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Investigations on the effect of entomopathogenic fungi on whiteflies

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TO MY PARENTS

WHO ALWAYS TAKE ME FOR WHAT I AM

AND NEVER ASK TOO MUCH OF ME

BUT NOT TOO LITTLE EITHER

THANK YOU!!!

Anke Skrobek

Investigations on the effect of entomopathogenic fungi on whiteflies

The entomopathogenic fungus *Paecilomyces fumosoroseus* is well-known for its broad host-spectrum and efficacy against many kinds of insects. Although its effectiveness against soil-borne arthropods has already been described over 100 years ago, *Metarhizium anisopliae* has only recently been cited as pathogenic to homopteran pests. In the present study different isolates of both microbial control agents were evaluated for their potential to control the whitefly species *Trialeurodes vaporariorum* and *Bemisia argentifolii*. Whiteflies are one of the most important arthropod pests of greenhouse and field crops, *B. argentifolii* occurring mostly in tropical and subtropical climates and *T. vaporariorum* being the predominant species in Northern Europe. Many isolates of *P. fumosoroseus* and *M. anisopliae*, originating in different climatic regions, were found to be effective against all larval stages of both whitefly species.

For the integration of this biological control agent into crop protection systems, a possible synergism between the entomopathogens and insecticides from the group of the chitin synthesis inhibitors was investigated. Although fungi and insecticide acted synergistically when applied with a time interval, control of whiteflies was not sufficient for an effective, practical pest control. In contrast, corresponding investigations on *Spodoptera littoralis*, the Egyptian cotton leafworm, gave high mortality levels and thus offered good prospects for reducing the pesticide input.

Different oils, waxes and polymeric additives were examined for their ability to enhance the efficacy of the biological control agents. Two polymeric additives were found to increase the shelf-life at room temperature of conidia of *M. anisopliae* when conidia were dried within the formulation. Microscopical studies using fluorescence, low temperature scanning electron and confocal laser scanning microscope indicated positive effects of Addit ® (Koppert, Netherlands) and a polymeric additive on the distribution of formulations – and hence conidia- on leaf and insect surfaces. Spore germination, speed of kill and mortality of the target insect were also found to be enhanced. Both additives offered good prospects for optimising the efficacy of entomopathogenic fungi against *B. argentifolii* and *T. vaporariorum*, thus indicating high potential for the integration in the framework of an IPM strategy.

Kurzfassung

Anke Skrobek

Untersuchungen zur Wirkung von entomopathogenen Pilzen auf die Weiße Fliege

Der entomopathogene Pilz *Paecilomyces fumosoroseus* ist bekannt für sein breites Wirtsspektrum und seine Wirksamkeit gegen verschiedene Insektenarten. Die Pathogenität von *Metarhizium anisopliae* gegen pflanzensaugende Insekten ist erst kürzlich bekannt geworden, obwohl seine Wirksamkeit gegen bodenbürtige Insekten bereits vor über 100 Jahren beschrieben wurde. In der vorliegenden Arbeit wurde das Potential verschiedener Isolate beider Pilze zur Kontrolle der Weißen Fliegen *Bemisia argentifolii* und *Trialeurodes vaporariorum* untersucht. Weiße Fliegen zählen weltweit zu den bedeutendsten Schädlingen, dabei tritt *B. argentifolii* hauptsächlich in tropischen und subtropischen Gebieten auf, während *T. vaporariorum* die dominierende Art in Nord Europa ist. Einige Isolate von *P. fumosoroseus* und *M. anisopliae* aus unterschiedlichen klimatischen Regionen zeigten hohe Wirksamkeit gegen alle Larvenstadien beider Insektenarten.

Ein potentieller Synergismus zwischen den Insektenpathogenen und Insektiziden aus der Gruppe der Chitin Synthesehemmer wurde untersucht, um die biologische Bekämpfungsmaßnahme in bestehende Pflanzenschutzsysteme zu integrieren. Obwohl bei einer zeitversetzten Applikation synergistische Effekte beobachtet wurden, war die Mortalität von *T. vaporariorum* zu niedrig für eine ausreichende Kontrolle des Insekts. Im Gegensatz dazu zeigten vergleichbare Versuche mit *Spodoptera littoralis*, dem Ägyptischen Baumwollkapselwurm, eine Steigerung in der Mortalität und somit eine gute Möglichkeit zur Reduzierung des Pestizideinsatzes.

Das Potential von verschiedenen Ölen und Polymerzusätzen, die Wirksamkeit der Antagonisten zu steigern, wurde untersucht. Zwei Polymere konnten die Lagerfähigkeit von *M. anisopliae* bei Raumtemperatur signifikant erhöhen. Mikroskopische Untersuchungen mittels Fluoreszenz, Raster Elektronen und Konfokaler Laser Scan Mikroskopie zeigten positive Effekte des Ölpräparates Addit ® und eines Polymers auf die Verteilung von Formulierung und Konidien auf Blättern und Insekten. Sporenkeimung, Geschwindigkeit der Pathogenese und Mortalität der Zielinsekten wurden ebenfalls erhöht. Beide Additive steigerten die Wirksamkeit entomopathogener Pilze gegen die Weiße Fliege und bieten somit die Möglichkeit einer Integration der biologischen Schädlingsbekämpfung in ein IPM-Programm unter Gewächshausbedingungen.

PREFACE

This project was carried out as a collaboration between the Agricultural Research Organisation, The Volcani Center, Bet Dagan, Israel, and the Institute for Plant Diseases, University of Bonn in Germany. Additionally, some parts were done in the School of Biological Sciences, University of Wales, Swansea, UK. Many people have supported me during my work but without the help of some special persons this project would have not been possible. I am most grateful and would like to thank:

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CONTENTS

1 Introduction

Whiteflies are minute, usually inconspicuous and one of the most important arthropod pest of greenhouse and field crops (OSBORNE & GERLING, 1992). Whitefly populations have drastically increased throughout the world since the 1970s, attacking previously uninfested plant species and becoming acclimatised to new environments (BROWN, 1994). The cause of this increase is unknown but it may be due to the extended use of synthetic organic insecticides and subsequent augmented resistance to pesticides, changing climatic conditions and international movement of plant materials in the nursery and horticultural trade (DUFFUS, 1994).

Their widespread distribution is attributed to their exceptionally wide host range and short generation time. Infestations can lead to severe economic losses as a result of crop yield reductions, which can arise from a number of whitefly activities (BECKER *et al.*, 1992). For example, direct damage occurs due to sucking plant sap from the phloem and in very heavy infestations leaves drop, maturing of fruits is prohibited and the plant dies. Also, the excretion of ample amounts of honeydew encourages the growth of sooty mould on leaves, thus inhibiting photosynthesis, and can foul crops like cotton, making them difficult to process or unsaleable. Additionally, the insects transmit about 20 important plant pathogenic viruses. According to WISLER *et al.* (1998) losses in many important crops have been reported due to whitefly-transmitted geminiviruses, including tomato yellow leaf curl virus (TYLCV) and bean golden mosaic virus (BGMV). Although the majority of diseases associated with whiteflies are caused by geminiviruses, which are transmitted by *Bemisia tabaci*, the sweetpotato whitefly, many other groups of viruses have whitefly vectors (DUFFUS, 1987). TYLCV, a closterovirus, has been cited as the most destructive and perhaps the most widespread tomato virus worldwide. The beet pseudo-yellows virus (BPYV), also a closterovirus, is only transmitted by *Trialeurodes vaporariorum*, the greenhouse whitefly, and has a broad host range of crops, weeds and ornamentals. Detailed listings are given by BYRNE *et al.* (1990).

Whiteflies, which belong to the order Homoptera and the family Aleyrodidae, are extremely polyphagous, feeding on a number of agricultural crops, amongst them cotton, tobacco, vegetables and ornamentals. Only a few of the many whitefly species found

are economically important. Six species out of the twenty found on citrus are pestiferous and only four species are serious pests row and glasshouse crops. The dominant whitefly species in Northern Europe and in colder climates is the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), which is mainly attacking greenhouse crops. In tropical and subtropical areas the silverleaf whitefly, *Bemisia argentifolii* (Bellows & Perring), former known as *B. tabaci*, strain B, and proposed as a new species by BELLOWS *et al.* (1994), is feeding on field and glasshouse crops. However, *B. tabaci* seems to have recently extended its range to 45 °N in France and onto greenhouse crops as far as Sweden (BINK-MOENEN & MOUND, 1990). Therefore, not only the control of *T. vaporariorum* but also of *B. tabaci* or *B. argentifolii* is of great importance in Northern Europe.

Details on the whitefly life cycle are cited from BYRNE & BELLOWS (1991) and GILL (1990). Whiteflies are able to reproduce all year round and can have a generation time of 28 days for *T. vaporariorum* or as short as 18 days for *B. argentifolii*, depending on environmental conditions. Adult longevity is about 10 to 22 days and fecundity is high at about 30 to 300 eggs per female. Eggs are attached by a stalk to the undersides of leaves where they are usually clustered in groups. Egg hatching takes place after eight to twelve days depending on environmental conditions. The insect goes through four larval instars, which are sedentary with the exception of the crawler (1st stage). The 1st instar moves a short distance away from the egg, usually on the same leaf, before it settles and starts feeding on a suitable minor leaf vein. Mouthparts are only removed during moulting in the transition to the next developmental stage. The 4th instar develops distinct red eyespots and is often referred to as pupal stage. When the adult whitefly emerges from the pupa it leaves a distinctive T-shaped split in the pupal case. All stages of the insect are covered in powdery white wax particles that differ in morphology as well as in chemical and physical properties depending on the species (NELSON *et al.*, 2000).

Despite important advances in the biology of the insect, only limited control measures are available. Plant protection measures with frequent applications of pesticides to protect valuable crops are typical of intensive agriculture. They cause a high selection pressure on the pests controlled which represents the most important factor contributing to resistance development (GEORGHIOU, 1983). The high population densities and short

generation times of the Aleyrodidae are responsible for rapid development of strains resistant to synthetic insecticides (BECKER *et al.*, 1992). Furthermore, resistant strains were found to produce up to more than three times as many eggs as the original strain (COCK, 1986). Consequently the need of biological alternatives has increased in the last years.

The natural enemies of whiteflies include a very wide range of predators, parasitoids and pathogens, so that alternatives to chemical control do exist (FRANSEN, 1990). Comprehensive listings on the former are given by COCK (1986, 1993) and in OSBORNE & GERLING (1992). Some, like the parasitoid *Encarsia formosa,* have been used successfully for biological control. However, introducing predators and parasitoids into new areas may involve a number of complex problems (ONILLON, 1990). Climatic conditions may be either permissive or prohibitive for the activity of the natural enemy. Therefore, a climate-species adaptation must be considered in the acclimatisation strategy as well as specific host-predator or host-parasitoid interactions. Predators are related to their prey by a wide spectrum of trophic relations, parasitoids exhibit relative specificity towards their hosts' species and instar.

Microbial control is another approach for biological plant protection. The use of pathogens in biological control can be integrated with other natural enemies and the immediate effect of a microbial control agent can protect the crop when parasitoids and predators are unable to maintain the pest population below the damage threshold.

Pathogens of Aleyrodidae are restricted to fungi, because they are the only group of organisms that can penetrate the cuticle and so infect these plant-sucking insects (FRANSEN, 1990). Infection processes vary slightly with the different fungal species. The infection of the insect host starts with the unspecific adhesion of the fungal conidia to the insect cuticle. Successful adhesion depends on the characteristics of mucilage, enzymes, lectins, hydrophobic bonding and electrostatic forces (BOUCIAS *et al.*, 1988). Spore germination is the second step of the infection process and many factors have been found to play an important role in conidial germination and behaviour whereby water is an obligatory parameter (BUTT, 1990). In most cases the germination is followed by the formation of an appressorium. Low levels of nutrients enhance the formation of appressoria whereas high levels do not (ST. LEGER *et al.*, 1989). The final pene-

tration is a combination of mechanic and enzymatic processes and occurs mainly through intact cuticle, as opposed to through wounds or natural orifices (BUTT, 1990). After penetration most of the entomopathogens change their structure into yeast-like propagules, which spread in the host haemolymph. The death of the insect host is a result of the penetration of the tissue, extensive growth in the haemolymph and the production of toxins (CHARNLEY, 1989). Presuming that adequate moisture is provided, the antagonist emerges through the cuticle of the dead host and sporulates on the cadaver thus providing an inoculum source for the infection of additional insects. If the environmental conditions are not suitable, the fungus can survive in the dead body for several months, finally emerging and sporulating when conditions become favourable (SAMSON *et al.*, 1988).

Already more than 100 years ago METSCHNIKOFF (1880) promoted entomopathogenic fungi for the control of arthropods and more than 750 fungi from 90 species have been described as entomopathogenic against different insect species since then (SAMSON *et al.*, 1988). Different products based on fungi against a variety of insects have been commercialised in different countries worldwide: e.g. Mycotal ® and Vertalec ® (both Koppert Biological Systems B.V., Netherlands) contain different strains of *Verticillium lecanii* and are used against homopteran pests in glasshouses; Mycotrol ® and BotaniGard ® (both Mycotech Corporation, US) are based on spores of a *Beauveria bassiana* strain for the control of leaf feeding insects and Brocaril ® (Laverlam, Columbia), which is used to control the coffee berry beetle, contains another strain of *B. bassiana.*

The fungus *Paecilomyces fumosoroseus* (Wize) Brown & Smith naturally occurs in most countries of the world. It belongs to the Deuteromycotina, order Hyphomycetales, and the section *Isarioidea* (SAMSON, 1974). It grows with white mycelium and forms brightly coloured conidia, colours ranging from yellow to pinkish with a size of 3-4 x 1-2 µm. The fungus can be found in the soil in very low densities and has frequently been isolated from various infected insects. Insects from many orders belong to its wide host range (SMITH, 1993). It was first described as pathogenic against the greenhouse whitefly by FANG *et al.* (1985) and against the sweetpotato whitefly by OSBORNE *et al.* (1990). The isolation of the strain 'Apopka 97' finally led to the commercialisation of the product PreFeRal ® (Biobest N.V., Belgium) for the control of *T. vaporariorum*

(BOLCKMANS *et al.*, 1995). Numerous strains with control potential for *B. argentifolii* can be found worldwide (WRAIGHT *et al.*, 1998).

Another important entomopathogenic fungus, *Metarhizium anisopliae* (Metschnikoff) Sorokin, was first described as entomopathogenic against the scarabeid beetle *Anisoplia austriaca* by METSCHNIKOFF (1880) and VEEN (1968) mentioned about 200 insect species as potential hosts. The taxonomy of the genus *Metarhizium* (Deuteromycotina: Hyphomycetales) has recently been reassessed on the basis of RAPD data. According to DRIVER *et al.* (2000) most of the strains belong to the species *M. anisopliae* and are subdivided into four clades: *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *majus*, *M. anisopliae* var. *lepidiotum* and *M. anisopliae* var. *acridum*. The former *M. album*, *M. flavoviride* var. *flavoviride* and *M. flavoviride* var. *minus* were redefined according to ITS sequence data so that three clades are now representing two new varieties, *M. flavoviride* var. *novazealandicum* and *M. flavoviride* var. *pemphigum*. The third is not yet named.

All species grow with white mycelium and form oval- to egg-shaped conidia whose size and colour depends on the variety. All species are pathogenic to insects and occur in soils and insects all over the world (DOMSCH *et al.*, 1980). Since the discovery of *M. anisopliae* a lot of research has been done on the control of different Coleoptera, Lepidoptera and Orthoptera (GOETTEL, 1992; IGNOFFO & GARCIA, 1979; MUELLER-KOEGLER, 1965; STEPHAN *et al.*, 1996). Among the products that have been commercialised on the basis of *M. anisopliae* are Bio-Path ® (EcoScience, US), a trap for cockroaches, Biogreen ® (Technology Pty. Ltd, Australia) which acts against *Adoryphorus couloni*, redheaded cockchafer and Bio 1020 ® (Bayer AG, Germany) for the control of *Otiorhynchus sulcatus*, the black vine weevil. Only recently, GreenMuscle ® (Biological Control Products, South Africa) was registered for the control of locusts in some African countries while GreenGuard ® (Seed Grain and Biotechnology, Australia) is now commercially available in Australia. The efficacy of *M. anisopliae* against homopteran pests was discovered only recently. VESTERGAARD *et al.* (1995) first described the potential of the fungus to control *Frankliniella occidentalis*, the western flower thrips. MALSAM *et al.* (1998) reported the efficacy against *T. vaporariorum* and investigated possibilities to increase the antagonistic potential.

Entomopathogenic fungi can serve as alternatives to broad-spectrum chemical insecticides. Efficacy and cost are usually the sole perspectives when comparing microbial control agents with conventional chemical pesticides. Numerous advantages can be found in the utilisation of entomopathogens in addition to efficacy. Advantages consist in safety for humans and other non-target organisms, reduction of pesticide residues in food, preservation of other natural enemies and increased biodiversity in managed ecosystems (LACEY *et al.*, 2001). However, many factors still limit the acceptance of entomopathogens by growers and general public. In order to increase their utilisation, research needs to concentrate on: (a) pathogen virulence and speed of kill, (b) pathogen performance under challenging environmental conditions (cool weather, dry conditions etc.), (c) efficiency in the production process, (d) formulations that enable ease of application, increased environmental persistence and longer shelf-life, (e) integration into managed ecosystems and interaction with the environment and other integrated pest management (IPM) components (LACEY *et al.*, 2001).

