage [COLEY-SMITH *et al.*, 1980]. *B. cinerea* is an important risk factor for quantity and quality of grapes and wine; disease severity of grey mould in grapevine normally range about 20 % and can reach up to 100 % [FARETRA & POLLASTRO, 2001]; comparably low severities of grey mould from 5 to 26 % were observed in the field experiments.

Adequate knowledge on the biology of *B. cinerea* is crucial to understanding its epidemics and for the evaluation of possibilities for antagonistic competition. Corresponding to the epidemiology of *B. cinerea* in grapevine frequently discussed in literature, symptoms of *B. cinerea* were observed in field experiments on different parts of grapevine, such as leaves, tendrils, inflorescences and canes and most frequently on mature berries (Figure 38).

It is usually said that *B. cinerea* reproduces only asexually and all strains are identical, regardless of the host, place or year [GIRAUD *et al*., 1998]. Formation of apothecia was not observed in field trials; they are rarely found in vineyards and are irrelevant in the epidemics of grey mould in grapevine [PEARSON & GOHEEN, 1988]. Nonetheless, molecular analysis of strains collected from Champagne, France, showed that there is great genetic diversity and that sexual reproduction is therefore efficient [GIRAUD *et al*., 1998].

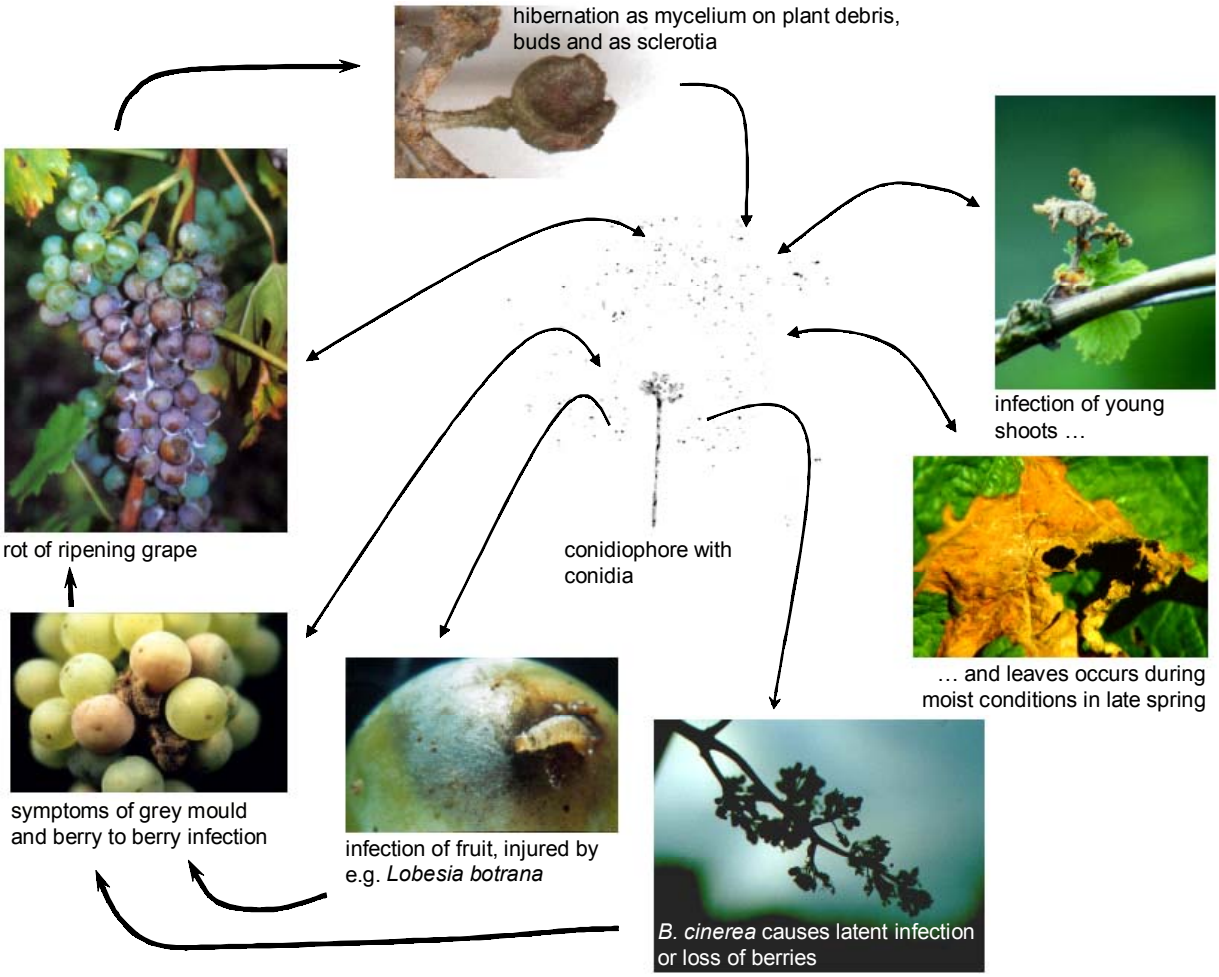


Figure 38: Epidemic spread of grey mould (*Botrytis cinerea*) in grapevine and nutrient sources possible for an antagonistic competition by *Ulocladium atrum*.

LEROUX *et al*. [1998] pointed out that there are at least two different sibling species normally identified as *B*. *cinerea* in Champagne named *B*. *vacuma* and *B*. *transposa*. This could explain why disease levels observed before *véraison* are not correlated with the grey mould level at harvest [DUBOS & Roudet, 2000]. An antagonistic fungus like *U*. *atrum* competing for nutrients would affect both species. Nonetheless, incidence of two or more species causing grey mould in grapevine would make a crop protection strategy more difficult. Generally, the pathogen causing grey mould in grapevine is still named *B*. *cinerea*, however and the possibility of two epidemic developments was therefore not taken into account in this study.

Epidemic development of grey mould in vineyards depends on primary inoculum and the subsequent spread by conidia and via mycelial growth [JARVIS, 1980]. *B*. *cinerea* hibernates as mycelium on many kinds of plant debris, on poorly hardened canes and on mummified berries; after winter dormancy, *B*. *cinerea* spreads by germination and spore production [COLEY-SMITH & COOKE, 1971]. Similar to studies of ROUDET & DUBOS [2000], *B*. *cinerea* also hibernated on the bark of grapevine. Sclerotia, another vegetative form of hibernation, most frequently germinate by formation of conidiophores [COLEY-SMITH, 1980]; these conidia represent an important source of primary inoculum of *B*. *cinerea* in grapevine, although sclerotia were observed rarely in vineyards [NAIR & NADTOTCHEI, 1987].

Young shoots are infected by *B*. *cinerea* and disease symptoms occur as leaf blight, which might be important for a subsequent spread [PEARSON & GOHEEN, 1988]. However, leaf blight was infrequently observed in the field experiments. More often, inflorescences of grapevine are infected by *B*. *cinerea*, which can cause loss of berries or total clusters as midseason bunch rot [NAIR & PARKER, 1985]. Furthermore, senescent flower debris colonised by *B*. *cinerea* are another important inoculum sources of grey mould in grapevine [DUBOS *et al*., 1982; WOLF *et al*., 1997]. An early flower infection can result in latent berry infection [MCCLELLAN & HEWITT, 1973]. Latent infections of berries can lead to grey mould occurring at *véraison* [JARVIS, 1980; BULIT & DUBOS, 1982; NAIR *et al*., 1995] and therefore, producing high levels of inoculum and a high risk of secondary infection of berries [NAIR, 1985]. A statistical quantitative relationship between saprophytic, parasitic or latent phases remains controversial however, and depends on many factors [FERMAUD & PIERI, 2000]. In field experiments, the relation between berries with symptoms of grey mould at harvest and incidence of latent infection was about 10 % to 25 %; latent infection has therefore an important implication on developing management systems for bunch rot [MCCLELLAN & HEWITT 1973; NAIR, 1985].

At *véraison*, grapes can be infected directly by conidia forming germtubes or by mycelium penetrating the epidermis or growing into wounds made by mechanical, osmotic damage or by insects [VERHOEFF, 1980]. Transmission from berry to berry represents therefore another possibility of *B. cinerea* infection in grapevine.

Berries wounded by pests, insects' webs and their debris represent a suitable niche for the saprophytic pathogen; especially larvae of the grape berry moth *Lobesia botrana* or the vine moth *Eupoecilia ambiguella* are known to be involved in the epidemic spread of *B. cinerea* [FERMAUD & LE MENN, 1989; MORANDO *et al*., 1985; KAST, 1993]. However, berries infected by *B. cinerea* may also be attractive for insects [VIDAL & FRANÇOIS, 1996; MONDY *et al*., 1998b]. It can therefore be concluded that there is a mutual relationship between pest and *B. cinerea* [MONDY *et al*., 1998a]. Nonetheless, incidence of insect pests was low in field experiments and an effect on *B. cinerea* epidemics could therefore be neglected.

The amount of conidia produced close to inflorescences and berries of grapevine is important for the epidemic spread of grey mould in grapevine. The air load of *B. cinerea* spores represents the sum of conidial production in the vineyard and its surrounding area [CORBAZ, 1972]; airborne *B. cinerea* spores are almost omnipresent due to its viability and reproductive potential [JARVIS, 1980; COERTZE *et al*., 2001]. Possibilities for a competitive antagonist to retard epidemics of grey mould should therefore be studied for these niches of *B. cinerea*: hibernating organs, necrotic and healthy leaves, inflorescences and berries (Figure 38).