The entomopathogens *P. fumosoroseus* and *M. anisopliae* have been reported to be effective against a wide range of insect pests. However, different aspects of the production and application process have to be taken into account for their increased utilisation. Different strains do have different potentials for the control of different insects and their adaptability to environmental conditions differs as well (BUTT *et al.*, 1995; HUXHAM *et al.*, 1989; VIDAL & FARGUES, 1998). Therefore, selection of the appropriate strain is the first important step.

Large-scale production of entomopathogenic fungi for the control of insects concentrates mainly on three types of propagules: (a) vegetative cells named blastospores, which grow in submerged, liquid culture (ADAMEK, 1965; CATROUX *et al.*, 1973; SAMSINAKOVA, 1966), (b) vegetative, multicellular mycelium, produced in liquid fermentation either in pellets or in hyphae with a filamentous morphology, which are fragmented afterwards (ANDERSCH, 1992; McCOY *et al.*, 1975; STENZEL, 1992), (c) conidia as so-called "resistant stage", which can be produced in a surface culture on solid medium, in a submerged culture in liquid medium or in a diphasic system which consists of inoculation of solid medium with blastospores produced in liquid fermenta-

tion (DE AQUINO *et al.*, 1975, 1977; FARGUES *et al.*, 1979; JENKINS & PRIOR, 1993; JENKINS & LOMER, 1994; MUELLER-KOEGLER, 1965).

While blastospores and conidia can infect the host directly, mycelium needs to grow and form infectious propagules first. Conidia can be produced easily and are more stable in challenging environmental conditions than blastospores. Therefore, the investigations of this project were carried out on conidia solely. Culture media and production conditions do not only affect the growth and sporulation of the fungi but also the spore viability and virulence (GILLESPIE & CRAWFORD, 1986; HUMPHREYS *et al.*, 1989; KLEESPIESS & ZIMMERMANN, 1998; KMITOWA & POPOWSKA-NOWAK, 1995). Hence, optimisation of the production conditions is necessary for successful biological control.

As pointed out before the penetration of fungal hyphae into the insect body occurs mostly through intact cuticle where chitin as main cuticle component presents a significant barrier for the invader (CHARNLEY, 1989). Thus chitin synthesis inhibitors, which interfere with chitin formation in insects, could act synergistically with fungal pathogens, weakening the insect prior to penetration. Chitin synthesis inhibitors, belonging to the group of insect growth regulators, have been used successfully and intensively for pest control. It is no wonder, therefore, that whitefly populations, resistant to most of them, have developed (DENHOLM *et al.*, 1999; GORMAN *et al.*, 2000). However, the efficacy of entomopathogenic fungi against insects could be increased distinctly by sublethal doses of these insecticides. HASSAN & CHARNLEY (1983) were the first to report synergistic effects of *M. anisopliae* and 'diflubenzuron' and hence successful control of *Manduca sexta*. Synergism of *Metarhizium* spp. and 'teflubenzuron' against *Schistocerca gregaria* was cited by JOSHI *et al.* (1992). Some further investigations on the control of Lepidoptera and Orthoptera with 'diflubenzuron' together with microbial antagonists have been published by DELGADO *et al.* (1999) and GUTIERREZ *et al.* (1995) but nothing can be found on homopteran pests.

Two chitin synthesis inhibitors were used for the investigations. Applaud ®, commercialised by Nihon Nohyaku, Japan, with the active ingredient 'buprofezin', acts specifically on homopteran pests such as whiteflies, planthoppers and scale insects (ISHAAYA *et al.*, 1988; IZAWA *et al.*, 1985; YAROM *et al.*, 1988; YASUI & FUKUDA, 1985). It is very

potent against both sweetpotato and silverleaf whitefly through contact as well as vapour toxicity and has been used successfully worldwide (DE COCK *et al.*, 1990; ISHAAYA *et al.*, 1988). Rimon® with the active ingredient 'novaluron' was developed by Makhteshim, Be'er Sheva, Israel. It is already commercialised in Israel and South America and about to be registered in Germany (MUEHLSCHLEGEL & BARAZANI, 2000).

Formulation of the antagonist is another parameter to focus on. In addition to the active ingredient (i.e. fungal spore) most formulations include one or more of the following basic components: a carrier, in most cases oil or water, diluent, binder, dispersant, UV protectants and virulence-enhancing factors (MOORE & CAULDWELL, 1997). For entomogenous fungi with hydrophobic spores like *Metarhizium* and *Paecilomyces*, a watery formulation with Tween® has always been used as standard. In the last years a lot of research has been done on oil formulations (AULD, 1992). Only recently, polymeric additives have been cited to enhance the efficacy of biological control measures (PIGGOT *et al.*, 2000; PUTERKA, 1999). Additives with which the spores are formulated can affect these in many aspects, the spore distribution and adhesion on the leaves and insects being one of them (INYANG *et al.*, 1998, 2000). Furthermore, the viability of spores on the leaf surface under environmental conditions such as temperature, UV radiation and humidity can be increased or decreased by different additives (ALVES *et al.*, 1998; DAOUST *et al.*, 1983). Conidial germination and appressoria formation on the insect cuticle is not only a question of climatic conditions but also of the presence or absence of chemical stimulants or inhibitors and nutrients. Additives have been found to be able to provide water and nutrients and to extract chemical substances from the insect cuticle that can act stimulatory or fungistatically (IBRAHIM *et al.*, 1999; INYANG *et al.*, 1999; MUGNIER & JUNG, 1985). Because of their effect on all these parameters additives can increase the control potential of antagonists towards their target (BATEMAN *et al.*, 1993; BURGES, 1999).

For practical use the shelf-life of microbial insecticides has to be considered and a lot of research has been done on this topic. A microbial insecticide must be produced, formulated and stabilised so that normal storage conditions do not affect insecticidal properties. Generally, at least 18 months stability under ambient storage conditions is required for servicing the agricultural markets (COUCH & IGNOFFO, 1981). If the pathogen is to

be supplied by contract for application at a specific time, shelf-life is less of a problem and stability for three to six months might be acceptable. Whether conidia are still viable after storage depends on the storage conditions (ABREU *et al.*, 1987; DAOUST & ROBERTS, 1983). Some additives have been shown to enhance the storage potential for longer periods and to overcome restrictions caused by humidity and temperature (ALVES *et al.*, 1987). However, the problem of developing a suitable formulation for entomopathogenic fungi which enhances their efficacy against the target insect whilst maintaining the fungus in a viable, virulent and stable state for a prolonged storage period still needs to be addressed and solved.

The present study was initiated as a collaboration between the University of Bonn and The Volcani Center, Agricultural Research Organisation, Israel, in order to select fungal strains virulent against *Bemisia argentifolii* and *Trialeurodes vaporariorum* and to optimise their efficacy. The potential of different isolates of *Paecilomyces fumosoroseus* and *Metarhizium anisopliae* for the control of both whitefly species should be evaluated, concentrating on *M. anisopliae* whose efficacy against *B. argentifolii* has not been investigated so far. While looking at different aspects of the production, formulation and application process, emphasis will be given to the effectiveness of formulations with focus on oils and polymeric additives. The potential of additives to increase the shelf-life and efficacy of entomopathogenic fungi should be determined and explained by looking at different stages of application and infection such as inoculum targeting, enhancement of spore germination and inoculum persistence on the leaves. The possibility of an integration of the antagonists into plant protection programmes with the insect growth regulators 'buprofezin' and 'novaluron' will be evaluated in order to implement a practical approach for the control of both insect species in the greenhouse. The overall objective is to demonstrate the potential of entomopathogenic fungi to be integrated into an effective and inexpensive IPM strategy.

2 Materials and methods

2.1 Organisms

This project was carried out in Israel and Germany to investigate the performance of the fungi under different climatic conditions. While the same fungal strains were used in both countries, the insect species and their host plants differed regarding the climate.

2.1.1 Fungi

Many species of fungi are described as pathogenic to insects. The investigations were carried out with *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith. Many strains of both species were already isolated worldwide. Origin and climatic zone from the strains that were used in the investigations are given in Table 2-1.

The strain *M. anisopliae* var. *anisopliae* 43 is also known as V127, 275 or F52 (ATTC number 90448) on which the biological insecticide Bio 1020® (Bayer AG, Germany) is based. *M. anisopliae* var. *anisopliae* 108 has also been called F590. *M. anisopliae* var. *acridum* 5 and *M. anisopliae* var. *acridum* are registered by the DSM-numbers 11336 and 11337.

Table 2-1. Isolate number, origin and location of isolates of entomopathogenic fungi**.**

no.	species	isolated from	location
M27[1]	*Metarhizium anisopliae* var. *anisopliae*	soil sample	Russia
M43[2]	*Metarhizium anisopliae* var. *anisopliae*	*Carpocapsa pomonella*	Austria
M97[2]	*Metarhizium anisopliae* var. *anisopliae*	semi-looper larva, Lepidoptera	Philippines
M108[2]	*Metarhizium anisopliae* var. *anisopliae*	*Aphodius* sp., Coleoptera	Germany
V242[3]	*Metarhizium anisopliae* var. *anisopliae*	potato field soil	Finland
V245[3]	*Metarhizium anisopliae* var. *anisopliae*	hay field soil	Finland
M5[2]	*Metarhizium anisopliae* var. *acridum*	*Locusta migratoria capito*	Madagascar
M11[2]	*Metarhizium anisopliae* var. *acridum*	*Kraussella amabile*	Senegal
P1[1]	*Paecilomyces fumosoroseus*	*Bemisia tabaci*, egg	Gaza
P2[1]	*Paecilomyces fumosoroseus*	*Boophilus giganteus*	Gaza

provided by: [1]Dr. G. Gindin, The Volcani Center, Israel, [2]Dr. G. Zimmermann, BBA, Darmstadt, Germany, [3] Dr. T.M. Butt, University of Wales, Swansea, UK.

2.1.2 Insects

Investigations were carried out on the two whitefly species *Bemisia argentifolii* and *Trialeurodes vaporariorum*. Experiments on the former were performed in Israel and on the latter in Germany. *Blaberus discoidales* was used for assessments on spore germination, providing cuticle pieces which were big enough and therefore easier to handle than cuticle of whitefly larvae. *Spodoptera littoralis* was chosen as a typical lepidopteran species for the Mediterranean climate.

The strain of *Bemisia argentifolii* (Bellows & Perring), the silverleaf whitefly, was collected initially from an Israeli cotton field in 1995 and reared under laboratory conditions in the Volcani Center since then (ISHAAYA, The Volcani Center, Israel, pers. comm.). The insects were feeding on cotton plants under greenhouse conditions of 28±7 °C, 60±35 % RH and a photoperiod of 16:8 (light:dark).

The strain of *Trialeurodes vaporariorum* (Westwood), the greenhouse whitefly, was obtained from the Bayer AG laboratories were it has been reared under standard laboratory conditions (MALSAM, Bayer AG, Germany, pers. comm.). Cucumber and tomato plants were used for rearing at 25±3 °C, 50±20 % RH and a photoperiod of 16:8 (light:dark).

Elytra of *Blaberus discoidalis* (American cockroach) were taken from adults of a strain that has been reared on artificial diet at 25 ± 3 °C, 50±20 % RH and a photoperiod of 16:8 (light:dark) in the School of Biological Sciences, University of Wales, Swansea (RATCLIFFE, University of Wales, UK, pers. comm.).

For larvae of *Spodoptera littoralis*, the Egyptian cotton leafworm, the strain was collected from a cotton field in Israel in 1990 and has been reared on leaves of the castor–oil plant under laboratory conditions in the Volcani Center since then (ISHAAYA, The Volcani Center, Israel, pers. comm.).

2.1.3 Plants

As the experiments were performed in different climatic regions on different insects, host-plant systems for experiments and rearing of the arthropods were chosen accordingly. Plants from typical crops for the climatic zones, which were likely to be infected by the insects, were used. The investigations were carried out on *Ricinus communis* (L.), the castor-oil plant, and on *Gossypium hirsutum* (L.), cotton 'Acala SJ2', in Israel. *Cucumeris sativus* (L.), cucumber 'Bella', and *Lycopersicon lycopersicum* (L.), tomato 'Rheinlands Ruhm', were used in Germany.

2.2 Cultivation

The entomopathogenic fungi were cultivated on cheap, easily available media under standardised laboratory conditions. Different types of media were used for cultivation and bioassays. Plants were reared in the greenhouse, temperature, humidity and light periods were adjusted as required.

2.2.1 Culture media

Metarhizium anisopliae and *Paecilomyces fumosoroseus* are easy to cultivate and grow on a variety of artificial media. The cultivation of the fungi and the experiments were carried out on different types of media. The micro-organisms were cultivated on Potato–Dextrose agar (PDA), in liquid cultures of Potato–Dextrose broth (PDB) with 0.01 % of yeast extract or on commercial long grain rice (Figure 2-1).

The assessments of mycelial growth were performed on PDA, Sabouraud–Dextrose agar (SDA) and Oatmeal agar (OMA). OMA contained 30 g instant oatflakes and 20 g agar per litre deionised water.

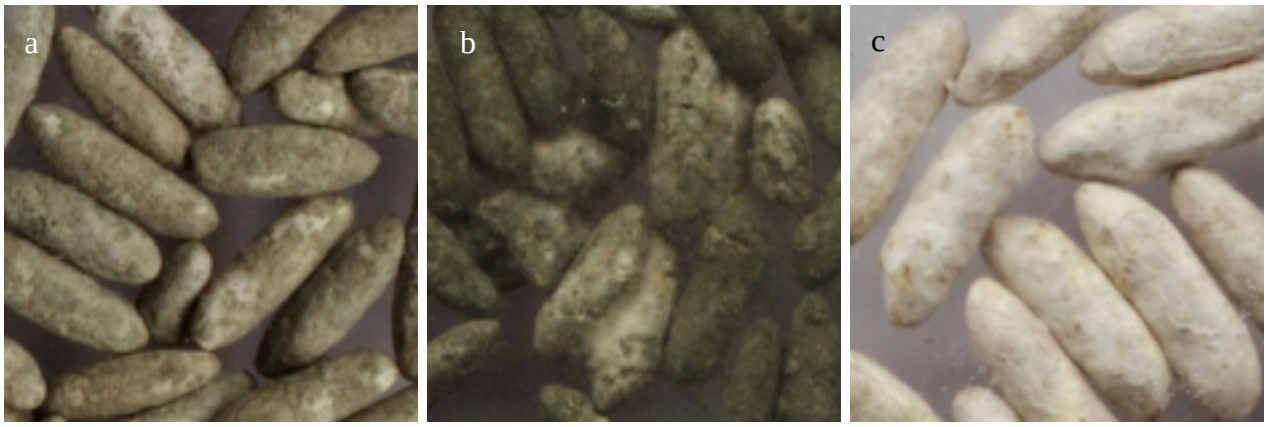

Figure 2-1. Cultures of *M. anisopliae* var. *anisopliae* V242 (a), *M. anisopliae* var. *acridum* 5 (b) and *P. fumosoroseus* 1 on rice grains (c).

The spore germination assays were carried out on water agar (WA, 1.5 %) and Yeast–Dextrose agar (YDA) with different water potentials (Table 2-2). Yeast extract (0.01 g), dextrose (0.2 g) and agar (1.5 g) were mixed with various quantities of glycerol (99.5 %) to obtain media with different water activities (A_w), which equate to different relative humidities (Table 2-2). The ingredients were mixed and made up to a total mass of 100 g with deionised water. All media were sterilised for 20 minutes at 121 °C before use.

Table 2-2. Amounts of glycerol in dependence of the water activity (A_w) and the equivalent relative humidity (RH), modified from DALLYN & FOX (1980).

glycerol (g)	**A_w**	**RH (%)**
0	1.00	100
10.15	0.98	98
20.5	0.96	96
30.7	0.94	94
40.95	0.92	92

2.2.2 Maintenance of fungal cultures and inoculum production

The fungal isolates were kept in glass vials, half-filled with a mixture of plantation soil and sand (1:1). The vials and soil were sterilised for 20 minutes at 121 °C and inoculated with fungal spore suspension after cooling down. Spore suspension was made up with 10^7 conidia per millilitre water containing 0.05 % Tween® 80 as surfactant. The vials were stored at –20 °C.

For the production of conidia, a few particles of the soil–fungus mixture were spread on PDA and incubated at 26 °C in the dark. After about two weeks small pieces of medium, covered with fungus, were taken from the petri dishes and put into sterile conical flasks containing PDB with yeast extract. The volume relation of medium and flask was about 1:10. The flasks were stoppered and shaken at 80 rpm at room temperature. *M. anisopliae* had formed blastospores after three days while *P. fumosoroseus* needed only two days. The blastospore suspension was poured onto rice grains. For that purpose conical flasks (100 ml) were filled with about 20 g of commercial rice. The grains were rinsed in deionised water and sterilised at 121 °C for 20 minutes. After cooling down the rice was incubated with about 2 ml of blastospore suspension. The flasks were closed with cotton wool and incubated at 26 °C in the dark for two weeks. Cultures were shaken daily to prevent the aggregation of grains. When the grains were colonised with conidia the flasks were sealed with aluminium foil and kept at 4 °C in the dark. Conidia were harvested from the cultures up to an age of three months.

Spore suspension for inoculation was obtained by harvesting conidia from colonised rice grains in Tween® 80 as a standard or in an aqueous formulation of the other additives. The suspensions were stirred for at least half an hour on a magnetic stirrer and thereafter the grains were removed by filtering through two layers of cotton gauze. The spore germination rate was determined before each experiment after incubation on WA for 24 hours at 26 °C. The spore concentration in the suspensions was adjusted with a Fuchs-Rosenthal haemocytometer to 10^6 conidia / ml for the investigations on spore germination, spore viability and spore adhesion while the bioassays on whiteflies were performed with a concentration of 10^7 conidia / ml.

2.2.3 Plants for bioassays and insect rearing

Plants were reared under greenhouse conditions, typical for the two countries. Cotton seeds were sown in plantation soil containing 30 % turf and 70 % tuff. Seedlings were grown under greenhouse conditions of 21±9 °C, 65±35 % RH and a photoperiod of 16:8 (light:dark). Cucumber and tomato plants were cultivated in standard plantation soil under greenhouse conditions of 25±3 °C, 50±20 % RH and a photoperiod of 16:8 (light:dark). Leaves from the castor–oil plant were collected from wild plants growing around the Volcani Center.

2.3 Chemicals

The potential of a combination of the entomogenous fungi with insecticides was investigated. The pesticides belonged to the group of insect growth regulators and acted as chitin synthesis inhibitors. Furthermore, the potential of different additives for the formulation of entomopathogenic spores was evaluated.

2.3.1 Insect growth regulators

The effect of two different insect growth regulators in combination with the entomopathogenic fungi was investigated. Both are inhibiting the chitin synthesis of insects. Applaud® with the active ingredient 'buprofezin' was synthesised by Nihon Nohyaku Co. Ltd., Tokyo, Japan. It is used intensively against homopteran pests worldwide. Rimon® with the active ingredient 'novaluron' was developed by Makhteshim, Be'er Sheva, Israel. It is already commercialised in Israel and South America and about to be registered in Germany. For the investigations a formulation that contains an emulsifiable concentration of 10 % of the active ingredient was used. The concentrations given are always referring to the actual 'novaluron' concentration.

2.3.2 Additives for the formulation of spores

Different oils, waxes and polymers were investigated for their effect as additives to the fungal spore suspension (Table 2-3). Tween® 80 is generally used for the formulation of entomopathogenic fungi with hydrophobic conidia. Addit® was developed as an additive for the product Mycotal® (Koppert, Netherlands), which is based on the entomogenous fungus *Verticillium lecanii*. It showed very promising results for enhancing the efficacy of the fungus for the control of whiteflies and thrips (VAN DER PAS *et al.*, 1998). Agrocer® and Stockosorb®Agro are commercially available as additives for pesticide formulations. PA1 and PA2 are not commercialised yet and still under registration process.

Table 2-3. Additives used for the formulation of fungi.

name	**company**
Addit®	Koppert, Netherlands
Agrocer®	Clariant, Germany
PA1[1]	Stockhausen, Germany
PA2[1]	Stockhausen, Germany
Stockosorb®Agro	Stockhausen, Germany
Tween® 80	Merck, Germany

[1] provided by the Department for Plant Diseases, Bonn University, Germany

2.4 Evaluation of fungal growth and viability

Different parameters for growth and viability of fungi can be determined. Fungal spores are viable if they are germinating. Germination can be evaluated by microscopical assessments. The viability of fungal cultures can be determined visibly by observing radial growth and sporulation.

2.4.1 Assessment of spore germination

A droplet of 0.1 ml spore suspension was pipetted onto WA in a petri dish with a diameter of 9 cm and spread evenly with a Drigalsky spatula. The dishes were incubated at 26 °C in the dark and 100 spores were examined for germination after 24 hours. A spore was considered to have germinated and hence be viable if the length of the germ tube was equal to or exceeded the breadth of the spore.

The spore germination under low relative humidity was assessed by a method from VESTERGAARD (1995). Three droplets of YDA medium based on a glycerol-water mixture were pipetted on sterile microscope slides. Each droplet had a volume of about 50 µl. 2-3 µl of spore suspension were applied to each droplet and after drying the slides were put into boxes that contained medium with the same water activity as the agar droplets on the slides. The boxes were sealed with lids and incubated at 26 °C in the dark. The proportion of germinated and non-germinated spores out of 100 per droplet was determined at 10, 15, 20 and 25 hours for A_w 1, at 10, 15, 20, 25 and 30 hours for A_w 0.98 and at 24, 48, 72 and 96 hours for A_w 0.96, 0.94 and 0.92.

The spore germination on insect cuticle under different relative humidities was investigated on elytra of *Blaberus discoidalis* (American cockroach). The wings were washed in deionised water and dried. Pieces of about 0.25 cm^2 were cut from the centre region of the wings and the uppersides were inoculated with 3-5 µl spore suspension per wingpiece. The wings were placed in boxes that contained YDA medium with water activities ranging from 1 to 0.92. Thereafter, the boxes were sealed with lids and incubated at 26 °C. After different periods depending on the water activity (see above), wingpieces were removed from the boxes and micro-organism propagules were stripped off the wings with Scotch ® Magic Tape. The strips were stained with lactophenol cottonblue,

rinsed in deionised water and attached to coverslips after drying. The coverslips were fixed onto microscope slides with nail polish and 100 spores per coverslip were examined for germination.

In this test it was assumed that all of the propagules were transferred from the wing surface to the tape. In order to test this assumption, wing pieces were mounted in Congo red (Serva) staining after stripping off (see chapter 2.7.3). Thereafter, they were rinsed in deionised water and examined under the fluorescence microscope for conidia (see chapter 2.7.1). Hereby it was proved that no conidia remained on the wing pieces.

2.4.2 Evaluation of mycelial growth and spore production

Homogenous fungal cultures were obtained by spreading of 0.1 ml spore suspension, containing 10^6 spores per ml in water with 0.05 % Tween® 80, evenly onto PDA medium in petri dishes (Ø 9 cm). The petri dishes were incubated at 26 °C in the dark. After two days mycelium plugs were removed with a sterile cork borer (Ø 0.6 cm) and transferred to petri dishes with either PDA, SDA and OMA or YDA with different water potentials. Measurements of the mycelial growth were taken after three and six days of incubation at 26 °C in the dark. The diameter of the colonies was estimated by calculating the mean of two perpendicular measurements. The sporulation rate was assessed after six days. The petri dishes were rinsed with 1 ml of Tween® 80 and the conidia were scraped off carefully with a spatula. The spore concentration was determined with a haemocytometer and the viability of the conidia was examined after incubation for 24 hours at 26 °C on WA.

2.4.3 Adhesion and viability of spores on leaf surfaces

Tomato plants in growth stage 14 were sprayed with about 20 ml of spore suspension with different additives (MEIER, 1997). The suspensions were applied mainly to the undersides of leaves with a hand venturi-type sprayer. After drying, leaf impressions of one leaflet from each leaf and treatment were taken on WA by the method of FRANSEN (1995). The undersides of the leaves were pressed gently onto the agar medium and removed again to achieve a representative sample of micro-organisms from the leaf surface. The number of *Metarhizium* conidia was determined under a microscope on 10 dif-

ferent parts of the impression resulting in an observation area of 1.5 mm^2. The plates were then incubated at 26 °C in the dark and the spore germination rate was assessed after 24 hours. Leaf impressions were taken on day 1-7, 10 and 14 after spraying.

In order to prove that fungal spores were completely transferred from the leaf surface, the undersides of the leaves were examined for remaining conidia after taking the impressions by the method of DRUMMOND & HEALE (1985). This method was modified by using Calcofluor white M2R (Sigma) as fluorescent dye (see chapter 2.7.1). Calcofluor was dissolved at a concentration of 0.01 % in 0.067 M potassium phosphate buffer, pH 8 (BUTT, 1987). Microscopical examination showed that no fungal propagules remained on the leaf surfaces.

2.5 Storage of formulated spores

Spore formulations were either stored in their liquid state or after drying. For liquid storage, the spore suspensions were poured into sterile glass vials with screw plugs and kept either at 4 °C or at 26 °C. Samples of 0.1 ml were taken for determination of the conidial viability on WA immediately and both seven days and one month following preparation.

For dry storage 1 ml of the formulated spores was spread on a watch glass and dried under the laminar flow hood until no more liquid was visible. The residues were scraped off and stored in Eppendorf caps at either 4 °C or 26 °C. Samples of 1 mg were taken out at day 0, day 7 and one and three months following preparation, suspended in deionised water and examined for spore germination after incubation on WA for 24 hours at 26 °C.

2.6 Design of bioassays

Bioassays were designed according to optimum environmental conditions for the insect hosts, *Bemisia argentifolii*, *Trialeurodes vaporariorum* and *Spodoptera littoralis*. Assays were either performed in the greenhouse on whole plants or on detached leaves in experimental chambers.

2.6.1 Investigations on Aleyrodidae

In order to obtain homogenous whitefly populations in the same developmental stage, whitefly adults were transferred with a tube from the rearing plants into small clip cages at a number of 30 adults per cage (Figure 2-2a). The cages had been developed by MELAMAD-MADJAR *et al.* (1984). They were attached to the undersides of the bioassay plants (Figure 2-2b). Thereafter, the whiteflies were allowed to lay eggs for 48 hours under greenhouse conditions. The cages and whiteflies were removed and the plants were kept under greenhouse conditions for larval development.

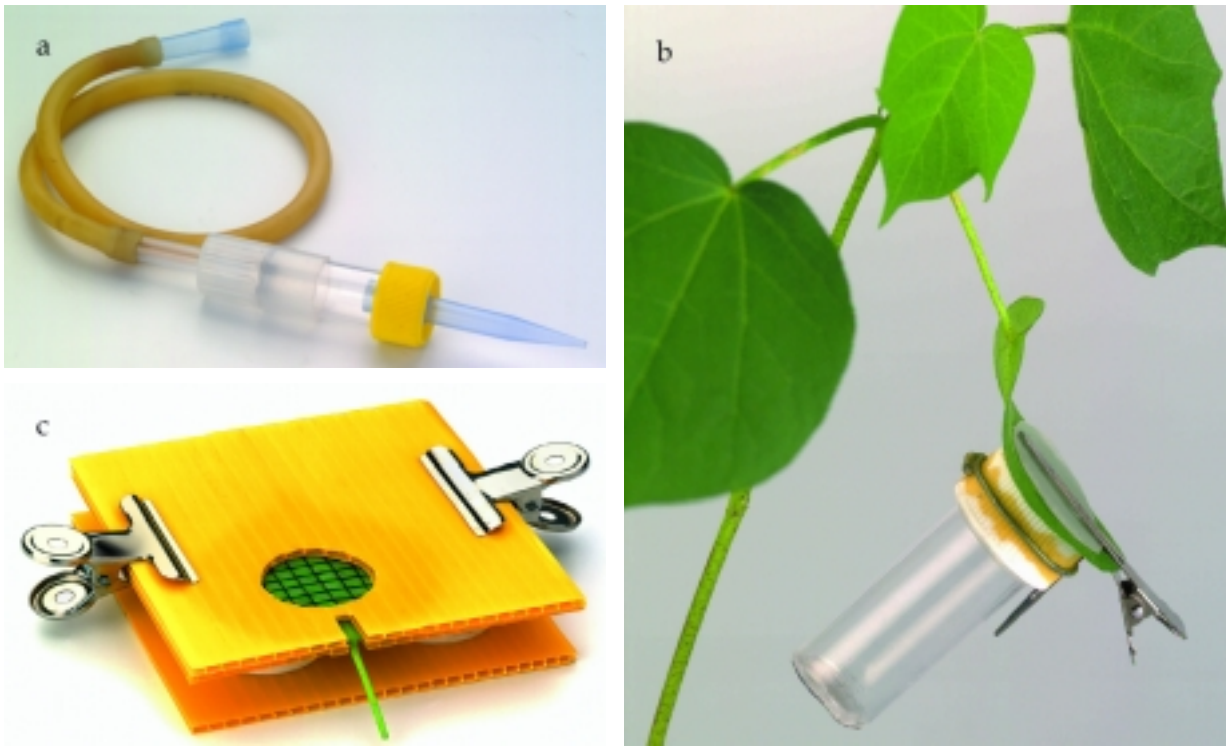

Figure 2-2. Devices for bioassays on whiteflies: cage with tube for collecting adults (a), clipcage attached to cotton leaf for infestation (b), counting chamber for evaluation (c).

The cage system facilitated infestation, removal of adults and evaluation because the target insects were concentrated in a defined area on the leaf. A counting chamber was used to visually divide the infested area into compartments under the binocular (Figure 2-2c). In preliminary experiments the cage system was compared to the bioassay system of MALSAM (1999) that involves the infestation of whole plants in a rearing chamber and simulates natural infestation. The efficacy of the fungi was not significantly different in either system.

The treatments were applied to eggs or different larval stages with a commercial venturi-type hand sprayer at a volume of 15 ml per plant. A spore concentration of 10^7 per millilitre was always used and the spore viability was determined before each experiment. The number of eggs, live and dead larvae was assessed under a binocular. Live larvae of *B. argentifolii* and *T. vaporariorum* are opaque or white-greenish, depending on their development stage, and shiny. Their bodies are oval-shaped and distinctly convex. Feeding activity is sometimes visible when honeydew droplets appear on the excretion organs. Dead whitefly larvae are mat, turn brownish and dry out depending on the relative humidity.

2.6.1.1 Experiments on detached leaves

Treatments were applied to the plants after infestation as described above. Single leaflets of tomato or single cotton leaves were left to dry in order to prevent saprophyte growth in the droplets and placed in boxes lined with moist filter paper. The boxes were closed with lids to maintain 100 % relative humidity and incubated at 26 °C in the dark. The larval mortality was determined at days 2, 4 and 6 post inoculation.

Investigations on the effect of different treatments on whitefly adults were carried out with choice and no-choice assays by placing two differently or equally treated tomato leaflets in the same box. About 30 adults were released inside the box that was closed with a lid with gauze-covered holes to provide air circulation. The number of whiteflies and the number of eggs on each leaflet were determined after 48 hours incubation at 26 °C in the dark.

2.6.1.2 Greenhouse experiments

The experiments were performed on cotton plants at growth stage 14 or on tomato plants at growth stage 16 (MEIER, 1997). Infestation and application of treatments were carried out as described above. The mortality rate was determined every one or two days for up to two weeks. After the last evaluation, the leaves were placed in boxes lined with moist filter paper, sealed and incubated at 26 °C in the dark for 72 hours to examine fungal growth and hence the number of larvae killed by the fungus.

Prophylactic treatments were applied to the plants 14 days, seven days or two hours before infestation. Application and infestation were carried out as described above. The number of eggs, hatched larvae and pupae was determined.

2.6.2 Investigations on *Spodoptera littoralis*

The experiments were performed on early 3rd instar larvae (0-24 hours after ecdysis) using a modified method of ISHAAYA *et al.* (1996). The larval weight was determined on day 0 and thereafter the insects were immersed in spore suspension made with 0.05 % Tween® 80 at a concentration of $5x10^7$ per ml for half a minute. An aqueous suspension of 0.05 % Tween® 80 was used as a control. After five minutes of drying on filter paper the insects were put into ventilated plastic boxes, which contained sawdust to avoid excess humidity, with a leaf of the castor-oil plant. For the investigations on 'novaluron', the castor-oil plant leaves were dipped into an aqueous suspension of 0.05 % Tween® 80 and 'novaluron' or of 0.05 % Tween® 80 alone for two minutes. After drying for two hours at room temperature the leaves were transferred to the boxes. The boxes were sealed with lids and incubated at 26 °C in the dark. After four days the larval weight gain was determined and fresh, untreated castor-oil plant leaves were added. The number of dead larvae was assessed at day 4 and day 8. The experiments were performed with ten larvae per box, six boxes per treatment and repeated three times.

2.7 Microscopy

Apart from conventional light microscopy three different microscopical methods were used for the assessments. Assessments on the distribution of formulations on the leaves and observations of remaining conidia on leaves and insect cuticle were performed with fluorescence microscopy. For having a closer look on the distribution and germination of entomopathogenic spores on insects low temperature scanning electron microscopy and confocal laser scanning microscopy were used.