Conidial spread of *B. cinerea* was investigated at Marienthal using a Burkhard® spore trap. Hirst-type samplers like these were often used for measurements of airborne fungal spore concentrations [HIRST, 1952; WARNER *et al*., 2000]. Conidia of *B. cinerea* were detected first at the time of flowering; incidence of spores was low until August, when the number increased. On the contrary, PAK [1992] reported the first peak of spore catches in August. This discrepancy might be explained by differences in location and climatic variations. Conidial spread of *B. cinerea* from diseased berries and the vintage resulted in high spore densities late in the season, similar to the results given by PAK [1992]. Especially in 1999, results of spore catches showed that a potential infection risk originates from *B. cinerea* conidia for almost the whole season, even if suitable climatic and physiological conditions finally facilitate infection [BROOME *et al*., 1995].

Senescent plant material is the major nutrient source of *B. cinerea*, and it is utilised by the saprophytic pathogen to grow, to form mycelium and conidia and to start its epidemic spread

[JARVIS, 1980]. Biocontrol of the pathogen in established lesions or the reduction of sporulation on necrotic plant tissues can minimise the epidemic spread [ELAD, 1996]. The saprophytic hyphomycet *U. atrum* degrades diverse organic materials, affects sporulation of pathogens e.g. *B. cinerea* and slows its epidemic development [KÖHL *et al.*, 1995b]. The potential of *U. atrum* to control *Botrytis* spp. was shown on senescent leaves of onions [KÖHL *et al.*, 1995c; KÖHL *et al.*, 1999], ornamentals [KÖHL *et al.*, 1995a; ELMER & KÖHL, 1998; KESSEL, 1999], strawberries [KÖHL & FOKKEMA, 1998; BOFF *et al.*, 1998; BERTO *et al.*, 2000], kiwifruits [BOYD-WILSON *et al.*, 1998] and tomatoes [FRUIT & NICOT, 1999]. Grapevine is known for its ability to synthesise fungicidal compounds like phytoalexins, glycolic acids, glycoproteins, phenolic compounds and proanthocyanidins [PEZET & PONT, 1992; GOETZ *et al.*, 1999]. However, *U. atrum* was able to colonise necrotic grapevine tissue, one prerequisite for a successful use of a saprophytic competitive biocontrol agent. Furthermore, conidia of the antagonist survived on the surface of grapevine bark, leaves, inflorescences and berries, and they were capable of germinating under moist condition.

Growth and sporulation of microorganisms depend on nutrients and on climatic conditions. Early attempts to use antagonists were often unsuccessful until environmental conditions were shown to be crucial to the balance of microorganisms in the phyllosphere [BLAKEMAN & FOKKEMA, 1982]. Conidia deposited in the phyllosphere are exposed to large and rapid changes in temperature and availability of nutrients and water; in addition to surviving these adverse conditions, antagonists must have the capacity to grow rapidly during the periods favourable for growth and be able to colonise the host substrate to compete effectively with the pathogen [ELMER & KÖHL, 1998]. Little information is available about fate of saprophytic antagonists in the phyllosphere, much less than on pathogens [KÖHL & MOLHOEK, 2001]; nevertheless, survival and permanent presence of biocontrol agents at sufficient density are important for their success, especially for *U. atrum* whose antagonistic effect depends on competition [KÖHL *et al.*, 1997].

Drought tolerance was one of the major selection criteria for *U. atrum* as an antagonist of *Botrytis* spp., testing interactions of fungi on necrotic onion leaves [KÖHL *et al.*, 1993]. Similar to tests on necrotic lily leaves [KÖHL *et al.*, 1995c], maximum number of spores was produced on grapevine leaves at high water potentials. In contrast to *B. cinerea*, *U. atrum* was able to grow and to sporulate at relative humidities lower than 96 %; this might give an edge to the antagonist in colonising necrotic tissue under field conditions. Results on necrotic

grapevine leaves and berries were similar to those described by NELSON [1951], who showed that *B*. *cinerea* requires at least 94 % r.h. for a successful infection of grapes.

Fungal growth in the phyllosphere is limited by constantly low humidities, but fluctuating water availability may also inhibit microorganisms. KÖHL & MOLHOEK [2001] showed that *U*. *atrum* is capable of adapting to these unfavourable conditions in the phyllosphere, a characteristic that is important for a successful establishment of an antagonist population in this niche. They showed that even if the germination speed was lower when moist periods were interrupted, maximum percentage of germination was the same for conidia incubated with or without interruption of moist conditions. *U*. *atrum* can tolerate water stress conditions during competitive substrate colonisation with *B*. *cinerea*, and *U*. *atrum* is therefore an attractive antagonist to be used in the phyllosphere [KÖHL & MOLHOEK, 2001]. Compared to hyaline spores, cell walls of dermatiaceous dark pigmented fungi as *U*. *atrum* can tolerate water stress and protect against transpiration [DIEM, 1971]. Ungerminated conidia of *U*. *atrum* are therefore capable of persisting in the phyllosphere, and, though only at low concentrations, *U*. *atrum* could be re-isolated from the bark of grapevine three years after application.

It is crucial for antagonistic microorganisms to be at least as eurythermic as their pathogenic target organism to guarantee antagonistic activity at a range of temperature as broad as possible. The effect of temperature on growth and sporulation on necrotic grapevine leaves was similar for *U*. *atrum* and *B*. *cinerea*; however, *U*. *atrum* was able to grow and to produce spores even at 5 °C. According to BLAKEMAN [1980] most studies have shown that *B*. *cinerea* has an optimum temperature of 20 to 25 °C, and so was the optimum for the isolate Bc 700 on necrotic leaves of grapevine. Spore production of *U*. *atrum* on necrotic leaves was maximal at 25 °C whereas the optimum for *B*. *cinerea* was 20 °C. Correspondingly, JALIL *et al*. [1997] described temperature ranges of the biocontrol agent *Trichoderma harzianum* isolate T39 and isolates of *B*. *cinerea*, and they concluded that this congruence of temperature ranges is approving the biocontrol agent. Differences between temperature optima of sporulation of the two fungi were emphasised in co-inoculation experiments, though *U*. *atrum* proved its antagonistic potential at all temperatures. Nevertheless, optimum temperature of antagonists should be adapted to environmental conditions at their target place.

Time of application is essential for colonisation of necrotic tissue, especially for a competitive and therefore protective antagonist. As WEEDS *et al*. [2000] described for competition between an aggressive and a non-aggressive strain of *B*. *cinerea* on leaves of French bean by

inoculating strains sequentially, the first isolate dominated the infection site. The effect of a time interval between inoculation of *U. atrum* and *B. cinerea*, respectively, was essential for leaf colonisation. Pre-inoculation of *U. atrum* for more than one day led to a reduction of the pathogen's spore production by more than 70 %, compared to the inoculation of *B. cinerea* alone. Spore production of the pathogen was reduced by more than 90 %, when the antagonist became well established on the leaves four days before application of *B. cinerea*. KESSEL [1999] reported similar results in multi-side inoculation tests in ornamentals. This emphasises the importance of permanent presence of antagonists to guarantee a rapid biodegradation of nutrients, which are essential for the pathogen's epidemic.

B. cinerea is capable of producing disease symptoms on grapevine leaves as leaf blight; the pathogen can infect leaves, thus producing necrosis which may be the origin of an epidemic spread onto berries [PEARSON & GOHEEN, 1988]. In contrast to the high impact of *Botrytis* leaf blight in other crops, especially in glasshouses [JARVIS, 1980; ELAD *et al*., 1992; KERSSIES, 1992], leaf blight is less important in grapevine [RIBÉREAU-GAYON *et al*., 1980]. However, this additional source of inoculum, very close to the grapevine inflorescences and berries, should not be neglected in the epidemiology of *B. cinerea*, although most studies in grapevine were conducted on grey mould of berries. In contrast to experiments with curative botryticides or compost extracts in grapevine [SACKENHEIM, 1993], incidence of leaf blight was not reduced, but *U. atrum* affected the pathogen's development. Even if *B. cinerea* is completely suppressed by *U. atrum* in some crops [FRUIT & NICOT, 1999; KÖHL *et al*., 1999], the antagonist was only capable of reducing severity of leaf blight symptoms in grapevine. Nonetheless, *U. atrum* affected leaf blight and is therefore able to reduce spore production.

Inflorescences of grapevine are susceptible to infection by *B. cinerea* and an early infection of flowers can either cause latent infection or loss of infected berries or even whole clusters, and latent infection can lead to grey mould occurring at *véraison* [JARVIS, 1980; BULIT & DUBOS, 1982; NAIR *et al*., 1995]. Even if it is an unusual phenomenon, grey mould occurring before *véraison* can be a severe problem in grapevine causing yield losses [NAIR & PARKER, 1985]. Midseason *Botrytis* disease was simulated on grapevine plants in moist chambers and in glasshouses. Due to the massive infection pressure, berries inoculated at early flowering 'trickled through' without any effect of *U. atrum*, but the antagonist had an effect on latent infection of berries inoculated at full flowering. At a very high disease pressure the antagonist in unable to protect fruits from *B. cinerea* infection. This failure of *U. atrum* was also

observed is field experiments in grapevine [ROUDET & DUBOS, 2000] and in strawberries [BOFF, 2001]; nevertheless, applied at late flowering stages, *U. atrum* affected latent infections of berries.

Transmission from berry to berry represents another possibility of *B. cinerea* infection in grapevine. Starting at *véraison*, grapes can be infected directly by conidia forming germtubes or by mycelium penetrating the epidermis or growing into wounds [VERHOEFF, 1980]. Therefore, the amount of spores produced on mature grapevine berries is important for the development of grey mould in the bunch and in the surroundings of infected bunches. Studies of SACKENHEIM [1993] on grapevine berries demonstrated the antagonistic potential of extracts from composted material on incidence of *B. cinerea in vitro*. In contrast to these tests, berries were wounded for experiments on the antagonistic effect of *U. atrum* to simulate injuries made by hail or insects and to ensure infection of all berries. Sporulation of *B. cinerea* was affected by *U. atrum*, but in contrast to studies of SACKENHEIM [1993] describing antibiotic effects of compost extracts there was no effect of *U. atrum* on the incidence of berry infection at these conditions very suitable for the pathogen.