2.7.1 Fluorescence microscopy

The distribution of formulations on the leaves was examined by staining the formulations with Nile red (Sigma). Nile red is not water-soluble and was therefore dissolved in acetone at a concentration of 1000 mg dye per millilitre organic solvent first. Then 10 µl dye solution were added to one millilitre of each formulation. Half a millilitre of stained formulation was applied to the underside of a tomato leaf with a hand venturi-type sprayer. The specimens were examined with a magnification of 50x after drying with EF 490 / 15 as exciter, 500 as chromatic beam splitter and BP 525 / 20 as barrier filter. Pictures were recorded digitally and the diameter of the droplets was measured.

Observations for conidia stained with Congo red (Serva) were made using the following combination of filters: EF 490 / 15 (exciter), 500 (chromatic beam splitter) and BP 525 / 20 (barrier). Specimens stained with Calcofluor (Sigma) were examined after drying with the following combination of exciter, chromatic beam splitter and barrier filters: BP 340-380, 400 and LP 425, respectively.

2.7.2 Low temperature scanning electron microscopy

The distribution of conidia of *M. anisopliae* on whitefly larvae was observed with a low temperature scanning electron microscope (LTSEM). Preparation for examination was performed by techniques described by BECKETT & READ (1986).

Droplets of 3 µl of the formulations tested were placed directly onto 4th instar larvae of *T. vaporariorum* on tomato leaves and left to dry. When no more liquid was visible, pieces of the leaf with whiteflies were cut out and placed on a brass stub. The specimens were quickly frozen-hydrated by immersing them in liquid nitrogen (-190 °C) for 2-3 minutes. Thereafter, the specimens were etched by raising the temperature to –65 or –55 °C for 2-15 minutes. Various amounts of water were removed by sublimation and the freeze-dried specimens were then transferred under vacuum to the coating chamber with a special transfer device. At a temperature below –130 °C and under dry argon atmosphere the specimens were coated with gold for 5 minutes in a spruter, SEM coating E5000, Polaron. Observations were made with a Phillips 501B scanning electron microscope (10 kV) at a low temperature (approximately –175 °C) and pictures were recorded digitally.

2.7.3 Confocal laser scanning microscopy

Specimens for the investigations on conidial distribution on the insect were prepared as for the LTSEM. Pieces of leaf were mounted in fixative at room temperature for at least 24 hours.

Recipe for fixative (GERLACH, 1984)

90 ml ethanol (70 %)
5 ml formaldehyde (35 %)
5 ml acetic acid (conc.)

The specimens were then rinsed in deionised water and mounted in a droplet of Congo red (Serva) for at least 30 minutes. Microscopical assessments were made after rinsing the specimens in deionised water and sticking them to microscope slides with double-sided tape. Examinations were made with the 543 nm laser of a CLSM (LSM 300, Zeiss, Germany) and pictures were recorded digitally.

Recipe for Congo red staining (MALSAM, 1999)

0.1 % Congo red (Serva)

0.1 % Tween® 20

0.05 % ethanol

Specimens for spore germination were taken from greenhouse assays at different periods after inoculation. Specimens were fixed, rinsed and stained as described above.

2.8 Biochemical assessments

Biochemical assessments on the larval cuticle of *Spodoptera littoralis* were carried out for chitin and protein content. Cuticle was obtained from 12-24 larvae per treatment by cutting off the heads with a razor blade and carefully squeezing out the gut on filter paper. The remaining cuticle was cut into small pieces and the fresh weight was determined. Thereafter, the samples were deep-frozen at –80 °C for one hour and freeze dried.

2.8.1 Evaluation of chitin content

Samples of freeze-dried cuticle with a weight of about 1 mg were weighed and processed by the method of HACKMAN & GOLDBERG (1981). Absorbances were read at 650 nm with a spectrophotometer. A reference curve was prepared by plotting the weight of definite amounts of pure chitin from crabshells (Sigma) against the corresponding absorbance, measured after processing the chitin accordingly.

2.8.2 Determination of protein content

Samples of about 15 mg of freeze-dried cuticle were weighed, made up to a volume of 1.5 ml with deionised water and homogenised with a tissue grinder. The samples were centrifuged for 10 minutes at 14000 rpm. 100 µl of the supernatant were mixed well with Coomassie brilliant blue as protein reagent and the protein content was determined by the method of BRADFORD (1976), preparing a standard curve from Bovine serum albumin.

2.9 Statistical analysis

For the droplet distribution of formulations, the average diameter of 10 droplets per picture was calculated. Results of 10 pictures were combined to give means and standard deviations so that the treatments could be analysed with the H-Test (Kruskal-Wallis Analysis, $p \leq 0.05$) for not normally distributed data. Significant differences were then determined with the DUNN'S-Test and indicated by different letters.

For the spore germination assays, estimated GT_{50} values (time required for the germination of 50 % of the conidia) were calculated for each water activity by probit analysis with the program Polo-PC (LeORA SOFTWARE, 1987). Results of three independent assessments with three replicates each were combined to give means and confidence limits. The treatments were compared amongst each other and different letters were used to indicate significant differences.

Studies on conidial adhesion, conidial viability, mycelial growth, storage, oviposition, egg hatch and prophylactic treatments were evaluated with the program SigmaStat 2.0 for Windows (SPSS Inc.). Results of three independent assessments with five replicates each were used to give means, which were analysed for normal distribution and equal variance ($p \leq 0.05$). Significant differences were determined by the t-Test in case of two groups or by the TUKEY-Test in case of more than two groups and indicated by different letters or ✶.

Data that were not distributed normally or showed unequal variance were analysed with the H-Test (Kruskal-Wallis Analysis, $p \leq 0.05$). Significant differences were then determined with the DUNN'S-Test and indicated by different letters.

The values for larval mortality were corrected by the natural mortality that occurred in the control treated with Tween ® 80 and the results were analysed by the same procedure as described for conidial adhesion (ABBOTT, 1925).

3 Results

3.1 The potential of entomopathogenic fungi for the control of *Bemisia argentifolii* and *Trialeurodes vaporariorum*

Investigations were carried out on the potential of *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* for the microbial control of whiteflies. The different isolates of *M. anisopliae* var. *anisopliae, M. anisopliae* var. *acridum* and *P. fumosoroseus* originated in different climatic zones were they had been isolated from different soil types or insect species (Table 2-1). They were tested for their virulence against *Bemisia argentifolii,* the silverleaf whitefly, and *Trialeurodes vaporariorum,* the greenhouse whitefly. The screening was carried out on detached leaves under optimum conditions for the fungi. High humidity was provided by using moist chambers and incubation was performed in the dark at 26 °C. Furthermore, the efficacy of *M. anisopliae* var. *anisopliae* against different developmental stages of *T. vaporariorum* was investigated in different experiments. Hence, the potential of the entomopathogens for the control of whitefly eggs, larvae and adults was evaluated to determine the influence of the insects' developmental stage on the success of the biological control method.

3.1.1 Pathogenicity of entomopathogenic fungi against *Bemisia argentifolii*

Ten fungal isolates were evaluated for their potential to control 1st instar larvae of the silverleaf whitefly. The investigations demonstrated significant differences in the virulence of the isolates concerning the total mortality after six days and the time required for obtaining different mortality rates (Figure 3-1).

After six days of incubation insect mortalities of the ranged from 15 % to 80 %. Two strains of *M. anisopliae* var. *anisopliae,* M27 and M43, strain M11 of *M. anisopliae* var. *acridum* and both strains of *P. fumosoroseus* were found to cause the highest mortality.

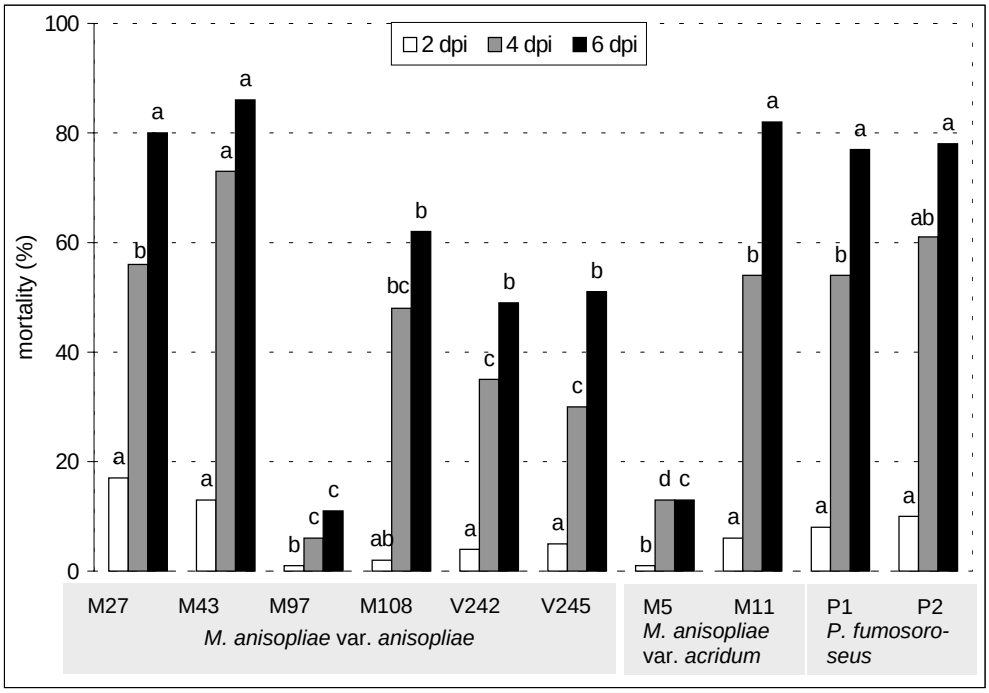

Figure 3-1. Pathogenicity of different isolates of *M. anisopliae* and *P. fumosoroseus* against larvae of *B. argentifolii* (1st stage) (cotton, 10^7 spores / ml, 26 °C, 100 % RH, dark, values corrected for controls by Abbott's formula (ABBOTT, 1925), significant differences, TUKEY, $p \leq 0.05$, on the same day of evaluation are indicated by different letters).

Out of 10 isolates eight were found to be virulent against larvae of *B. argentifolii*. Virulent isolates originated in different climatic regions and had been isolated from different insect species. Intra- and interspecific variation could be observed in the speed of kill and in the total mortality caused.

3.1.2 Pathogenicity of entomopathogenic fungi against *Trialeurodes vaporariorum*

Investigations on the potential of *M. anisopliae* and *P. fumosoroseus* for the control of the greenhouse whitefly were carried out accordingly. In general, the results for *T. vaporariorum* corresponded with those for *B. argentifolii* (Figure 3-2). *M. anisopliae* var. *anisopliae* 97 and *M. anisopliae* var. *acridum* 5 did not cause more than 20 % larval mortality after six days. About 100 % of the larvae died from the application of *M. anisopliae* var. *anisopliae* 27 or of the two *P. fumosoroseus* strains and about 90 % mortality were achieved with *M. anisopliae* var. *anisopliae* 43 or *M. anisopliae* var. *acridum* 11. Fungal emergence and sporulation was visible on the cadavers (Figure 3-3, Figure 3-4).

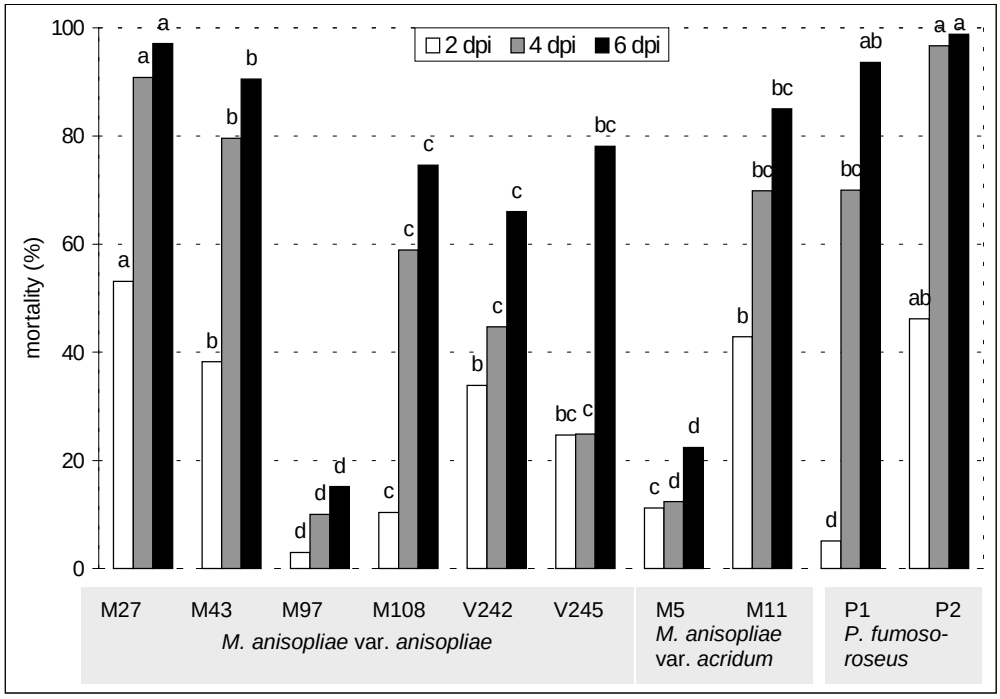

Figure 3-2. Pathogenicity of different isolates of *M. anisopliae* and *P. fumosoroseus* against larvae of *T. vaporariorum* (1st stage) (tomato, 10^7 spores / ml, 26 °C, 100 % RH, dark, values corrected for controls by Abbott's formula (ABBOTT, 1925), significant differences, TUKEY, $p \leq 0.05$, on the same day of evaluation are indicated by different letters).

The application of *M. anisopliae* var. *anisopliae* 27 or *P. fumosoroseus* 2 resulted in 40-50 % mortality after two days already and more than 90 % of the larvae were killed after four days. Although only about 10 % of the larvae were killed by *M. anisopliae* var. *anisopliae* 108 or *P. fumosoroseus* 1 after two days, their application resulted in about 60 % mortality for *M. anisopliae* var. *anisopliae* 108 and about 75 % for *P. fumosoroseus* 1 after four days, respectively.

Isolates of entomopathogenic fungi with an evenly high control potential for *T. vaporariorum* as for *B. argentifolii* were detected. Speed of kill was as important as total mortality. It has to be taken into account that environmental conditions were adjusted for optimum fungal growth so that these results need to be extended to investigations on the performance of the fungi under greenhouse conditions.

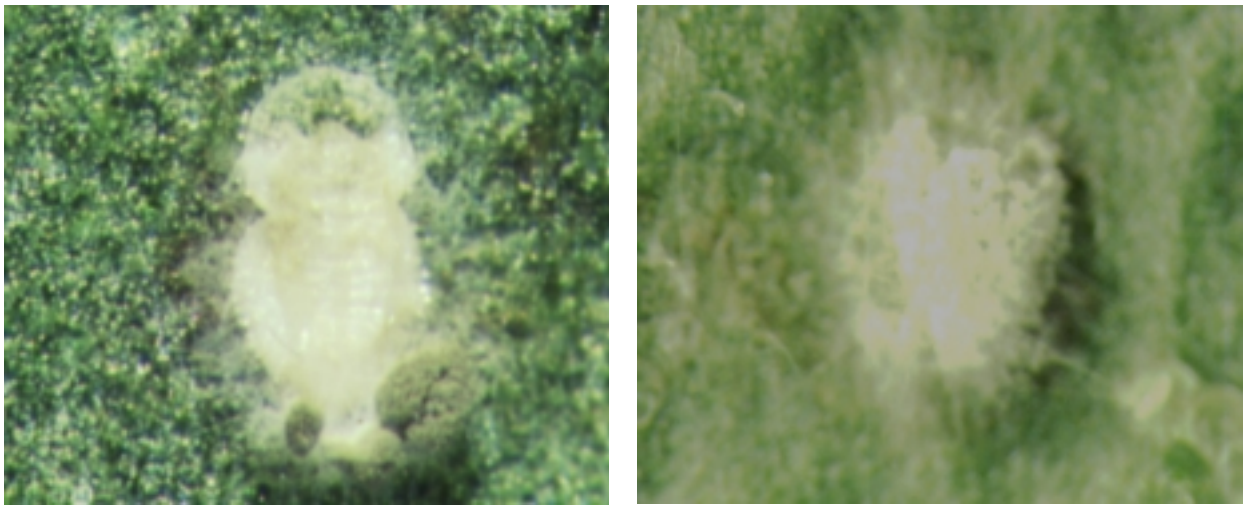

Figure 3-3. Fungal colonisation on 4th instar larvae of *T. vaporariorum*: *M. anisopliae* var. *anisopliae* 27 (left) and *P. fumosoroseus* 2 (right), 6 days post inoculation.

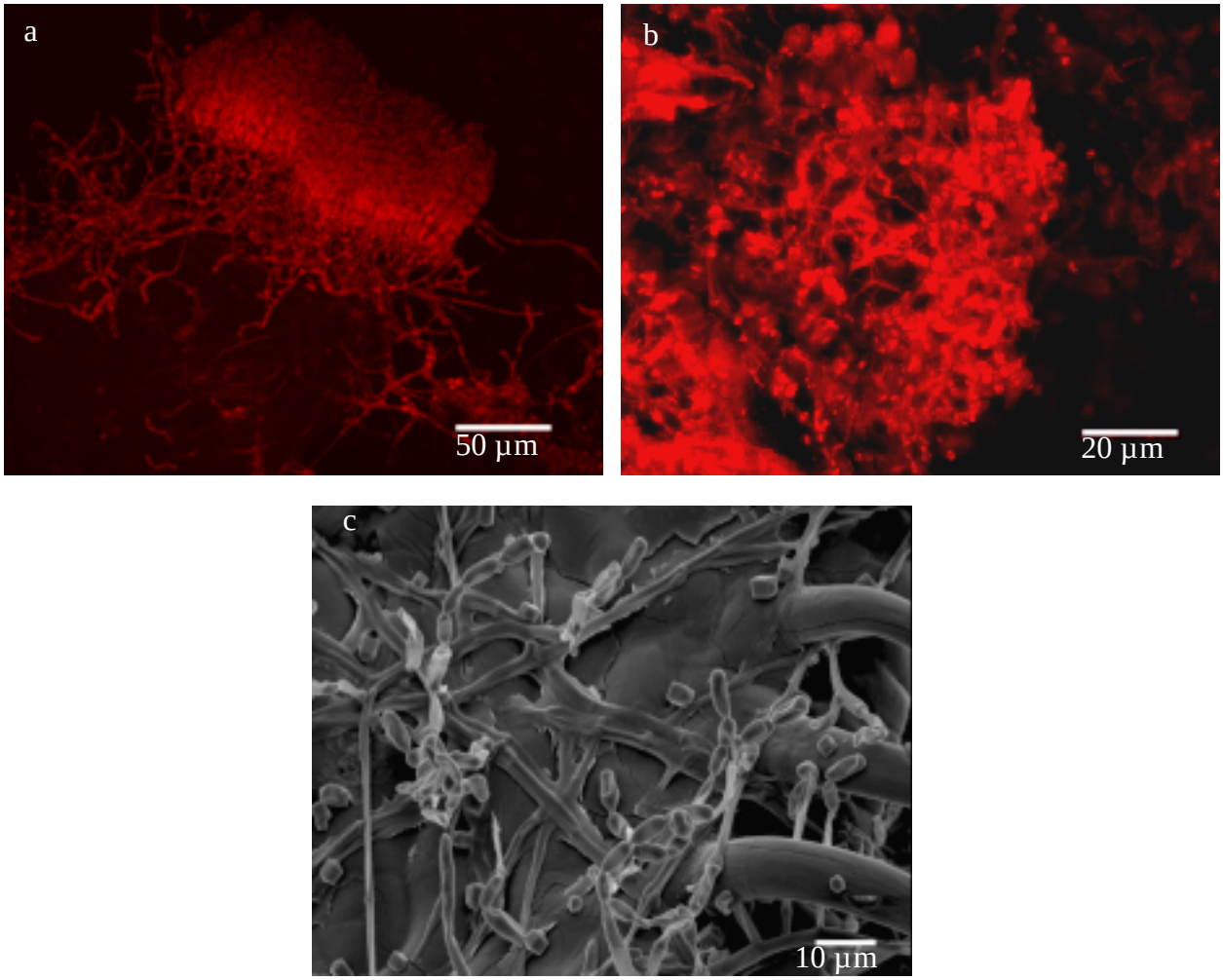

Figure 3-4. Sporulation of entomopathogenic fungi on 4th instar larvae of *T. vaporariorum: M. anisopliae* (a) and (c), *P. fumosoroseus* (b) (moist chamber, 6 days after inoculation, images (a) and (b) by CLSM, image (c) by LTSEM).

3.1.3 Effect of the whiteflies' developmental stage on the efficacy of the antagonist

In a whitefly population all developmental stages can be found and, therefore, need to be controlled. Adults and 1st larval instars are mobile while eggs and the other larval stages are sedentary. Cuticle thickness is different for all the stages. For a successful pest control, the antagonist should affect all the developmental stages of the insect despite their differences. The susceptibility of eggs, 1st instar larvae, 2nd instar larvae, pupae and adults to *M. anisopliae* var. *anisopliae* was investigated in different experiments.

3.1.3.1 Pathogenicity of *M. anisopliae* against whitefly eggs

Published results on the efficacy of entomopathogenic fungi against eggs are contradictory. Therefore, the control potential of *M. anisopliae* var. *anisopliae* for eggs of *B. argentifolii* was evaluated in an experiment on detached leaves. Spore suspension was applied to three days-old eggs and the egg hatch was determined on day 6 after inoculation. Furthermore, the mortality of the hatched larvae was assessed (Table 3-1).

It was observed that the fungus had not only an effect on egg hatch but was also able to infect 1st instar larvae of the silverleaf whitefly after hatching. Only about 33 % of the larvae had hatched from the eggs which were treated with *M. anisopliae* and fungal growth could be observed on the ones that were not hatched. The mortality of the hatched 1st instar larvae was 45 % for the eggs treated with fungus while 97 % of the larvae in the control survived.

Table 3-1. Efficacy of *M. anisopliae* var. *anisopliae* 108 on egg hatch and larval mortality of *B. argentifolii* (cotton, 10^7 spores / ml, 26 °C, 100 % RH).

treatment	hatched larvae, 6 dpi (%)	larval mortality, 10 dpi (%)
Tween® 80	98	3
M108	33*	45*

* significant differences within the columns (t-Test, $p \leq 0.05$)

3.1.3.2 Susceptibility of different whitefly larval stages to the antagonist

Looking at chemical pest control, different larval stages have different susceptibilities to insecticides and higher concentrations are required to control the older larval stages (ISHAAYA *et al.*, 1993). Therefore, the control potential of the antagonist for the 1st, 2nd and 4th larval stage of *Trialeurodes vaporariorum* was evaluated under greenhouse conditions. Differences were observed in the potential of *M. anisopliae* var. *anisopliae* 43 to control the different larval stages. The 1st instar was the most, the 4th instar the least susceptible growth stage (Figure 3-5).

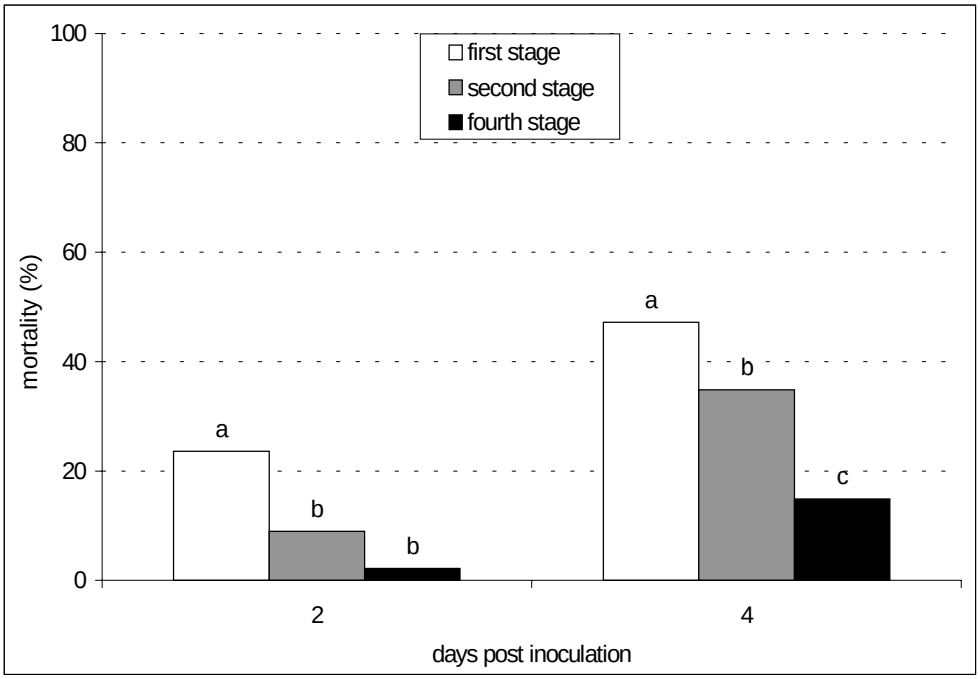

Figure 3-5. Efficacy of *M. anisopliae* var. *anisopliae* 43 against different larval stages of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters on the same day of evaluation indicate significant differences, DUNN'S, $p \leq 0.05$).

3.1.3.3 Effect of *M. anisopliae* on whitefly adults

Whitefly adults are usually found on the undersides of leaves. Compared to the larvae, they are mobile and start flying when they come in contact with the plant protection treatment. Therefore, the effect of the antagonist on adults was investigated in a choice and no-choice assay with a prophylactic treatment on detached leaves. The location of probing and the fertility, expressed by the number of laid eggs, were used as parameters for evaluation of the control success (Table 3-2).

Whitefly adults were released inside a box that contained two leaves, one previously treated with an aqueous suspension of Tween® 80 only and one treated with conidia of *M. anisopliae* var. *anisopliae* 43 formulated in the same aqueous suspension. The number of adults and eggs on each leaf was determined after 48 hours at 26 °C.

The prophylactic treatment with the fungus had no effect on the location of probing of whitefly adults. For the reproduction no significant differences could be observed between the treatments either although the number of eggs on the leaves treated with the antagonist decreased slightly in comparison to the one on the leaves treated with surfactant only. After 48 hours, all of the whitefly adults were still alive.

Table 3-2. Effect of a prophylactic treatment with *M. anisopliae* var. *anisopliae* 43 on the location of probing and the reproduction of *T. vaporariorum* adults (tomato, 10^7 spores / ml, 26 °C, 80 % RH, evaluation 48 hours post infestation).

parameter	**leaf treatment**	
	Tween® 80	M43
no. of adults	65	54
no. of eggs	483	316
eggs per adult	7.4	5.6

no significant differences between treatments (t-Test, $p \leq 0.05$)

3.2 Investigations on the antagonists' cultivation conditions

Within the production process different factors do affect the cultivation of entomopathogenic fungi. Type of culture media, amount of available water, temperature and humidity can have an impact on mycelial growth, spore production and conidial viability of the antagonists. It was investigated whether the requirements could differ for different species and strains. The effect of different solid culture media and of media with different water activities on the cultivation of *M. anisopliae* and *P. fumosoroseus* was determined in order to characterise the strains and to receive preliminary information about their performance under challenging environmental conditions.

3.2.1 Effect of different culture media on fungal growth parameters

The antagonists have been reported to grow on a variety of media but requirements for different species were varying (IGNOFFO, 1988). Three current solid culture media, Potato-Dextrose agar (PDA), Sabouraud-Dextrose agar (SDA) and Oatmeal agar (OMA), were compared for their effect on mycelial growth, spore production and spore viability of different isolates of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* (Table 3-3, Table 3-4).

Concerning mycelial growth no interspecific variation could be observed. No similarities were found for the different species or varieties, the differences only occurred between the strains of *M. anisopliae* var. *anisopliae* and of *P. fumosoroseus.*

Results for sporulation and viability of conidia were found to be similar. Differences between the species or varieties did not occur but differences between the strains of *M. anisopliae* var. *anisopliae* were observed. The culture media did not affect sporulation or viability of spores of *P. fumosoroseus* while conidial viability of *M. anisopliae* var. *acridum* was higher on OMA than on the other two media.

Table 3-3. Effect of different culture media on the mycelial growth of different isolates of *M. anisopliae* var. *anisopliae, M. anisopliae* var. *acridum* and *P. fumosoroseus* at 26 °C in the dark.

isolate	mycelial growth after 3 days (cm)			mycelial growth after 6 days (cm)		
	PDA	SDA	OMA	PDA	SDA	OMA
M. anisopliae var. *anisopliae*						
M27	1.1	1.0	1.0	2.1	2.1	2.4*
M43	1.2	1.0	1.2	2.9	2.6	2.2*
M108	1.3*	1.1	1.1	2.9	2.1*	2.5
M. anisopliae var. *acridum*						
M5	0.9	0.8	0.8	1.6	1.7	1.7
M11	1.3	1.2	1.1	2.4	2.4	1.9
P. fumosoroseus						
P1	1.3	1.2	0.9*	1.9	2.2	1.9
P2	1.3	1.3	1.1	2.2	2.2	2.3

* significant difference to other culture media (DUNN'S, $p \leq 0.05$)

It has to be taken into account that only a few strains were tested so that results and tendencies might be different if the influence of culture media on entomopathogenic fungi would be investigated on a higher number of strains.

Table 3-4. Effect of different culture media on sporulation and conidial viability of different isolates of *M. anisopliae* var. *anisopliae, M. anisopliae* var. *acridum* and *P. fumosoroseus* at 26 °C in the dark.

isolate	sporulation after 6 days (10^6 spores/ml)			conidial viability (%)		
	PDA	SDA	OMA	PDA	SDA	OMA
M. anisopliae var. *anisopliae*						
M27	1*	0.3	0.2	93	84*	90
M43	8	10	10	73	75	63
M108	20	20	10*	80	77	90
M. anisopliae var. *acridum*						
M5	10	10	6	82	90	95*
M11	0.3	0.02	0.8	31*	85	98
P. fumosoroseus						
P1	2	0.2	3	100	100	100
P2	3	0.8	20*	100	100	100

* significant difference to other culture media (DUNN'S, $p \leq 0.05$)

3.2.2 Effect of water availability on fungal growth

Water is an obligatory parameter for fungal growth. The availability of water in culture media can be limited by the glycerol content (DALLYN & FOX, 1980). Response of different species and strains of entomopathogenic fungi to a limited amount of available water was investigated to determine inter- and intraspecific variations. The amount of available water was expressed by the water activity (A_w) of the medium. Mycelial growth and sporulation of different strains of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* were examined on Yeast-Glucose agar (YGA) with different water activities, corresponding to relative humidities ranging from 92 % to 100% (Figure 3-6, Figure 3-7). Differences in the response to decreasing water activity were observed between the isolates but not between the species. Mycelial growth was decreasing with decreasing water availability. Sporulation was also decreasing except for one isolate of *M. anisopliae* var. *acridum* and one strain of *P. fumosoroseus* where sporulation was observed to increase slightly with decreasing water activity.

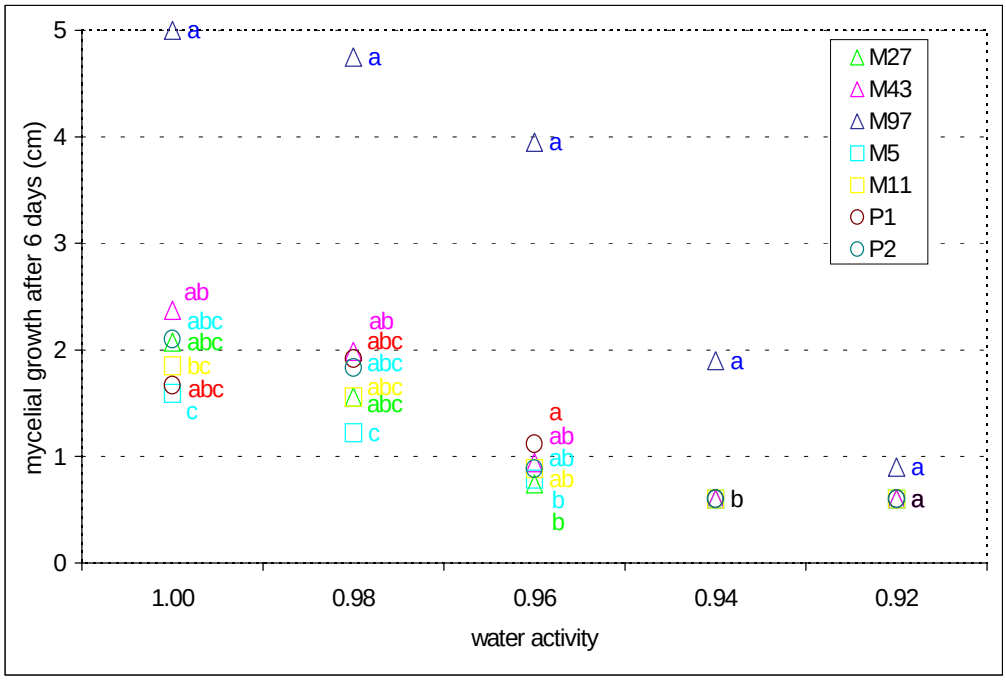

Figure 3-6. Effect of different water activities on mycelial growth of different isolates of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* at 26 °C in the dark (different letters indicate significant differences between values for the same water activity, DUNN'S, $p \leq 0.05$).

Strain *M. anisopliae* var. *anisopliae* 97 was the most tolerant to low humidity. While no other isolate grew below 96 % RH, growth of strain 97 could still be observed at 94 % RH already three days after incubation. No mycelial growth could be detected at 92 % RH. No sporulation could be observed below 96 % RH except for *M. anisopliae* var. *anisopliae* 97 which was still sporulating at 94 % RH. A high sporulation rate was monitored with this strain at 96 % RH while the other isolates did only produce few spores. *M. anisopliae* var. *anisopliae* 27 did not sporulate below 100 % RH and *M. anisopliae* var. *acridum* 11 did not produce spores at all after six days. No sporulation was observed for both strains of *P. fumosoroseus* below 98 % RH.