Possibility of spontaneous recovery of berries was described by GÄRTEL [1970] and could be simulated by an inoculation of *B. cinerea* two days after injury. Reduction of sporulation was enhanced by a two days interval between injury and inoculation of the pathogen, but berry infection was also not prevented. The antagonist colonised wounds without pathological symptoms inside the berry and proved not to be a pathogen of grapevine, though conditions were highly favourable to *U. atrum*. FRUIT [INRA Avignon, France, pers. comm.] reported similar results for stem wounds of tomato where mycelial growth and sporulation of *U. atrum* was macroscopically visible without showing disease symptoms.

Sclerotia of *B. cinerea* occur as vegetative hibernating organs on many kinds of plant debris and on poorly hardened canes in vineyards; they can germinate in myceliogenic, sporogenic and carpogenic ways [COLEY-SMITH & COOKE, 1971]. Especially in production of grafted vines, sclerotia can cause infections of grapevine wood, but direct economical significant losses in viticulture are uncommon [GÄRTEL, 1970]. However, formation of conidiophores is most frequent for germinating sclerotia [COLEY-SMITH, 1980] and these conidia represent an important source of primary inoculum of *B. cinerea* in grapevine [NAIR & NADTOTCHEI, 1987]. KÖHL & SCHLÖSSER [1988] described the antagonistic potential of *Trichoderma* spp. against *B. cinerea* to macerate and overgrow sclerotia. *U. atrum* was capable of growing on

sclerotia of all tested *B. cinerea* isolates, but in contrast to experiments of KÖHL & SCHLÖSSER [1989], sclerotia were still viable and only sporulation was reduced by the antagonist. Due to their various modes of action, isolates of *T. harzianum* are the more efficient antagonists of *B. cinerea* sclerotia [KÖHL & SCHLÖSSER, 1988]. Thus far, the most common biological control agents of the genus *Trichoderma* have been reported to be strains of *T. harzianum* acting as mycoparasites, competitor, by producing antibiotics or inducing systemic resistance; nonetheless, recent studies using molecular biological techniques have shown that some of the previously identified *T. harzianum* have to be classified as other species of *Trichoderma*, also corresponding with their different modes of action [HERMOSA *et al*., 2000].

Co-inoculation with *U. atrum* reduced spore production of *B. cinerea* growing on various necrotic plant materials as summarised by KÖHL & FOKKEMA [1998] and halved sporulation of the pathogen on necrotic grapevine leaves and mature berries. Furthermore, the antagonist reduced severity of leaf blight and affected spore production from sclerotia. These results only show one quantitative effect of antagonism of *U. atrum*. Viability and pathogenicity of spores produced in co-culture with the antagonist, and therefore grown at reduced nutrient availability, might also be affected [MACFOY & SMITH, 1986], but such an effect has not been shown for *U. atrum* so far.

It is essential for a successful use of antagonistic microorganism to know its mode of action. Besides hyperparasitism and induced systemic resistance, antagonistic activity of fungi and bacteria used in grapevine against *B. cinerea* often depends on suppression of pathogens' spore germination based on antibiosis [DUBOS *et al*., 1982; PAUL *et al*., 1997; GUETSKY *et al*., 2001]. Furthermore, competition for nutrients is possible as a biocontrol strategy since germination of *B. cinerea* conidia depends on the presence of external nutrients [ELAD, 1996]. In contrast, COLE *et al*. [1996] showed that conidia were infective without exogenous nutrients after dry inoculation. The mode of action of *U. atrum* was described as a rapid and extensive competition for necrotic tissue excluding the competitors without any antagonistic action like parasitism or antibiosis [KÖHL *et al*, 1997]. It was shown that *U. atrum* is capable of germinating without additional nutrients, whilst *B. cinerea* needs external nutrient sources. Only if the antagonist was inoculated before the pathogen, and just at low sucrose concentrations, *U. atrum* suppressed sporulation of *B. cinerea*, which indicates that one antagonistic activity of *U. atrum* is to metabolise nutrients *B. cinerea* needs for germination. Correspondingly, FILONOW [1998; 2001] deduced that competition for sugars is essential for

the yeasts *Sporobolomyces roseus* and *Cryptococcus laurentii* as biocontrol agents against *B. cinerea* in apple, but there are usually also other factors involved in the efficacy of successful antagonists of *B. cinerea* as postulated by ELAD *et al*. [1994].

Retardation of the epidemiological development is a promising alternative strategy to the elimination of pathogens by direct antagonism such as antibiosis or hyperparasitism [FOKKEMA, 1990]. *U. atrum* reduced the epidemic development of *Botrytis* spp., especially its spore production, by competition for nutrients and space [KÖHL *et al*., 1995b]. This efficacy of *U. atrum* was demonstrated *in vitro* on oatmeal agar where the antagonist was capable of competing for space. Inoculated three days before *B. cinerea*, *U. atrum* almost completely covered the media after ten days of incubation. An over-growing or a zone of inhibition was not observed; neither a change of morphological structures of the pathogen was observed for co-inoculations with *U. atrum* nor was direct contact necessary for the antagonistic effect.

Correspondingly, CASTORIA *et al*. [1997, 2001] reported that neither antibiosis nor direct physical interaction did appear to be involved in the antagonistic activity of two saprophytic yeasts against *B. cinerea*; nonetheless, they found cell wall-degrading enzymes being involved in the antagonism. Generally, excretion of these enzymes is identified as a mycoparasitic mode of action as shown for a *T. harzianum* isolate [SCHIRMBOCK *et al*., 1994; LORITO *et al*., 1996]. Furthermore, these enzymes are involved in the response of grapevine leaves or other plants to the incidence of plant pathogens, especially *B. cinerea* [RENAULT *et al*., 2000]. As with the efficacy of *Pichia membranifaciens* [MASIH & PAUL, 2002], BERTO *et al*. [2000] suggested that these enzymes, especially β-1,3-glucanase, play a complementary role in the competitive colonisation of necrotic strawberry leaves and in antagonism of *U. atrum* against *B. cinerea*. Furthermore, fungal antagonists can be stimulated by *B. cinerea* [SCHIRMBOCK *et al*., 1994]. Correspondingly, *U. atrum* produced more plant and fungal cell wall-degrading enzymes in the presence of *B. cinerea*, and isolate Ua 385, most successful in biocontrol, excreted more of these enzymes than the other tested isolates of *U. atrum*; Ua 385 was therefore best in metabolising nutrient sources and in the biocontrol of *B. cinerea* [BERTO *et al*., 2001]. Growth-stimulation of the antagonist by the pathogen could not be observed on necrotic grapevine leaves due to mutual antagonistic effects of both fungi. There might be a direct interaction between these two fungi, even if, corresponding to results of KÖHL *et al*. [1997], no antagonistic actions such as parasitism or antibiosis were observed. Nonetheless, competition for space and/or nutrients seems to be the main antagonistic effect of *U. atrum*.

Antagonistic activity of many microorganisms was proved in tests under controlled conditions; however, inconsistency in efficacy is one of the major limitations with biocontrol agents, which often arises when antagonistic microorganisms reach the state of large-scale testing [WHIPPS & LUMSDEN, 2001]. Essentially the antagonist must first survive application and then stay active throughout the period control is required. This may be several months for some crops and four to five month for the protection of grapevine bunches from flowering until *véraison*.

Applications of biocontrol agents and chemical pesticides often differ in their required technical parameters, e.g., application pressure and droplet size [SUTTON & PENG, 1993; COPPING & MENN, 2000]. The optimal droplet size was evaluated for many biofungicides and summarised by BATEMAN & CHAPPLE [2001]. They concluded that biofungicides require the greatest efficiency of coverage of all pesticides, which can be achieved by small droplets. Nevertheless, application pressure also determines the survival rate of applied organisms, and techniques of applying biocontrol agents need much more attention in the future [MATTHEWS, 2000]. Spores of *U. atrum* tolerated a spray pressure of 2 to 6 bar, whilst an application pressure of 2 bar was required for an even spread of the suspension and the viability of spores, and the conidia of *U. atrum* showed a high germination rate after being sprayed at a pressure up to 5 bar. It was decided not to exceed a pressure of 4 bar, to have evenly spread conidia with a high survival rate, and to apply *U. atrum* with conventional sprayers also used for chemical pesticides.

Germination rate of *U. atrum* in the field was very similar to those reported in lily [KÖHL *et al*., 1995a], in wheat [LENNARTZ, 2001] and in strawberry [BOFF, 2001]. Furthermore, the antagonist used in tank mixtures applied with conventional spray equipment showed a high vitality. However, antagonistic activity was better in field experiments when *U. atrum* was applied with knapsack sprayers separately from general crop protection measures; tank mixtures therefore have to be developed to take account of the specific requirements of this biocontrol agent.

An even more sparing application was used in strawberries by KOVACH *et al*. [2000], who disseminated *Trichoderma harzianum* by honeybees and bumblebees. This method resulted in a better *Botrytis* control than spray application, though density of *T. harzianum* on flowers collected from bee-delivered plants was half the density on sprayed strawberry flowers. An application of *U. atrum* via insects could be possible, but as the antagonist should also be

present on parts of the plant not headed by these insects; therefore, application by spray equipment seems to be unavoidable.