In general, variation was found to be intra- but not interspecific. As mentioned before the number of isolates tested was low. The isolate *M. anisopliae* var. *anisopliae* 97 was the most tolerant strain to low humidity in growth and sporulation.

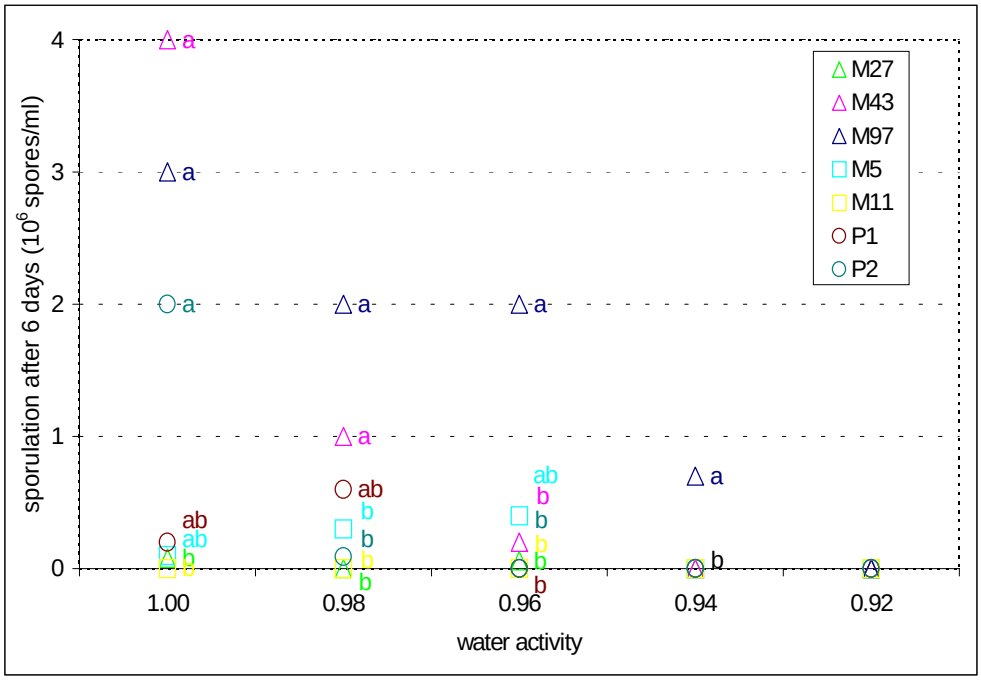

Figure 3-7. Effect of different water activities on sporulation of different isolates of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* at 26 °C in the dark (different letters indicate significant differences between values for the same water activity, DUNN'S, $p \leq 0.05$).

3.3 Combination of biological antagonists with insect growth regulators

The insect cuticle serves as a barrier for the penetration of entomopathogenic fungi. Pesticides, which kill the insects by inhibiting the chitin synthesis, are found in the group of insect growth regulators. As chitin is one of the main cuticle components a reduced level of chitin might increase the efficacy of entomopathogenic fungi. Therefore, a possible synergism between *M. anisopliae* or *P. fumosoroseus* and 'buprofezin' or 'novaluron' was investigated. Sublethal doses of the insecticides were used in order to prevent the development of resistances. Experiments were carried out on larval stages of *B. argentifolii* and *T. vaporariorum*. Additionally, the potential of a combined treatment of insecticide and antagonist for the control of *Spodoptera littoralis*, the Egyptian cotton leafworm, was evaluated for its uptake of the insecticide differs from that of the whitefly. Studies on the mode of action were performed by assessing the chitin and protein level of *S. littoralis* cuticle.

3.3.1 Compatibility of *M. anisopliae* and *P. fumosoroseus* with chitin synthesis inhibitors

Chitin is not only found in the insect cuticle but is also one of the main components of most fungal cell walls. The compatibility of the fungi with 'novaluron' or 'buprofezin' was assessed by investigating spore germination and mycelial growth.

Fungal spores were formulated in Tween® 80, different amounts of the chitin synthesis inhibitors were added and the viability of the spores was evaluated at different periods after the preparation, keeping the formulation at 26 °C (Table 3-5). Fungal spore suspension with Tween® 80 was also spread on PDA medium containing the above-mentioned amounts of insecticides (Table 3-6).

Table 3-5. Spore germination of *M. anisopliae* var. *anisopliae* 108 and *P. fumosoroseus* in a formulation with different concentrations of 'novaluron' ('buprofezin') at 0, 3, 10 and 24 hours post preparation (germination on WA after 24 hours of incubation at 26 °C).

isolate	hours post preparation	spore germination (%) 'novaluron' ('buprofezin') content (ppm)				
		0	0.25 (2.5)	0.5 (5)	1 (10)	4 (40)
M108	0	31 (35)	33 (36)	49 (38)	41 (32)	38 (40)
	3	30 (33)	29 (27)	34 (36)	36 (37)	48 (33)
	10	32 (31)	34 (40)	47 (42)	29 (33)	44 (37)
	24	68 (72)	59 (65)	61 (61)	76 (69)	66 (70)
P2	0	55 (56)	56 (54)	55 (56)	52 (53)	57 (54)
	3	49 (52)	48 (53)	52 (56)	56 (56)	52 (55)
	10	49 (55)	47 (52)	48 (53)	53 (49)	56 (48)
	24	77 (72)	82 (83)	80 (79)	82 (79)	84 (86)

no significant differences between concentrations (TUKEY, $p \leq 0.05$)

Results were similar for 'novaluron' and 'buprofezin' as well as for both fungal species. No significant differences could be found between the different preparations. When the spore viability was assessed at 24 hours after the preparation the spore germination rate was generally about twice as high as at 0, 3 and 10 hours after preparation. Spore germination of both strains was similar for all the treatments. Incubating the plates at 26 °C for six days after the first evaluation led to mats of mycelium, which covered all plates equally.

Table 3-6. Spore germination of *M. anisopliae* var. *anisopliae* and *P. fumosoroseus* formulated with Tween® 80 on PDA containing 'novaluron' ('buprofezin').

isolate	spore germination (%) 'novaluron' ('buprofezin') content (ppm)				
	0	0.25 (2.5)	0.5 (5)	1 (10)	4 (40)
M108	98 (98)	97 (98)	99 (99)	98 (99)	97 (99)
P2	99 (95)	99 (99)	95 (98)	95 (97)	95 (98)

no significant differences between concentrations (TUKEY, $p \leq 0.05$)

3.3.2 Efficacy of entomopathogens on whiteflies in a combined treatment with insect growth regulators

The efficacy of *M. anisopliae* var. *anisopliae* in combination with sublethal doses of 'buprofezin' and 'novaluron' was determined. For this purpose the fungal spores were formulated with different concentrations of the insect growth regulators. Experiments were carried out with *B. argentifolii* on detached leaves in moist chambers (Table 3-7).

The combinatory treatments consisting of 'novaluron' and fungus resulted in significantly higher mortalities for the investigated concentrations while the combination of 'buprofezin' and fungus showed similar results as 'buprofezin' alone. Therefore only 'novaluron' was tested under greenhouse conditions. No significant differences could be observed between the treatments with insecticide alone and with insecticide and antagonist under greenhouse conditions.

Table 3-7. Efficacy of *M. anisopliae* var. *anisopliae* 108 combined with 'buprofezin' and 'novaluron' on 1st instar larvae of *B. argentifolii* in a moist chamber and under greenhouse conditions (cotton, 10^7 spores / ml, evaluation on day 4 post treatment, values corrected for controls by Abbot's formula (ABBOTT, 1925)).

treatment	larval mortality (%)			
	moist chamber		greenhouse	
	without fungus	with fungus	without fungus	with fungus
Tween® 80	3	37*	10	36*
'novaluron' 0.025 ppm	35	65*	37	48
'novaluron' 0.05 ppm	36	53*	41	48
'buprofezin' 1 ppm	54	57	n.d.	n.d.
'buprofezin' 2 ppm	44	61	n.d.	n.d.

* significant difference between the treatment with and without fungus under the same environmental conditions (t-Test, $p \leq 0.05$)

Trialeurodes vaporariorum is less susceptible to 'novaluron' than *B. argentifolii* (ISHAAYA *et al.*, 1996; ISHAAYA *et al.*, 1998). Therefore, the effect of a combinatory treatment with 'novaluron' in ten fold higher concentrations than in the previous experiments and the antagonist was investigated on detached tomato leaves in moist chambers (Table 3-8).

Adding the fungal conidia to the insect growth regulator resulted in significantly higher mortalities than applying the insecticide alone. For 0.25 ppm 'novaluron' the mortality was increased from 19 % to 41 % but 42 % mortality of the insect were already achieved by applying the fungus alone. For a 'novaluron' concentration of 0.5 ppm the mortality was increased by 27 % so that only the effect of the higher 'novaluron' concentration was investigated under greenhouse conditions.

The mortality rate of *T. vaporariorum* decreased strongly under greenhouse conditions. Only 10 % of the larvae had died on day 4 after the application of the antagonist. The same mortality was achieved with 'novaluron' alone. 16 % mortality were obtained with the combined treatment but the difference could not be confirmed statistically.

Table 3-8. Efficacy of *M. anisopliae* var. *anisopliae* 108 in a combinatory treatment with 'novaluron' on 1st instar larvae of *T. vaporariorum* in a moist chamber and under greenhouse conditions (tomato, 10^7 spores/ml, evaluation on day 4 post treatment, values corrected for controls by Abbot's formula (ABBOTT, 1925)).

treatment	**larval mortality (%)**			
	moist chamber		**greenhouse**	
	without fungus	**with fungus**	**without fungus**	**with fungus**
Tween® 80	7	42*	5	10*
'novaluron' 0.25 ppm	19	41*	-	-
'novaluron' 0.5 ppm	32	59*	10	16

* significant difference between the treatment with and without fungus under the same environmental conditions (t-Test, $p \leq 0.05$)

Chitin synthesis inhibitors act on the newly synthesised chitin. After moulting the chitin level of a larva treated with the insecticide will be lower than the chitin level of an untreated larva. Thus, the chitin level is only reduced if the insecticide treatment has taken place prior to moulting. Therefore, a possible synergism of chitin synthesis inhibitor and entomopathogenic fungus is more likely to occur when the insecticide has been applied before moulting and fungal inoculation has taken place thereafter.

A combined treatment of *M. anisopliae* var. *anisopliae* 108 and 'novaluron' was compared to single applications with a time interval in between (Figure 3-8). The treatments were applied on the same day and with a two day interval. The larval mortality was evaluated after eight days and fungal emergence from the dead larvae was examined after three days of incubation in a moist chamber.

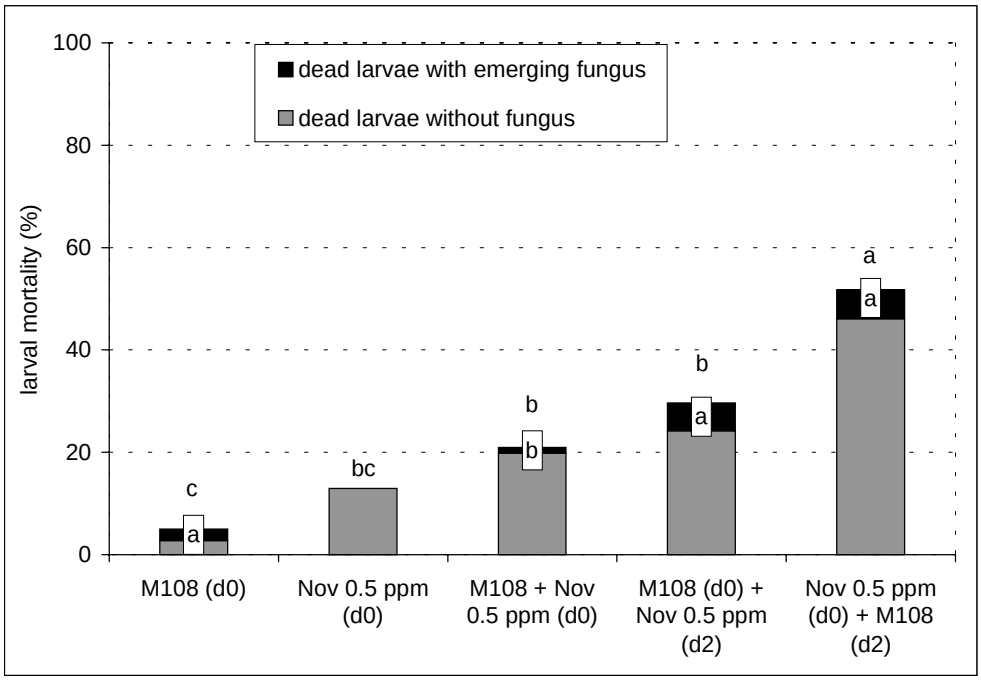

Figure 3-8. Efficacy of *M. anisopliae* var. *anisopliae* 108 in a combined treatment with 'novaluron' (0.5 ppm), applied together or with two days interval, on 1st instar larvae of *T. vaporariorum* under greenhouse conditions (tomato**,** 10^7 spores/ml, evaluation on day 8 after first application and after three days in a moist chamber, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters indicate significant differences, TUKEY, $p \leq 0.05$).

As already assumed the application of 'novaluron' two days before the fungus resulted in about 50 % mortality, significantly higher than in all the other treatments. Applying the antagonist and 'novaluron' together led to a significantly lower mortality and so did the application of *M. anisopliae* var. *anisopliae* 108 on day 0 and of 'novaluron' on day 2 with about 20 % and about 28 %, respectively. Fungal emergence was observed for 2 % of the dead larvae when the antagonist was applied alone and for 5 % and 6 % for the successive treatments. A significantly lower value (1 %) was found if the treatments were applied together.

Older larval stages are less susceptible than the 1st instar (compare 3.1.3.2). Therefore, investigations were carried out on 2nd instar larvae with 1.5 ppm 'novaluron' and a more virulent strain of *M. anisopliae* var. *anisopliae* (Figure 3-9). Additionally antagonist or 'novaluron' alone were applied twice with the same time interval than the combined treatments.

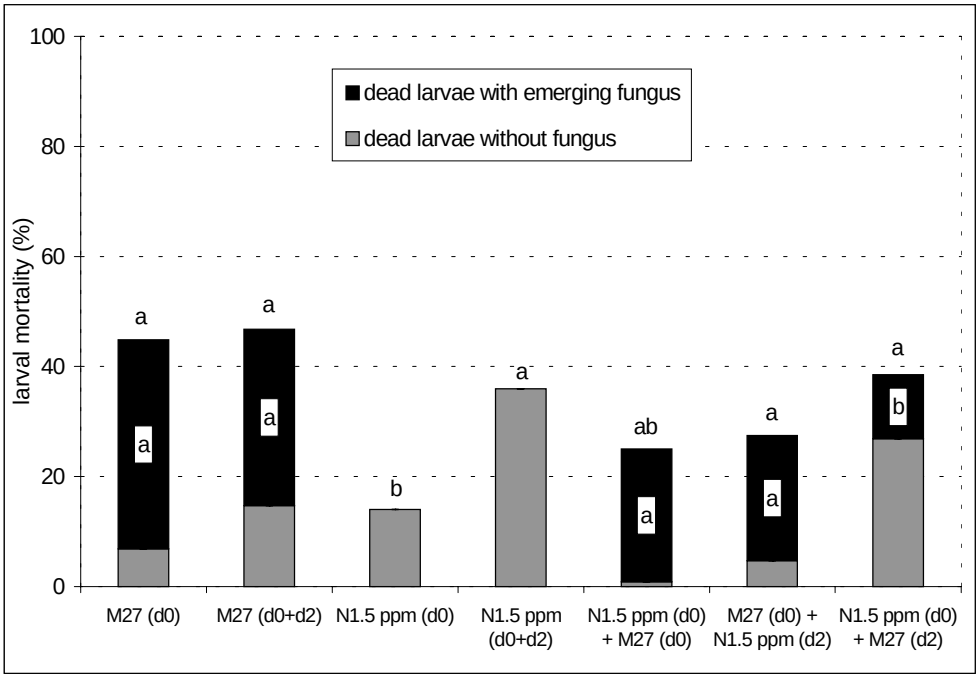

Figure 3-9. Efficacy of *M. anisopliae* var. *anisopliae* 27 in a combined treatment with 'novaluron' (1.5 ppm), applied together or with two days interval, on 2nd instar larvae of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores/ml, evaluation on day 8 after first application and after three days in a moist chamber, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters indicate significant differences, DUNN'S, $p \leq 0.05$).

No significant differences could be observed between the treatments except for the single application of the insect growth regulator alone that caused a significantly lower mortality than all the other variations. In general, not more than 45 % mortality were achieved with either treatment. For the emergence of the antagonist from the dead larvae 90 up to 98 % were colonised in all the variations except for the combined treatment of 'novaluron' on day 0 and *M. anisopliae* var. *anisopliae* 27 on day 2. The fungus was found to emergence only from about 30 % of the insects.