Microorganisms of the phyllosphere are exposed to changes in climatic conditions and radiation; ultraviolet (UV) radiation can affect fungal structures on the plant surface and due to variation in pigmentation, genera of fungi differ in their susceptibility to this radiation [ENGLISH & GERHARDT, 1946]. The effect of UV also depends on the wavelength; whilst near-UV radiation has been shown to promote grey mould [REUVENI *et al*., 1989; NICOT *et al*., 1996], a negative effect of UV-C on the epidemics of grey mould in grapevine were observed and correlated to direct affection of the pathogen or induced resistance of the plant [PEZET & PONT, 1992; NIGRO *et al*., 1998]. Therefore, a negative effect of UV on the survival of the soil-borne fungus *U. atrum*, applied onto the phyllosphere, might also be possible. However, melanin, a dark pigment of the black walled dermatiaceous spores including *U. atrum*, may provide protection against UV [ROTEM *et al*., 1985]. BOYD-WILSON *et al*. [1998] showed that spores of *Ulocladium* spp. were affected five times less than the lighter pigmented *B. cinerea* spores when exposed to short wavelength radiation *in vitro*. They also described that conidia of *Ulocladium* spp. survived among other saprophytic antagonistic fungi within the kiwifruit canopy for at least 16 weeks without a measurable effect of natural UV radiation. Flowers and berries of grapevine are also partially protected from direct sunlight by leaves, and *U. atrum* proved its ability to persist in the phyllosphere of grapevine for a suitable period, and re-isolation of *U. atrum* from grapes was possible more than 16 weeks after application.

Plant pathogens are dispersed by rain splash [FITT *et al*., 1989] or they are removed from aerial plant surfaces [BUTTERWORTH & MC CARTNEY, 1991]. Thus, inoculum of antagonistic fungi may also be washed off by rain; even supposing that fungi have many mechanisms to solve this problem, much of the inoculum therefore expires due to climatic conditions [DICKINSON, 1986]. Nonetheless, a reduction of the antagonist's density was also measurable on berry and bark samples in field experiments of grapevine, but the decline was low and could not be correlated to heavy showers. Differences of splash and other climatic conditions between canopy levels have been reported: lower plant parts are better protected from rain than upper leaves [ELMER & KÖHL, 1998; LENNARTZ, 2001]. In contrast to experiments with *U. atrum* on leaves of ornamentals or in wheat, major targets in grapevine were inflorescences and berries, which are protected by leaves similar as lower plant parts in wheat or ornamentals.

Another requirement for a biocontrol agent is harmlessness to non-target organisms, especially to crop plants [LYNCH, 1992]. A potential effect of the antagonist on the plant was studied, because *U. atrum* has also been described as a plant pathogen causing achene blemish of sunflower [SHTIENBERG, 1994] and leaf spot disease in potato [TURKENSTEEN, 1979] and in cucumber [BUTLER *et al*., 1979]; conversely, *U. atrum* was used successfully as an antagonist of *B. cinerea* in cucumber [DIK & ELAD, 1999]. Furthermore, *U. atrum* was described by LINKE *et al*. [1992] as a bioherbicide against *Orobanche* spp. in beans, infecting and destroying tubercles and emerged shoots of the parasitic plant. Other *Ulocladium* species are mentioned as plant pathogenic: *U. chartarum* causing storage rot on apple and pawpaw [SHARMA, 1991], *U. consortiale* on cotton [SVIRIDOV *et al*., 1991] and on parsley seeds [NOWICKI, 1997] and *U. cucurbitae* causing leaf spots in cucumber [ZITTER & HSU, 1990]. In some cases, like in pine, *Ulocladium* spp. was only isolated from damping-off seedlings but was not pathogenic in tests according to Koch's postulates [LILJA *et al*., 1995]. This shows the generally saprophytic habit of *Ulocladium* spp., which can become pathogenic under certain circumstances and on some plant species. *Ulocladium* spp. has not been described as a grapevine pathogen so far. Under conditions, either highly suitable or unsuitable for the growth of *U. atrum*, the isolate Ua 385 did not cause any pathological symptoms in grapevine. Similar observations were made in experiments in onions, ornamentals, strawberries, tomatoes, wheat, or grapevine in France, conducted in studies of the EU-Project this thesis was integrated.

Another essential presupposition to introduce a biocontrol microorganism into general practice is its harmlessness for the non-target fauna, especially for mammals and man. Confirming results of KÖHL *et al*. [1999], conidia of *U. atrum* persisted for a short time at temperatures above 40 °C, and the fungus was neither able to grow nor to sporulate at temperatures above 36 °C. Aerobiological studies in warm climatic zones have shown that *U. atrum* is present in house dust samples, without any indication of being human-pathogenic, nonetheless, the fungus was correlated to produce allergens [AL FRAYH *et al*., 1988; KWAASI *et al*., 1998]. Furthermore, *Ulocladium* spp. was found in cutaneous infections of dogs with non-specific dermatitis, but the hyphomycet was not described as causing pathological symptoms [BAUMGÄRTNER & POSSELT, 1983]. Tests with rats have shown that *U. atrum* did not cause any symptoms after oral application of spores and the antagonist was classified as safe for handling [BERG, Bayer CropScience, Germany, pers. comm.]. *Ulocladium* spp. was

used in studies on toxigenic moulds and mycotoxins in feeds within an identification of isolates of moulds where many were able to produce toxic metabolites; however, *Ulocladium* spp. did not excrete toxins [BENKERROUM & TANTAOUI ELARAKI, 2001]. However, 5% of patients were tested positive in human skin prick test on extracts of *Ulocladium* spp. prepared from airborne isolates from Saudi Arabia; a possible role for this fungus as an allergen is therefore indicated and inclusion of *Ulocladium s*pp. antigens in allergy tests is recommended [HASNAIN *et al*., 1995].

Using antagonistic microorganisms cannot be seen isolated from other organisms in the phyllosphere. *U. atrum* applied as spore suspension has proved to persist on many plants and to be antagonistic to *B. cinerea* and to the naturally occurring saprophytes *Alternaria* spp. and *Cladosporium* spp. [ELMER & Köhl, 1998]. *Alternaria* spp. is described as an antagonist of *B. cinerea* in grapevine [MACHOWICZ-STEFANIAK, 1998; STEWART *et al*., 2000]. Capability of *U. atrum* of competing with commonly occurring saprophytic fungi in grapevine must therefore also be seen as a reduction in the antagonistic potential of an intact phyllosphere. Even if persistence of an introduced antagonist is wanted in the biological control, ecological balance should not be disturbed by organisms, which could be potentially damaging to the environment [LYNCH, 1992]. Survival of *U. atrum* on stems of grapevine was less than one colony-forming unit per cm^2 in the season after application. In the following season, density of *U. atrum* decreased to a fifth, and the antagonist could not be isolated from samples of new canes nor from inflorescences nor from berries. An epidemic spread of *U. atrum* on grapevine plants is therefore believed improbable. Nevertheless, ROUDET & DUBOS [2000] have shown that *U. atrum* hibernates on plant debris on the ground; ecological studies have therefore to be done to ensure that the antagonist is not damaging the ecosystem.

Another presumption to use pesticides for viniculture is its innocuousness for vinification, and with decreasing limits of detectability, consumers awareness is increasing; though for all grapevine pesticides tested, the time necessary to reach the concentration of recommended maximum residue limit was below their designated days to harvest time [NAVARRO *et al*., 2001]. Residues of fungicides such as folpet, iprodione, procymidone and vinclozolin can affect fermentation of grapevine musts [MLIKOTA *et al*., 1996]. Studies of FLORI & CARBAS [1990] showed a complete decomposition of folpet and other pesticides during fermentation; differences may be due to a diversity of yeasts used for vinification though residues of pesticides were measurable. Some pesticides such as fenhexamid did not cause problems

during vinification, nonetheless, they remained inert [CABRAS *et al*., 2001]; others stimulated yeasts to produce more alcohol [CABRAS *et al*., 1999], whereas wine from grapes treated with MycoSan® (clay mineral product, Andermatt Biocontrol AG, Swiss) smelled of bad eggs [VIVIANI-NAUER *et al*., 1995]. Microorganisms, antagonistic or pathogenic, are able to produce metabolites, which ensure their survival affecting other organisms. Grapevine pathogens such as *B. cinerea*, *Penicillium expansum* and *Trichothecium roseum* are capable of producing mycotoxins, and grapes moulded by *P. expansum* or *T. roseum* should not be used for vinification [ALTMAYER *et al*., 1985]. Laccase, an enzyme found in *B. cinerea* infected grapes, affects quality of wine and its degradation process [NAUDIN, 1989; DUBOS *et al*., 1996]. Must from grapes sprayed with Trichodex® fermented without any problems [TOPOLOVEC-PINTARIC *et al*., 1999]. *U. atrum* did not affect vinification, similar to results reported by ROUDET & DUBOS [2000]. Furthermore, no negative effect of *U. atrum* on gustatory quality or on ripening of wine was observed, which completes the prerequisites of a safe use of *U. atrum* as a biocontrol agent of grey mould in grapevine.

It is desirable for a biological agent to control many pathogens, however, biocontrol agents cannot solve all disease problems due to their specificity [TÖRMÄLÄ, 1995]. Other important fungal grapevine diseases occurring beside grey mould are downy mildew, powdery mildew, *Phomopsis* cane and leaf spot, *Penicillium* rot and *Rotbrenner*. Commercial biobotryticides as Serenade® (*Bacillus subtilis*, AgraQuest Inc., USA) are recommended for downy and powdery mildew in grapevine and various other diseases in many crops. Similar side-effects of yeasts used as biocontrol agents against grey mould were observed in grapes [LIMA *et al*., 1997].