3.3.3 Effect of entomopathogenic fungi and 'novaluron' on *Spodoptera littoralis*

Spodoptera littoralis is an important lepidopteran pest in tropical and subtropical regions. In these bioassays 'novaluron' acted on ingestion instead of contact. Hence, investigations on the potential of the entomopathogenic fungal isolates for the control of the Egyptian cotton leafworm were performed. The possibility to enhance the efficacy of the fungi with sublethal doses of 'novaluron' was evaluated. In order to investigate the mode of action of 'novaluron' in combination with entomopathogenic fungi, biochemical assessments on protein and chitin content of the insect cuticle were carried out.

3.3.3.1 Susceptibility of *Spodoptera littoralis* to entomopathogenic fungi

A screening for virulence of the different isolates against *S. littoralis* larvae in the 3rd instar did not show any effect of the treatments after four days (Table 3-9). Significant differences to the untreated control could not be found either in the larval weight gain, expressing the feeding activity, or in the larval mortality. After eight days none of the strains led to more than 16 % mortality.

Table 3-9. Efficacy of different isolates of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* on 3rd instar larvae of *Spodoptera littoralis* (castor-oil plant, 26 °C, dark, $5x10^7$ spores / ml, mortality values corrected for controls by Abbott's formula (ABBOTT, 1925)).

fungal isolate	average larval weight gain in difference to control, 4 dpi (mg)	larval mortality, 4 dpi (%)	larval mortality, 8 dpi (%)
M. anisopliae var. *anisopliae*			
M27	-13.2	0	6
M43	-4.1	0	15*
M97	-6.8	0	0
M108	-38.8	8	15*
V245	+2	0	5
M. anisopliae var. *acridum*			
M2	-10.2	0	0
M5	-10.2	6	9
P. fumosoroseus			
P2	+17.3	0	0

* significantly different (DUNN'S, $p \leq 0.05$)

3.3.3.2 Efficacy of entomopathogens on *S. littoralis* in a combined treatment with 'novaluron'

M. anisopliae var. *anisopliae* 43 was chosen as one of the most virulent strains to *S. littoralis* for further investigations. When immersed in spore suspension and fed on leaves treated with 'novaluron', the larval weight gain after four days decreased significantly compared to the untreated control or to the larvae treated with the fungus alone (Table 3-10). With increasing 'novaluron' concentrations the larvae became smaller (e.g. the weight gain was reduced by about 120 mg after treatment with 0.2 ppm) but additional treatment with fungal spores did not result in a significantly lesser weight gain than with the corresponding amount of 'novaluron' alone. Concerning the mortality after four days the combined treatment with fungus and 'novaluron' increased the number of dead larvae significantly compared to each corresponding 'novaluron' concentration (Figure 3-10). The speed of kill was significantly increased. After eight days the mortality of the larvae exposed to the combined treatment had increased but was not significantly different to those treated with 'novaluron' alone in the higher concentrations (Figure 3-11). A significant difference between the combined and the single insecticide treatment could only be found for 0.1 ppm insecticide. The speed of kill as well as the efficacy of the fungus were increased so that a high potential for pesticide reduction could be determined.

Table 3-10. Efficacy of a combined treatment of 'novaluron' and *M. anisopliae* var. *anisopliae* 43 on 3rd instar larvae of *Spodoptera littoralis* (castor-oil plant, 26 °C, dark, $5x10^7$ spores / ml, mortality values corrected for controls by Abbott's formula (ABBOTT, 1925)).

treatment	average larval weight loss in difference to control, 4 dpi (mg)
Tween® 80 + M43	-7 d
'novaluron' 0.1 ppm	-74 c
'novaluron' 0.1 ppm + M43	-98 bc
'novaluron' 0.2 ppm	-120 ab
'novaluron' 0.2 ppm + M43	-120 ab
'novaluron' 0.4 ppm	-120 ab
'novaluron' 0.4 ppm + M43	-125 a

different letters indicate significant differences within columns (TUKEY, $p \leq 0.05$)

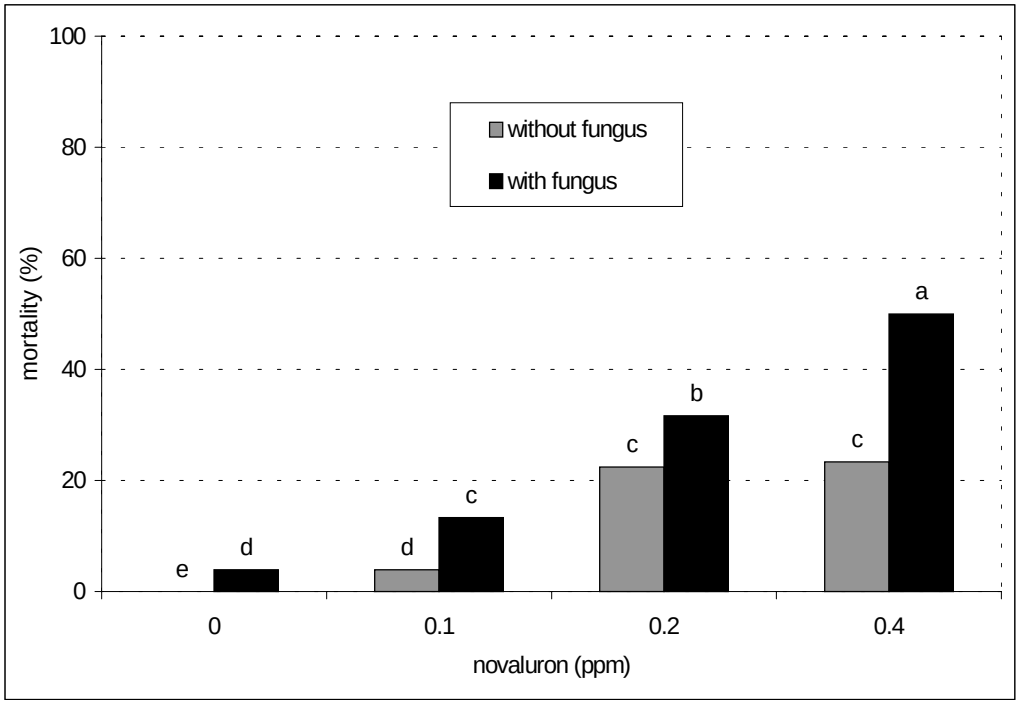

Figure 3-10. Efficacy of a combined treatment of 'novaluron' and *M. anisopliae* var. *anisopliae* 43 on 3rd instar larvae of *Spodoptera littoralis*, 4 days post inoculation (castor-oil plant, 26 °C, dark, $5x10^7$ spores / ml, different letters indicate significant differences, DUNN'S, $p \leq 0.05$).

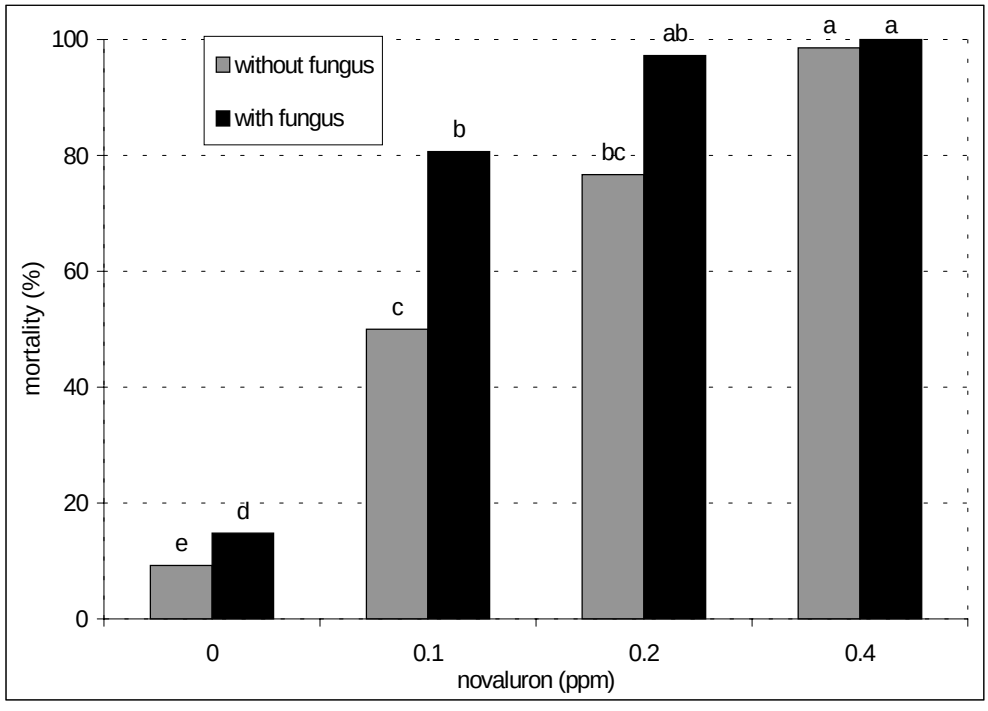

Figure 3-11. Efficacy of a combined treatment of 'novaluron' and *M. anisopliae* var. *anisopliae* 43 on 3rd instar larvae of *Spodoptera littoralis*, 8 days post inoculation (castor-oil plant, 26 °C, dark, $5x10^7$ spores / ml, different letters indicate significant differences, DUNN'S, $p \leq 0.05$).

3.3.3.3 Effect of the antagonist and 'novaluron' on components of the insect cuticle

Biochemical assessments for the chitin and the protein content of *S. littoralis* cuticle were made to investigate the mode of action of the insecticide alone and in combination with the fungus (Table 3-11). No significant differences could be observed between the different variations neither for the chitin nor for the protein content of the insect cuticle.

Table 3-11. Effect of a combined treatment with 'novaluron' and *M. anisopliae* var. *anisopliae* 43 on the chitin and protein content of the cuticle of 3rd instar larvae of *Spodoptera littoralis* (castor-oil plant, 26 °C, dark, $5x10^7$ spores / ml).

treatment	**chitin in % of cuticle fresh weight**	**protein in % of cuticle fresh weight**
Tween® 80	2.48	2.24
Tween® 80 + M43	2.90	2.16
'novaluron' 0.1 ppm	2.35	1.89
'novaluron' 0.1 ppm + M43	2.76	2.16
'novaluron' 0.2 ppm	2.21	2.24
'novaluron' 0.2 ppm + M43	3.22	1.77
'novaluron' 0.4 ppm	3.07	2.62
'novaluron' 0.4 ppm + M43	3.38	2.21

no significant differences between the treatments (TUKEY, $p \leq 0.05$)

3.4 Effect of additives on the efficacy of entomopathogenic fungi

Water is normally used as a carrier for fungal inoculum in a spray application. Conidia of *M. anisopliae* and *P. fumosoroseus* are hydrophobic so that the surfactant Tween ® is usually added for suspending them in water. Improvements to the formulation of fungal inoculum might enhance their effectiveness for crop protection. Different workers have reported oil formulations to be superior to water-based suspensions (BATEMAN *et al.*, 1993; MALSAM *et al.*, 2001; SINGH, 1977). The potential of polymeric additives to enhance the success of biological control has only been cited recently (PIGGOT *et al.*, 2000; PUTERKA, 1999). Conidia of *M. anisopliae* and *P. fumosoroseus* were formulated with different oils, waxes and polymeric additives in order to investigate the effect of the formulation on the antagonist and its efficacy at several stages of the production and application process.

3.4.1 Potential for storage of formulated conidia

Shelf-life is a crucial factor for the acceptance of microbial insecticide by growers and public. Insecticidal properties of the microbial control agent must not be affected by normal storage conditions, so that appropriate production, formulation and stabilisation is necessary.

The viability of fungal spores formulated with different additives was examined after they had been stored in their formulation at 4 °C and 26 °C. Spore suspensions of *M. anisopliae* var. *anisopliae* 27 or strain 43 in Tween ® 80, PA1, PA2 or Stockosorb ®Agro were stored either in their liquid state (Figure 3-12, Figure 3-13) or dried beforehand (Figure 3-14, Figure 3-15) and checked for conidial viability after different periods of storage. In all formulations the viability of the spores exceeded 96 % before storage.

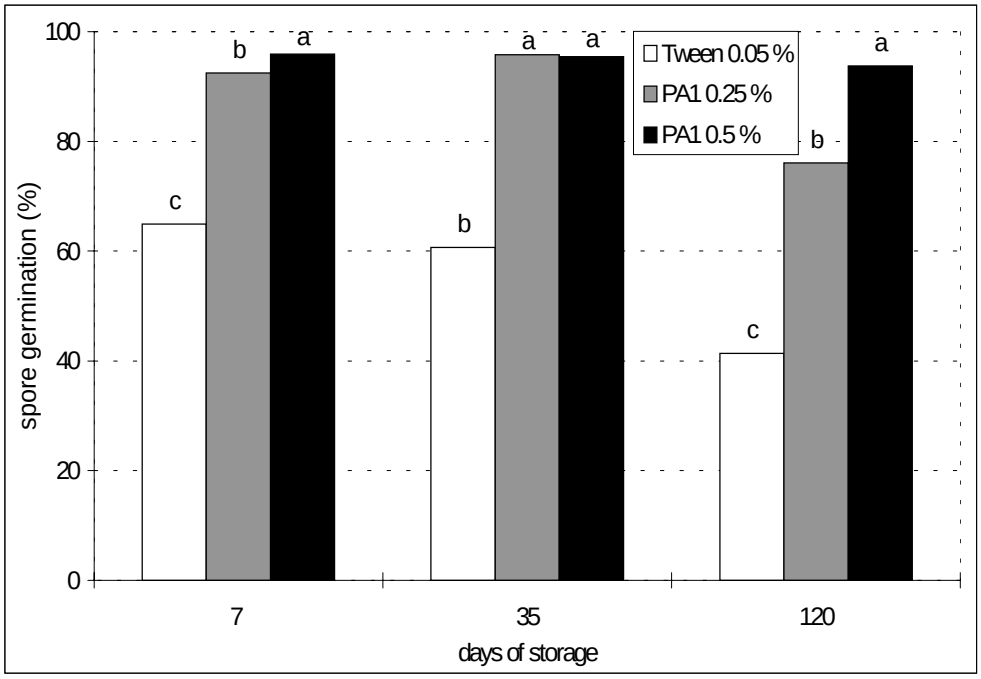

Figure 3-12. Effect of different additives on the viability of formulated conidia of *M. anisopliae* var. *anisopliae* 27, stored in liquid state at 4 °C (different letters indicate significant differences between values on the same day of evaluation, DUNN'S, $p \leq 0.05$).

The spore viability after storage at 4 °C in the suspensions formulated with PA1 was significantly higher than in the one formulated with Tween ® 80 (Figure 3-12). After seven days of storage about 65 % of the spores in the Tween ® 80 formulation were still viable, a decrease to about 40 % was observed after 120 days. Formulating the conidia with PA1 (0.25 %) resulted in about 95 % viability after 35 days but the number of viable spores decreased to about 85 % after four months of storage. Almost all conidia remained viable for four months when formulated in PA1 (0.5%) and stored at 4 °C.

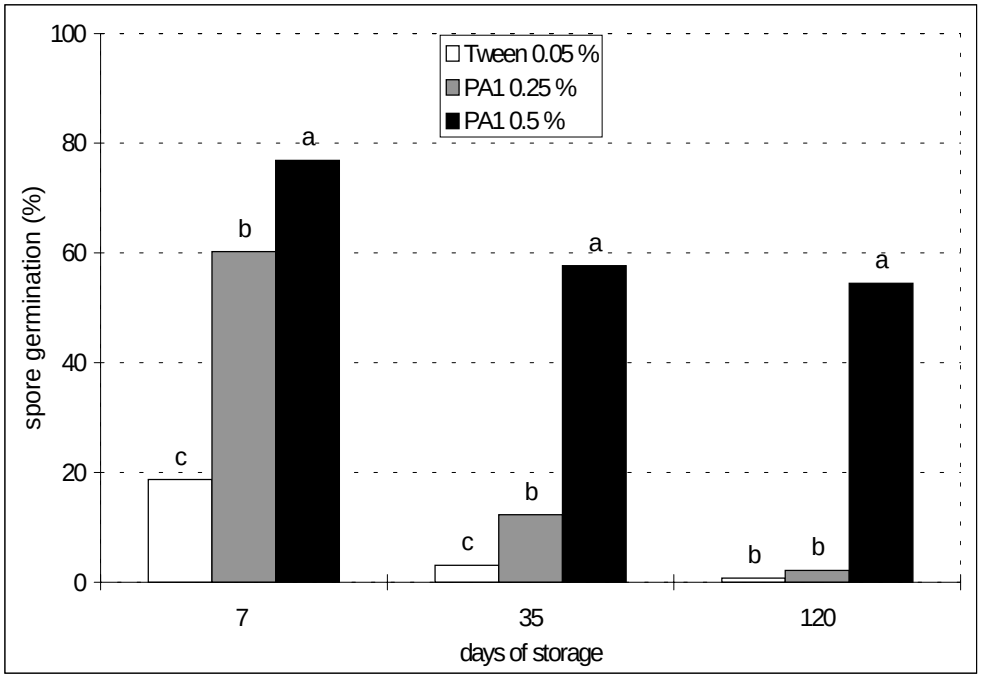

Figure 3-13. Effect of different additives on the viability of conidia of *M. anisopliae* var. *anisopliae* 27 stored in liquid state at 26 °C (different letters indicate significant differences between values on the same day of evaluation, DUNN'S, $p \leq 0.05$).