Hitherto, *U. atrum* was described as an antagonist of *Botrytis* spp. [KÖHL *et al*., 1993] and *Septoria* spp. [LENNARTZ *et al*., 1998] and its competitive side-effect on *Alternaria* spp. [ELMER & KÖHL, 1998]. *A. alternata* is often associated with grapevine bunch rot [NAIR, 1985; DUNCAN *et al*., 1995] and it can cause problems in viniculture [FARETRA *et al*., 1986]. Nonetheless, side-effects of pesticides generally provide a sufficient control and negative effects of this fungus occur rarely; it might become a problem when applications of fungicides are reduced [SCHWENK *et al*., 1989; SCHIEFER & KAST, 2002]. The antagonistic potential of *U. atrum* against saprophytes is therefore appreciated in biological controlled viticulture.

In 1997, a side-effect of *U. atrum* on *Phomopsis* cane spots was observed. This disease occurs all over the vine growing areas and it is usually controlled by side-effects of chemical fungicides against downy mildew [EGGER, 1999]. An effect of *U. atrum* on *Phomopsis* cane

spots would therefore be beneficial in biological controlled viticulture. Nevertheless, disease severity was low and results have to be confirmed in experiments without using fungicides effective against *Phomopsis* cane spots. *U. atrum* showed no antagonistic effect against *U. necator*, neither during the growing season nor on pathogens hibernation and its outbreak in the following season,. Similarly, *U. atrum* did not affect the biotrophic pathogens of wheat *Blumeria graminis* and *Puccinia recondita* [LENNARTZ, 2001], due to the biology of this saprophytic antagonist. Hence, *U. atrum* will have to be applied with chemical pesticides to guarantee healthy development of the crop as it was postulated as a successful biocontrol strategy for *Trichoderma* spp. [ELAD *et al*., 1993; 1997].

Pesticides with a broad spectrum of pathogens also reduce number and variety of saprophytic microorganisms in the phyllosphere [MANGIAROTTI *et al*., 1987; FOKKEMA, 1990]. To date, there are only few highly specific fungicides, and side-effects against naturally occurring saprophytes are often observed [SCHIEFER & KAST, 2002]. Possibilities of integration of *U. atrum* into crop protection programmes with grapevine fungicides were therefore evaluated.

Low sensitivity of *U. atrum* to fungicides was observed for pesticides used in conventional control of grapevine pathogens. Similarly, *U. atrum* proved to be insensitive against most pesticides used in wheat [SCHOENE *et al*, 1998]. *In vitro*-tests on the sensitivity of *U. atrum* were carried out to provide a survey of pesticides which may be applied in combination with the antagonist under field conditions, knowing difficulties of transferring results to field condition as mentioned by FENN & COFFEY [1984]. The meaning of an EC_{50} value of pesticides must be seen differently in relation to their approved basic concentrations; e.g. the DMIs fenarimol or penconazole are used in the vineyard at a concentration 240-times lower than sulphur. Furthermore, they can penetrate into the plant whilst inorganic pesticides remain at a high concentration on the phylloplane the habitat of the epiphytic antagonist. Non-systemic pesticides remain on the plant surface for longer periods at high concentrations than systemic pesticides, and chemicals with a high vapour pressure are only temporarily active in the phyllosphere. Nonetheless, *in vitro*-tests on the pesticide sensitivity of *U. atrum* gave an overview of chemicals, which can be combined with the antagonist under field conditions.

The inorganic fungicides copper and sulphur and also propineb, fenhexamid, dimethomorph, dichlofluanid and even the combination of mancozeb with Al-fosetyl did not reduce mycelial growth of *U. atrum* at any concentration tested. Most EC_{50} values of the fungicides for

germination of the antagonist were above 50 ppm, only dithianon, a pesticide with a multi-site activity, and pyrimethanil with membrane activity, had an effect on spore germination and mycelial growth of the antagonist at concentrations lower than 10 ppm. Mycelial growth was affected at lower dosages by some of the pesticides tested than germination was, independently from their mode of action. On the other hand, EC_{50} values of fungicides with multi-mode activity were lower for germination than for the mycelial growth of *U. atrum*. The insecticide parathion-methyl is known to have a fungicidal activity [KEES & OBST, 1972; ROSS & BRADY, 1985] and affected germination and mycelial growth of *U. atrum*; *B. cinerea* was more sensitive to this insecticide than the antagonist was, however. All pesticides, except dithianon and pyrimethanil, were at least as effective against *B. cinerea* as against *U. atrum*. Under field conditions, side-effects of these pesticides on the antagonist can be expected to be as low as on *B. cinerea*. Therefore, it is feasible to combine these pesticides and *U. atrum* in the vineyard.

Some pesticides affect introduced antagonists under field conditions as fungicide sensitivity of *Trichoderma* spp. impeded biological control of *B. cinerea* in grapevine [MEZZALAMA *et al*., 1985]. This problem was solved by fungicide-resistant mutants, which fortunately showed antagonistic activity against *B. cinerea* on grapes under laboratory and field conditions similar to the isolates of origin [KAY & STEWARD, 1994]. If problems occur in integrating *U. atrum* in conventional crop protection, this option of a desensitisation will also exist for this antagonist. HARMAN *et al*. [1996] showed that biocontrol agents could be integrated in conventional control programmes of grey mould in grapevine either to replace or to reduce pesticide treatments as alternating or combined applications. Botryticide applications could be replaced by *U. atrum*, correspondingly to the model chosen by SHTIENBERG & ELAD [1997], who replaced applications of chemical pesticides by 50 %. Combined application will only be successful if a pesticide does not affect the antagonistic fungus that can colonise the vacated niche of the eliminated pathogen. A reduced fungicide concentration was used for the control of postharvest diseases in combination with saprophytic yeasts [DROBY *et al*., 1993; CHAND-GOYAL & SPOTTS, 1996]. However, using a reduced fungicide dose in the field can abet onset of resistance to active ingredients [WADE, 1988]; for that reason, a reduced dose of fungicides was not tested in combination with *U. atrum*. Combined applications of chemical fungicides with tolerant biocontrol agent can have twofold advantages: a high level of plant protection is

provided early by the chemical component and then the biological agent becomes active later in the season [HARMAN & BJÖRKMAN, 1998].

Options of combining *U. atrum* with botryticides were investigated under field conditions with special respect to the time botryticides had to be applied before *U. atrum* without affecting the antagonist. Applied simultaneously at the highest approved concentration, *U. atrum* was affected by almost all botryticides except fenhexamid the only botryticide a combination within one application can be advised unrestrictedly. After a period of maximum 14 days, none of the botryticides had an effect of more than 50 % on mycelial growth of applied spores, and three weeks after application of botryticides the antagonist was able to germinate and grow on the leaf surface without an effect of any botryticide.

Pyrimethanil, the botryticide with the strongest *in vitro* effect on *U. atrum*, had a long-lasting effect on its spore germination and mycelial growth in the vineyard. For dichlofluanid, a negative effect on *U. atrum* similar to pyrimethanil was observed under field condition though dichlofluanid did only have a moderate *in vitro* effect. The non-systemic activity and the high concentration recommended for application of this botryticide resulted in this effect *in vivo*. This demonstrates that it is difficult to transfer *in vitro* effects to the situation under field condition and that field experiments are indispensable for testing a successful integration of *U. atrum* in conventional crop protection.

Nevertheless, a positive effect of chemical *Botrytis* control combined with colonisation of the niche by the antagonist three weeks after treatment with the botryticide is doubtful, because of the fate of pesticides and pathogen's regeneration. These botryticides are therefore required for an alternating combination with *U. atrum*. Thus, fenhexamid and probably tolylfluanid and vinclozolin are candidates for a mixed application with the antagonist.

In addition, survival on grapevine bark was monitored during the field experiments to determine the effect of the pesticides on the antagonist under practical conditions. The antagonist was strongly reduced after using a combination of cymoxanil and dithianon, and after application in a tank mixture with spiroxamine and parathion-methyl. Negative effects of other pesticides on the persistence of the antagonist were not observed, which enables an application of *U. atrum* and its integration in conventional crop protection programmes.

Looking for reference values for fungicide sensitivity of fungi related to *U. atrum*, *U. cucurbitae*, a pathogen in cucumber, is affected by iprodione and other pesticides against *Alternaria* spp. [ZITTER & HSU, 1992]. A strong effect of folpet on germination and of

iprodione on mycelial growth of *Alternaria* spp. were described by VAKALOUNAKIS & MALATHRAKIS [1988] and dichlofluanid affected both germination and mycelial growth of *A. alternata*. However, *U. atrum* was insensitive to some commercial products controlling *Alternaria* spp. such as copper, mancozeb and Al-fosetyl. Other fungicides used for the control of *Alternaria* spp., e.g., iprodione, metiram, pyrimethanil and the combination of cyprodinil and fludioxonil also affected the antagonist. Therefore, notwithstanding the close relationship, fungicide sensitivities of these two fungi were not always comparable; even fungicides of one chemical group such as metiram and mancozeb, both dithiocarbamates, which affect *Alternaria* spp., differ in their activity against *U. atrum*. Hence, experiences concerning fungicide sensitivities of other fungi cannot be transferred to the antagonist.

Host-pathogen relationship must also be considered in biocontrol strategies. Susceptibility of grapevine to grey mould depends on the variety [JEANDET *et al*., 1992; HOLZ *et al*., 2000]. The most common white wine varieties were chosen with moderate susceptibilities to grey mould. *Vitis vinifera cv*. 'Müller-Thurgau' was used at Marienthal. This hybrid of 'Riesling' and 'Chasselas de Courtiller' is the most common white wine variety in Germany and is a high-yielding variety highly susceptible to frost, of moderately low acid content of must, having an early *véraison* and being moderately susceptible to *B. cinerea* [ANONYMOUS, 2000]. Susceptibility to frost assumed significance when late frosts at the end of May reduced plant vigour and resulted in a low number of leaves and bunches per plant. The early and short duration of *véraison* and, as a result, an early harvest of grapevine *cv*. 'Müller-Thurgau' required a spray programme with only three applications after the beginning of flowering, different from the other cultivars where four applications were needed to ensure a sufficient density of the antagonist. Like *Vitis vinifera cv*. 'Müller-Thurgau', cultivar 'Kerner' a hybrid of 'Trollinger' and 'Riesling' is described as a variety that has a moderately low acid content of must and a high yield, but its susceptibility to grey mould is moderately high and *véraison* is moderately late [ANONYMOUS, 2000]. The white wine cultivar 'Riesling' is described as a moderate to low-yield crop with a high acid content of must, and *véraison* of this cultivar is late in the season [ANONYMOUS, 2000]. Susceptibility of cultivar 'Riesling' to grey mould is therefore fairly low [VASUDEVAN *et al*., 1998].

All grapevine cultivars used in field experiments have their origin in *cv*. 'Riesling' and the main difference between them - in respect of the grey mould epidemics - is the delayed epidemic of grey mould and the ability of the cultivar 'Riesling' to be harvested late in the

season. Furthermore, cluster tightness has an effect on grey mould [VAIL & MAROIS, 1991], nonetheless, the three cultivars differ only very slightly in this characteristic, and its influence can therefore be neglected in these studies.

The epidemic spread of *B. cinerea* in grapevine is very important for timing applications of an antagonist. In field trials, symptoms of *B. cinerea* occurred on different parts of grapevine, such as leaves, tendrils, inflorescences and canes and most frequently on mature berries. These niches were taken into account determining the antagonistic potential of *U. atrum*. The antagonist was capable of colonising these niches except for the latent infected berries. ROUDET & DUBOS [2000] reported that *U. atrum* is able to penetrate juvenile berries through abscission wounds of inflorescences. In contrast to this study, the antagonist could not be re-isolated from surface sterilised berries taken from field experiments. When *B. cinerea* invaded a berry, the antagonist is therefore not capable of reducing incidence of latent infection. Nonetheless, severity of grey mould on berries and spore production of *B. cinerea* can be affected by *U. atrum*, which reduces risk of a secondary infection.

Sufficient density of viable conidia of the antagonist close to the pathogen is essential in competition of *U. atrum* with *B. cinerea* in strawberries [BOFF *et al.*, 2001]. Timing of application is important, because thinning of spore density and loss in their viability reduce activity of an antagonist. As a result of above-mentioned epidemiological studies on *B. cinerea* and co-determined by incidence of *B. cinerea* as airborne conidia or the presence on grapevine bark, *U. atrum* was applied before flowering, to dissimilate nutrients *B. cinerea* needs for flower infection. Another application was conducted after flowering to colonise floral debris. Before cluster-closure, the antagonist can be applied into clusters to decompose debris and exudates of the grapevine plant. Last application was conducted before *véraison*, because *U. atrum* proved to reduce grey mould by colonising wounds of mature berries. Despite different modes of action of the biocontrol agents, these application dates correspond with the manufacturer's recommendation for the biobotryticide Serenade® (*Bacillus subtilis*, AgraQuest Inc., USA), demonstrating necessity for *Botrytis* control at these stages.

An early application at bud burst was tested at Marienthal where plots of three rows enabled a measurement of the effect of *U. atrum* on the epidemic development of *B. cinerea* reducing edge effects, even though plots were too small for excluding these effects. In order to show an antagonistic effect of *U. atrum* on the epidemic spread of *B. cinerea* in the vineyard, plot size has to be sufficiently large and plots have to be isolated in order that conidia of *B. cinerea*

invading the plots are negligible. This is not practicable, however, because airborne spores of *B. cinerea* are almost omnipresent due to the viability and the reproductive potential of this pathogen [JARVIS, 1980; COERTZE *et al*., 2001]. Even if an effect of *U. atrum* on air-load of *B. cinerea* conidia was detected in 1997, an edge effect on the epidemic spread of *B. cinerea* in plots could be measured neither at Marienthal nor at Kirrweiler where four similarly treated rows were next to each other. Reduction of sporulation of *B. cinerea* therefore seems to be not the only effect of *U. atrum* on the epidemics of grey mould. Among other modes of action described by BERTO *et al*. [2000], competition for nutrients enables *U. atrum* to affect germination and infectivity of *B. cinerea* in the phyllosphere of grapevine.

Number of applications of *U. atrum* required to achieve a suitable control of *B. cinerea* is crucial for the economic success of biocontrol agents. *U. atrum* proved to persist on necrotic and healthy leaves of onion and lily [KÖHL *et al*., 1995a; c] under field conditions and could be re-isolated from the bark, inflorescences and berries of grapevine, but fate of the antagonist required re-applications. Sole applications of *U. atrum* could not reduce significantly incidence of *B. cinerea*. Therefore, importance of each treatment could not be shown. Only the total of all antagonistic effects, reduction of primary inoculum, dissimilation of nutrients *B. cinerea* needs for flower infection, colonisation of floral debris and reduction of sporulation on mature berries affected the epidemics of grey mould efficiently.

Compared to treatments with two or three applications, grey mould infection was better reduced by four applications of *U. atrum*. In seasons with short times of flowering and *véraison* as in 1998, the treatment after flowering could be pooled with the application at cluster closure without losing efficacy, but normally only two applications before and after flowering guarantee sufficient density of the antagonist. Furthermore, additional applications of *U. atrum* could increase its population density and may enhance its antagonistic success. Nevertheless, an economic benefit is doubtful since disease rate of grey mould was already similar to the success of chemical botryticides and below damage threshold of 2-20 % disease severity, depending on the grapevine variety.

Application frequency of *U. atrum* was moderate low compared to the number of treatments of other biocontrol agents against grey mould. Compost extracts had to be applied every 14 days [KETTERER *et al*., 1992] and manufacturer's recommendation advice applying Trichodex® (*T. harzianum*, Makhteshim Chemical Works Ltd, Israel) six times, and Serenade® (*Bacillus subtilis*, AgraQuest Inc., USA) and the plant defence elicitor Elexa®

(Chitosan, GlycoGenesys Inc., USA) has to be sprayed four times a season. Nonetheless, biocontrol agents have to be applied more often than chemical botryticides, and their use as tank mixtures is often restricted. Biocontrol agents therefore imply a higher amount of work and costs, which will have to be reduced if biocontrol agents are to persist on the market among other pesticides [COPPING & MENN, 2000].

Sustained phytosanitary effects are important for crop protection in perennial crops. Sclerotia or plant material infected by *B. cinerea* represents an important source of primary inoculum in grapevine [NAIR & NADTOTCHEI, 1987]. Degree of infestation depends on the variety, provenance and weather conditions [GARIBALDI *et al.*, 1981]. Carry-over infection from the previous season increased flower infections, whilst a satisfactory disease control in one year retards disease development in the following season [NAIR *et al.*, 1995]. This phytosanitary effect can be achieved by chemical control eliminating the pathogen; nonetheless, due to restrictions of chemical pesticides active ingredients have to be disappeared after a certain time. In contrast, biological control organisms are able to persist in the phyllosphere, and they can therefore affect the epidemic development pathogens for a longer period.

The long-term effects of crop protection programmes for the control of grey mould were shown in 1998 at Marienthal, where *U. atrum* applied in 1997 resulted in a reduction of *B. cinerea* on the bark and of flower and berry infection in 1998, irrespectively of treatments in 1998. In plots without an application of *U. atrum* in 1998, the antagonist was rarely found on inflorescences. Direct competition on flowers was therefore negligible and the long-term effect resulted from a phytosanitary effect of *U. atrum*, affecting hibernation of *B. cinerea*. This antagonistic effect of suppressing mycelial growth and sporulation of the pathogen was also shown *in vitro*. Similarly, ROUDET & DUBOS [2000] reported that *U. atrum* affects hibernating sclerotia and sub-epidermal mycelium of *B. cinerea* on shoots of grapevine in the Bordeaux region. *U. atrum* was therefore capable of reducing sporulation of *B. cinerea*, which may initiate primary infections, and consecutively the antagonist affected the epidemic development of grey mould.

Another conidial inoculum originates from grey mould infected mature berries. For detailed examination of air-loads of *B. cinerea* spores at the end of the season, density of airborne spores was measured within the plots. Rotorods® were used at berry level within the crop to minimise horizontal differences in air load between spore traps and the target [AYLOR & QIU, 1996]. In 1997, four applications of *U. atrum* or botryticides reduced the number of airborne

B. cinerea spores. Due to their eradicative effect the efficacy of botryticides was highest until early October and declined at the end of the season resulting in a considerable increase in the spore load also in these plots. Spore catches corresponded with the intensity of grey mould in 1997. Though Rotorods® are suitable for measurements of airborne fungal spores under different climatic conditions [EDMONDS, 1972], frequent rainfalls impaired measurements and wind speeds over 2 $m \cdot s^{-1}$ spread *B. cinerea* spores all over the plots in 1998 and 1999. Despite visible differences in severity of grey mould within plots, differences in air load of *B. cinerea* were not detected between treatments in these seasons. A multiplying effect, reducing air-load of *B. cinerea* inside the experimental plots, was not measurable and remains hypothetical. These results demonstrated that plots have to be larger to prevent incursion of *B. cinerea* conidia; the effect of *U. atrum* on the epidemic development of *B. cinerea* could thus be increased and biological control of the pathogen by suppression of spore production could be more successful, if the antagonist is used in large-scale trials or even in a total vineyard.

Yield parameters such as weight or acid and sugar content are as important for the value of grapes as their health status. Due to moderate disease rates of grey mould, neither applications of botryticides nor *U. atrum* affected yield parameters of sugar and acid content. Only grapes were harvested fulfilling the health requirement of a standard vintage, yield quantity was therefore affected in almost the same percentage as the severity of grey mould. Nonetheless, severity of grey mould also determines the date of picking the grapes; more healthy grapes could therefore be harvested later allowing them to accumulate sugars additionally. This advantage of a healthy crop was not utilised, because all plots were harvested at the same time. Nevertheless, this positive effect of *U. atrum* on yield parameters may be possible.

Hitherto, the price of *U. atrum* applied at the concentration of $2 \cdot 10^9$ spores/l and 600 l/ha would be still a multiple of adequate chemical botryticides [RAVENSBERG, Koppert, The Netherlands, pers. comm.], which cost 50-110 €/ha per application [ANONYMOUS, 2001b]. Hence, a sufficient antagonistic presence without leaving out economic considerations must be ensured. In 1998 only three dates of application were conducted at the double concentration to test a work ease, but in 1999 the antagonist was applied four times to ensure incidence of the antagonist on all berries. Furthermore, dates of application were adapted in 1999 to the other field experiments in order to standardise the application method, and the experiment resulted in an antagonistic success of *U. atrum* like in the years before. Advanced ecological

fitness of the antagonist in the vineyard and its efficiency at lower concentrations could help to develop an economically profitable biobotryticide.

Besides their costliness, problems occurring in production and storage of biocontrol agents are reasons why biocontrol is often less accepted on the market than chemical pesticides [REINECKE, 1990]. Formulations and physiological manipulation of fungal growth conditions improved ecological fitness of *U. atrum* for field applications, and enhanced the efficiency of inoculum production [FREY & MAGAN, 1998a; 2001]. Before *U. atrum* can be commercialised methods have to be developed to prepare a biocontrol agent that can be stored and easily used by the farmer. According to LENNARTZ [2001], *U. atrum* can be frozen and defrosted or dried and rehydrated several times without losing viability. This would simplify using the *U. atrum* as a biocontrol agent compared to an application of freshly made spore suspensions.

Effective *Botrytis* control was achieved by *U. atrum in vitro*, and the antagonist was used under practical conditions integrated in conventional crop protection as a biocontrol agent. Field experiments showed that only the sum of all four *U. atrum* treatments ensured a suitable density of the antagonist and a sufficient efficacy of grey mould control. Using tank mixtures with other pesticides at Kirrweiler affected the antagonistic potential of *U. atrum* showing problems that may arise from integration of the antagonist in conventional crop protection. Furthermore, extremely high disease pressure, due to the very poor condition of skin of the berries, lead to an explosive disease development and no significant control effect of *U. atrum* was found in grapevine; using a chemical botryticide was therefore inevitable to guarantee yield quantity and quality [ROUDET & DUBOS, 2000]. Under these conditions, a balanced fertilisation and pruning might enrich the biocontrol strategy for controlling grey mould efficiently [ENGLISH *et al*., 1993; WOLF *et al*., 1997; R'HOUMA *et al*., 1998].

Additive effects of chemical and biological control of grey mould were not observed, though the antagonist was not affected by the botryticide. Nonetheless, both chemical and biological control reduced grey mould below damage threshold. A combination of *U. atrum* with curative botryticides should therefore be envisioned for seasons highly suitable for grey mould. However, botryticides could be replaced by *U. atrum*; corresponding to the model chosen by SHTIENBERG & ELAD [1997], pesticides were reduced by 50 %. Nonetheless, improvements of the biocontrol with *U. atrum* are feasible in inoculum production, formulation and application.

Cultivation temperature of microorganisms can influence optimum temperature of their growth, and physiological manipulation of biocontrol agents can improve its tolerance to adverse environmental conditions [TEIXIDÓ *et al*., 1998]. The antagonist was cultivated on oat kernels at 20 °C to give a suitable spore yield [FREY & MAGAN, 1998b]. Temperatures in German vineyards are often below 20 °C; antagonists highly adapted to these conditions or tolerating at least the same temperature range as the pathogen are therefore expected to be a superior antagonist. Otherwise, the antagonist may be active for only a short time each year as reported for *Trichoderma viride* used as an antagonist in forest soils [INTINI, 1979]; this restricted use would not be acceptable for a biocontrol agent. *Gliocladium roseum* lost its efficacy against *B. cinerea* in cyclamen leaves below 20 °C, though it was highly effective at higher temperatures, whilst *U. atrum* suppressed sporulation of the pathogen at all tested temperatures and proved to be a eurythermic antagonist corresponding to results reported by KÖHL *et al*. [1999; 2000]. It could be advantageous to cultivate *U. atrum* at a lower temperature to have an antagonist with the same optimum temperature as the pathogen, even if cultivation is not as profitable as at its optimum temperature.

The wetting agent Tween® 20 was used to facilitate an even dispersion of the hydrophobic spores, and it allowed a suitable survival rate of *U. atrum*, though other additives have to be tested to improve the survival and fitness of the antagonist. Improving formulations used for application of the antagonistic fungi might diminish problems occurring with climatic conditions. By using additives like oils, spore-dispersion of the antagonistic hyperparasite *Ampelomyces quisqualis* and adhesion to plant surface can be enhanced [PHILIPP *et al*., 1984]; nonetheless, protection from negative environmental factors under field condition is still negligible [PHILIPP *et al*., 1990]. Additives such as detergents or oil derivatives used at high concentrations can affect vitality of *U. atrum* [LENNARTZ, 2001]. In that case, additives and dead spores or hyphae of the antagonist represent an additional nutrient source for saprophytic pathogens, and diseases might be even intensified [DANDURAND & KNUDSEN, 1994].

Additives such as Ca^{2+} or Mg^{2+} ions can also improve survival and fitness of antagonists and their antagonistic potential against *B. cinerea* [WISNIEWSKI *et al*., 1995]. Especially hyperparasitic antagonist utilise lytic enzymes such as chitinases, lipases, cellobiase, pectate lyase or β-1,3-glucanase [LORITO *et al*., 1996; JIJAKLI & LEPOIVRE, 1998]. The role of these cell wall-degrading enzymes in the colonisation of necrotic strawberry leaves by *U. atrum* and the ability to control *B. cinerea* was studied by BERTO *et al*. [2000]. They suggested that these

enzymes might play a complementary role in antagonism of *U. atrum* against *B. cinerea*. Agents were found stimulating the antagonist synthesising lytic enzymes and the antagonistic potential of *U. atrum* could be improved by application of these agents [BERTO *et al*., 2001].

As another class of additives, nutrient sources not digestible by the pathogen like the amino acids L-asparagine and L-proline or a nutrient analogous like the carbohydrate 2-deoxy-D-glucose proved to enhance survival and fitness of antagonists and therefore biological control of *B. cinerea* [JANISIEWICZ *et al*., 1992; 1994]. These additives might also have a beneficial effect on *U. atrum*. However, the antagonist has to be promoted without being overstrained and exhausted too fast, otherwise the long-lasting effect of the microorganism, one advantage of biocontrol agents, is vitiated. Furthermore, price of a biocontrol product also determines market acceptance and economic profit; the amount and costs for formulation therefore have to be as low as possible [LEWIS & PAPAVIZAS, 1991].

Combinations of biological control agents reduce the probability of control failure and could increase reliability of biological control of *B. cinerea* [JANISIEWICZ, 1988; GUETSKY *et al*., 2001]. Single bacterial or fungal antagonists, combinations of two antagonists, natural compounds and antagonists or even compost extracts were used successfully against grey mould in grapevine. Nevertheless, enhancement of antagonistic potential will only be achieved if biocontrol agents enhance each other and complete their modes of action [FALCONI & MENDGEN, 1994].

Combination of *U. atrum* and plant extracts could enrich biological options to control *B. cinerea*, as some natural compounds proved efficacy against *B. cinerea* [ESTERIO, 1992; HAMILTON KEMP *et al*., 1992; SAKS & BARKAI-GOLAN, 1995; MEKURIA *et al*., 1998; SEDDON & SCHMITT, 1998; WALTER *et al*., 2001]. Nevertheless, the combination of the antagonist and pesticides or additives has to be synergistic; a combination with an antifungal compound like the extract of *Ginkgo biloba* seems to be unsuitable since it also affects *Ulocladium* spp. [MOUREY *et al*., 1985]. Other natural compounds affecting *Alternaria* spp. such as Trilogy® (Neem oil, Thermo Trilogy, USA) or extracts from *Aloe vera* [SAKS & BARKAI-GOLAN, 1995] or *Bryophytes* [MEKURIA *et al*., 1998] must be chosen carefully. A mixture of *U. atrum* with the antagonistic fungus *Gliocladium catenulatum* was shown to enhance biocontrol [Köhl *et al*., 1995a]. LENNARTZ [2001] showed that the inhibitory effect of *Chaetomium cochliodes* on *U. atrum* resulted in a non-synergistic effect of a combination of these fungi, however. A mixture with the biobotryticide Serenade® (*Bacillus subtilis*, AgraQuest Inc., USA) has to

be checked carefully, because Serenade® is recommended against the pathogenic genus *Alternaria* closely related to *U. atrum*. Even so, alternating combination of antagonists could also complete their modes of action and this method is therefore more promising in enhancing biological control options.

Other biological control microorganisms like *Trichoderma* spp. had to 'climb many fences' in the last twenty years such as mass-production, formulation, desensitisation against pesticides, combination with additives or pesticides, integration into crop protection measures and forecasting systems and registration to end up in a marketable biological product. Almost ten years ago, *U. atrum* isolate Ua 385 was first described as a biocontrol agent against *Botrytis* spp. and proved its effectiveness in many field experiments [KÖHL, 2000]. This project was one step to develop a biological control agent.

With advances, more questions arise, and problems need to be solved, however. Many advances have been made in biocontrol; nonetheless, microbial antagonists are not yet fully accepted. Beside their price, biocontrol agents have to contend with problems occurring in production, formulation, storage and their unconvincing efficacy [REINECKE, 1990; COPPING & MENN, 2000]. Pollution of the environment and residues of pesticides make consumers desire biologically produced food, but it is often too expensive and quality standards cannot be achieved sometimes. *U. atrum* isolate Ua 385 proved its antagonistic potential in grapevine, it was integrated into conventional crop protection measures successfully and did not show any negative effects on non target organisms. With improvements in production and formulation, economical profit of a biocontrol agent based on this antagonist can be achieved.

Biological control should not only be seen as a replacement of chemical control, although *U. atrum* could also be combined with other biological and organic crop protection measures to complete the spectrum of biological disease management. An integrated approach with chemical control should be furthered to enrich variety of control methods, however, and to delay or prevent selection of pesticide resistant pathogens, to back pesticides that are safe for beneficials, to reduce pesticide input into the environment and into the food and, last but not least, to support biological control. These advances will be mutually beneficial for both control methods and they will be advantageous for farmers and consumers. Therefore, biocontrol is a powerful option and should be utilised over a much larger area than at present.

5 Summary

Grapevine, *Vitis vinifera* L., is cultivated as an extended crop at many places in temperate regions. As with all intensively cultivated crops, plant diseases impede growing grapevine and viticulture without any crop protection is improbable. *B. cinerea* appears in many field and greenhouse crops and occurs pathogenic as grey mould in grapevine. The pathogen is capable of infecting leaves, stems, flowers and fruits, and causes economically significant losses in the production phase as well as in storage. A combination of balanced fertilisation, pruning and fungicide treatment is important for controlling grey mould efficiently.

The saprophytic hyphomycet *U. atrum* degrades various organic materials, and its antagonistic potential against *Botrytis* spp. was described in some crops. The antagonistic potential of *U. atrum* was evaluated in the EU-project, 'biological control of airborne necrotrophic plant pathogens by suppression of spore production'. The aim of the research described in this thesis was to study ecology of *U. atrum* and its antagonistic potential against *B. cinerea* under controlled conditions and in field experiments in grapevine, with special regard to integration into existing crop protection programmes. This knowledge can help to simplify application of *U. atrum* and to optimise a strategy of biological control of grey mould with the antagonist in grapevine.

1. *U. atrum* was capable of colonising necrotic grapevine tissue, one prerequisite of a successful use as a saprophytic competitor. Furthermore, *U. atrum* colonised the surfaces of inflorescences and berries of grapevine and entered ecological niches of *B. cinerea*.
2. Drought tolerance was a major criterion in selecting *U. atrum* as an antagonist of *Botrytis* spp., although maximum number of spores was produced on grapevine leaves at high water potentials. In contrast to *B. cinerea*, *U. atrum* was able to grow and sporulate at relative humidities lower than 76 %; this might be advantageous to the antagonist in colonising necrotic tissue under field conditions.
3. Senescent plant material is the major nutrient source of *B. cinerea*, and is utilised by the necrotrophic pathogen to grow, to form mycelium and conidia, and to start epidemic spread. Co-inoculation with *U. atrum* reduced spore production of *B. cinerea* growing on various necrotic plant materials and halved sporulation of the pathogen on necrotic grapevine leaves. Spore production of *B. cinerea* was reduced on mature berries and/or sclerotia, and flower infection leading to latent infection of berries was affected by *U. atrum*. Hence, the antagonist is capable of affecting the epidemic spread of *B. cinerea* in grapevine.

4. For integration of *U. atrum* into existing crop protection programmes the sensitivity of the antagonist to pesticides was investigated. Under controlled conditions and in field experiments, *U. atrum* proved to be insensitive to most pesticides used in grapevine. This means that an integration of *U. atrum* into crop protection measures is feasible in the vineyard, and it enables a *Botrytis*-control with *U. atrum* combined with chemical crop protection programmes.
5. Applications of a biocontrol agent and a chemical pesticide often differ in the technical parameters required. In investigations on the optimisation of application technique, spores of *U. atrum* tolerated a spray pressure of 2 to 6 bar, whilst an application pressure of 2 bar was required for an even spread of the suspension, and the viability of spores of *U. atrum* showed a high germination rate up to a pressure of 5 bar. Pressure of 4 bar provided an evenly spreading of conidia with a high survival rate and the opportunity applying *U. atrum* like a chemical pesticide with conventional sprayers.
6. Safety to non-target organisms, especially crop plants, is another requirement for an antagonist. A potential effect of the antagonist on the plant was tested, because *Ulocladium* species were described as plant pathogens. Under all conditions, the isolate Ua 385 did not cause disease symptoms on leaves, inflorescences or fruits of grapevine.
7. Biocontrol microorganisms have to be harmless to non-target animals, mammals and man. Though spores of *U. atrum* persisted for a short time at temperatures above 40 °C, the fungus was not capable of growing or sporulating above 36 °C. This is important for practical use of a microbial antagonist not to be a potential mammal pathogen.
8. *U. atrum* was applied at the concentration of $2 \cdot 10^{9}$ spores/l and 600 l/ha used for field experiments would be still a multiple of adequate chemical botryticides. In 1998 only three dates of application were conducted to test work ease, but in 1999 the antagonist was applied four times to ensure a sufficient incidence of the antagonist on all berries. Advances in shelf life of the antagonist in the vineyard and its efficiency at lower concentrations could help to develop an economically profitable biocontrol agent.
9. As a result of various epidemiological studies on *B. cinerea* from literature and co-determined by incidence of *B. cinerea* as airborne conidia and its presence on grapevine bark, *U. atrum* should be applied before flowering to dissimilate nutrients the pathogen needs for flower infection, after flowering to colonise floral debris, before cluster closure to decompose debris and exudates inside clusters and before *véraison*, because *U. atrum* proved to reduce spore production of *B. cinerea* by colonising wounds of mature berries. Furthermore, an application at bud burst reduced primary inoculum of *B. cinerea*.
10. Sustained phytosanitary effect is important for crop protection in perennial crops. Sclerotia or plant material colonised by *B. cinerea* represent important sources of

primary inoculum in grapevine. The long-term effect of *U. atrum* against grey mould was shown in 1998 at Marienthal; the presence of *B. cinerea* on the bark and both, flower and berry infection were reduced by application of *U. atrum* in the previous season. In plots without an application of the antagonist in 1998, the antagonist was rarely found on inflorescences. Therefore direct competition on flowers can be neglected and long-term effects can be seen as a phytosanitary effect of *U. atrum*, affecting hibernation of *B. cinerea* by suppressing growth and sporulation of the pathogen as proved *in vitro*.

11. Multiplying microorganisms or their enzymes were found to affect vinification, quality of wine and its degradation process. The effect of antagonists on vinification was therefore investigated. Must from grapes sprayed with *U. atrum* fermented without problems. A negative effect of the antagonist on gustatory quality or on ripening of wine was not observed, which completes the prerequisites of a successful use of *U. atrum* as a biobotryticide in grapevine.
12. Effective *Botrytis* control was achieved by *U. atrum in vitro*, and the antagonist was integrated in conventional crop protection under practical conditions. Applied three or four times, separate from the general spray programme, the antagonist was as efficient as chemical botryticides and proved its antagonistic potential in the vineyard. *U. atrum* affected hibernation of *B. cinerea*, suppressing growth and sporulation of the pathogen on the bark, which represents a niche for *B. cinerea* as a source of inoculum. Both, under low disease pressure and at moderately high infection rates, the antagonist was capable of reducing incidence and severity of flower infections, latent infection and grey mould, as well as air load of *B. cinerea*.

The investigations demonstrated that *U. atrum*, which has been selected for biological control of *Botrytis* spp., has a great potential as a biocontrol agent in grapevine. The antagonist proved its effectiveness for biological crop protection via competition for nutrients and/or space. In seasons with low to moderately high disease pressure, *U. atrum* reduced grey mould alike chemical botryticide treatments. Nevertheless, at a high disease pressure of grey mould, combination of chemical control and biological control measures should be envisioned. The prospect for integrating *U. atrum* as a biocontrol agent into chemical crop protection programmes for the control of *B. cinerea* and possibilities of enhancement are discussed.

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Curriculum vitae

Name	**Philip Schoene**
	* Dec. 05 1967, in Paderborn, Germany
	Dec. 23 1997, married to Judith Schoene, née Strathmann
	July 28 2001, 1st child, Anna Julie Schoene
1974 – 1987	**Primary and Secondary school**, Paderborn
	Final German Secondary school examination
1988 – 1989	**Civil Service**
1989 – 1990	**Diploma studies in Chemistry**
	Rheinische Friedrich-Wilhelms-University of Bonn,
1990 – 1996	**Diploma studies in Biology,**
	Johannes Gutenberg University of Mayency and
	University of Constance
	Diploma thesis:
	University of Constance, Department for Plant Pathology
	'Erhöhung der Wirkung von Mischungen pilzlicher und bakterieller Antagonisten in der Biologischen Bekämpfung von *Penicillium expansum*'
1997 – 2002	**PhD studies**:
	Rheinische Friedrich-Wilhelms-University of Bonn,
	Institute of Plant Diseases, Department for Plant Diseases
	Scientific assistant (February 1997 – May 2000)
2000	Novartis Agro GmbH, Frankfort on the Main
	Developer of herbicides in rape (June 2000 – September 2000)