In general, a lower number of viable spores was observed when storing them at 26 °C instead of 4 °C (Figure 3-13). Corresponding to the results at the lower temperature less viable conidia were found when Tween® 80 had been added to *M. anisopliae* var. *anisopliae* 27. Only about 20 % of the conidia formulated in Tween® 80 were still viable after one week of storage and the number decreased to 2 % after four months. When formulated in PA1 (0.25 %) about 60 % of the spores were still able to germinate after one week but the rate of viable spores decreased to about 10 % after 35 days and to 5 % after 120 days of storage. Formulating the spores in a higher concentration of PA1 (0.5 %) led to about 80 % viable spores after one week. After four months of storage still more than half of the conidia were found to be viable.

Drying the conidia in their formulations after preparation and storing them in their dry state led to different results (Figure 3-14, Figure 3-15). In general, spore viability decreased within the storage period.

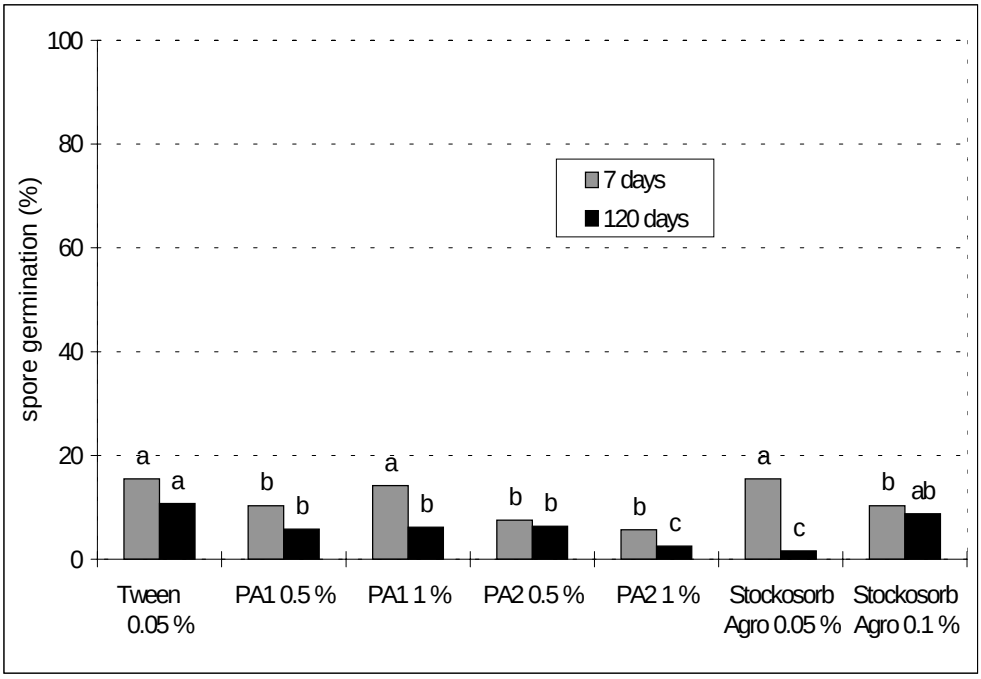

Figure 3-14. Effect of different additives on the viability of conidia of *M. anisopliae* var. *anisopliae* 27 stored in dry state at 4 °C (different letters indicate significant differences between values on the same day of evaluation, DUNN'S, $p \leq 0.05$).

Only 6 - 16 % of the spores stored at 4 °C were still viable after seven days and viability decreased to 2 - 11 % after 120 days. Significant differences were observed between the formulations, Tween® 80 and Stockosorb®Agro (0.1 %) were found to preserve viability after four months of storage while conidia formulated in PA2 (1 %) and Stockosorb®Agro (0.05 %) lost their viability faster than the ones formulated differently.

Except for Tween® 80 and Stockosorb®Agro (0.05 %) the number of viable conidia remained higher after storage at 26 °C than at 4 °C. The viability of the spores stored in PA1 and PA2 increased with the concentration of the additive and was generally higher in PA1. The best preservation of viability was achieved with PA1 (1 %) where 93 % of the spores were still viable after seven days and viability decreased to 84 % after 120 days. With Stockosorb®Agro (1 %) 72 % of the conidia were able to germinate after four months, while no spores were viable in the same formulation at a lower concentration (0.05 %). In PA1 (0.5 %) and PA2 (0.5 and 1 %) 60 %, 18 % and 39 % of the conidia remained viable after 120 days, respectively.

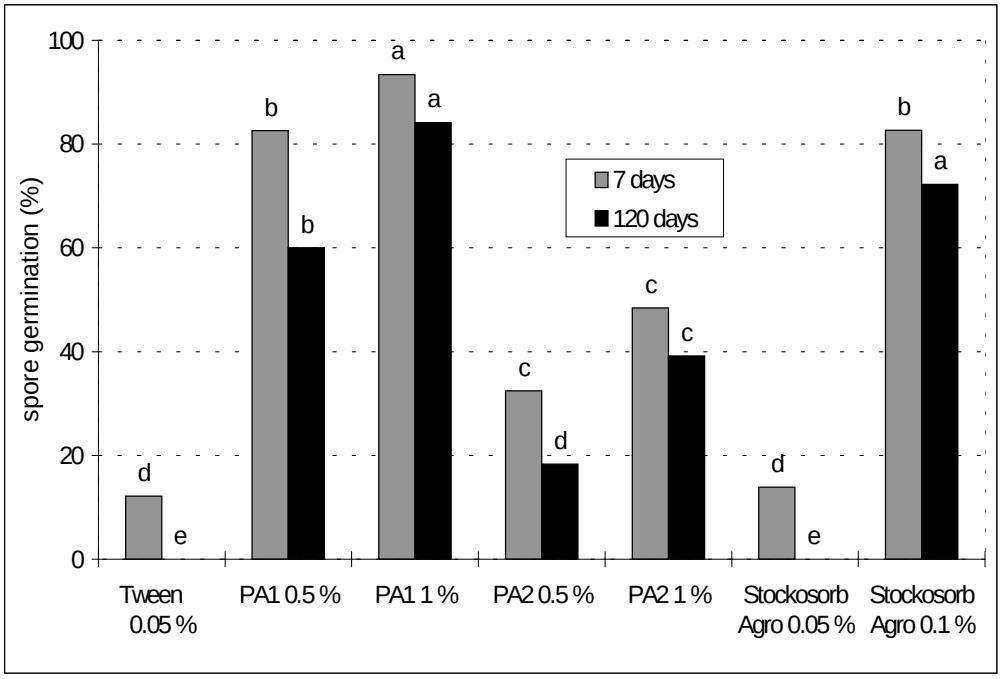

Figure 3-15. Effect of different additives on the viability of conidia of *M. anisopliae* var. *anisopliae* 27 stored in dry state at 26 °C (different letters indicate significant differences between values on the same day of evaluation, DUNN'S, $p \leq 0.05$).

The additives PA1 and Stockosorb®Agro increased the storage potential of *M. anisopliae* and showed perspectives for an acceptable shelf-life of the microbial control agent at room temperature.

3.4.2 Effect of additives on the distribution of formulations on leaves

The distribution of formulations on the leaf surface is dependent on the properties of the formulation and the host plant surface. Nevertheless, an even distribution is necessary for hitting the target and reducing the amount of spray. Microscopical assessments of the coverage of leaves after spraying and measurements of average droplet diameters were performed by staining the formulations with Nile red before spraying and examining them with a fluorescence microscope after drying. The use of different additives led to visible differences in the droplet size and the spreading of the formulations on the surface of tomato leaves (Figure 3-16, Figure 3-17).

With Tween® 80 mainly small droplets were spread evenly all over the surface (Figure 3-16 a). Using Addit® as additive resulted in small and partially in bigger droplets (Figure 3-16 b). The same results were found for Agrocer® (Figure 3-16 c). With PA1 the leaf surface was more or less covered completely with big droplets depending on the concentration of the additive (Figure 3-16 d and e). The droplet size and distribution of PA2 (Figure 3-16 f) were very much the same as those of PA1. Significant differences were found between the formulations when the droplet diameters were measured (Figure 3-17). The droplets of PA1 (0.5 %) were the biggest with about 900 µm in diameter while Tween® 80 was spread in very small droplets of about 200 µm in diameter. The lower concentration of PA1 (0.25 %) formed smaller droplets than the higher concentration but bigger ones than Addit® with an average diameter of about 370 µm.

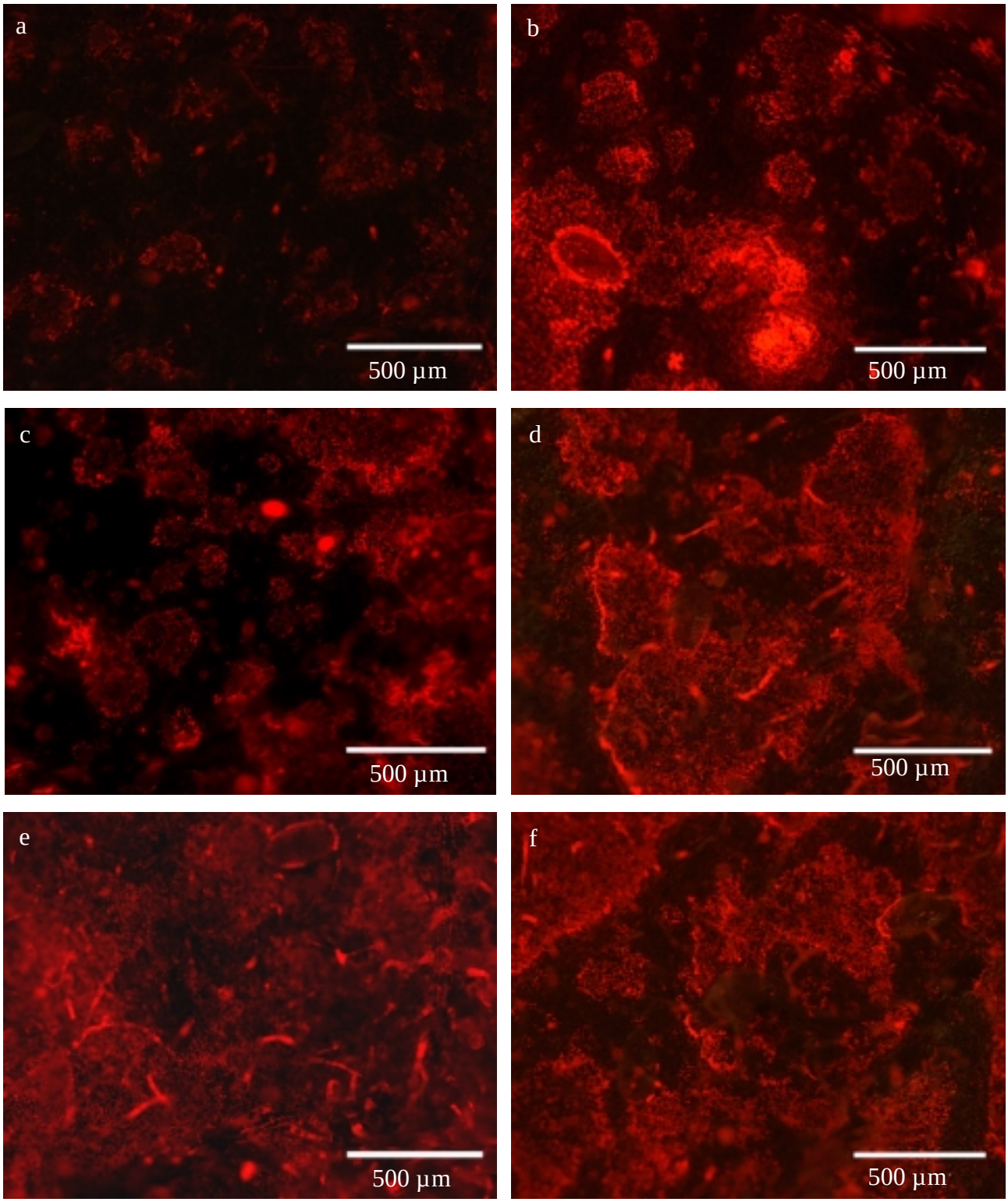

Figure 3-16. Fluorescence microscope images of the distribution of different formulations on tomato leaf surfaces: Tween® 80 (a), Addit® (b), Agrocer® (c), PA1, 0.25 % (d), PA1, 0.5 % (e) and PA2, 0.25 % (f) (formulations stained with Nile red).

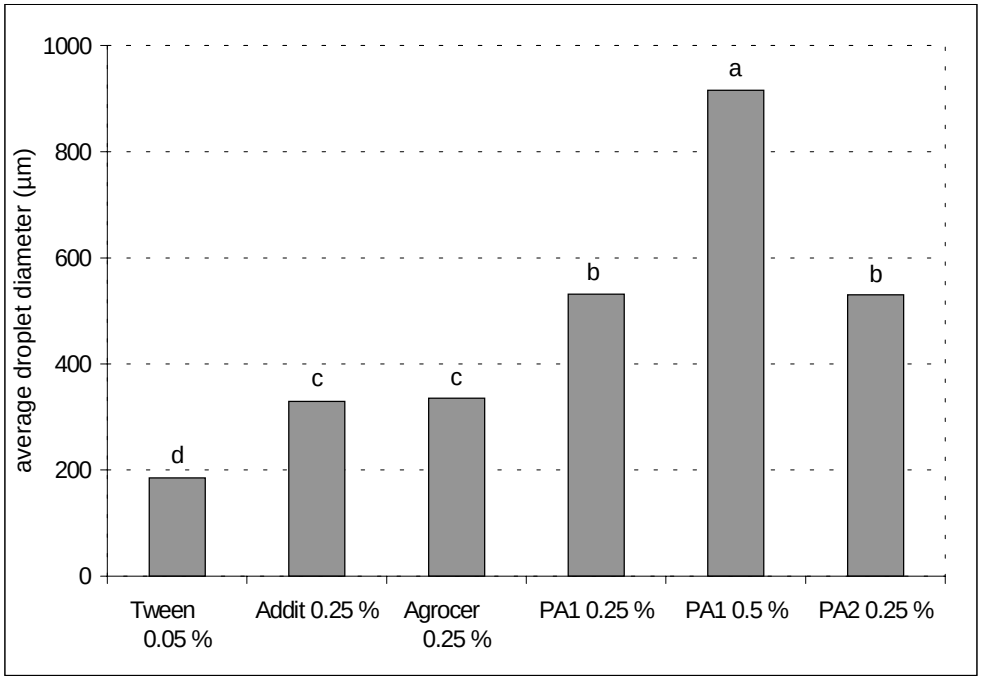

Figure 3-17. Effect of different additives on the droplet diameter of formulations on tomato leaf surfaces (different letters indicate significant differences, DUNN'S, $p \leq 0.05$).

3.4.3 Spore adhesion and viability on leaf surfaces

The effectiveness of entomopathogenic fungi for crop protection partly depends on the persistence of applied inoculum on the leaf surfaces. Only a portion of conidia applied on the crop will germinate and infect insects. Inoculum might be inactivated by UV radiation or washed off by irrigation. Inoculum is also diluted as a result of leaf expansion (INYANG *et al.* 1998). These factors reduce the success of pest control but might be corrected by improving the formulation.

The number of spores sticking to the abaxial leaf surface was determined by examining leaf impressions on WA for conidia of *Metarhizium anisopliae* (Figure 3-18). Leaf impressions were taken over a period of 14 days, beginning with the day of the application. The decrease in the number of spores that remained on the leaf surface was very fast when the fungus was formulated with Addit ®. Appointing the number of conidia on the day of application as 100 %, only 35 % of the conidia were still found on the leaf surface on day 1. After seven days about 10 % of the spores were left and only 2 % were found after two weeks. In the standard formulation with Tween ® 80 still more than 80 % of the conidia were stuck to the abaxial leaf surface on day 1 and about 50% were found after four days, but after two weeks the number had decreased to about 7 %.

With Agrocer® more conidia remained on the leaf surface compared to the Tween® 80 and the Addit® formulation so that still about 60 % of the spores were found after one week. After 14 days the number had decreased to about 17 %, significantly more than with the other two formulations.

The additive PA1 in a 0.25 % and a 0.5 % concentration caused a very low decrease in the number of spores of *M. anisopliae* var. *anisopliae* 43. With the 0.25 % formulation, about 80 % of the conidia remained on the leaf surface after 14 days while more than 95 % were still attached to the leaf with the 0.5 % formulation. Microscopical assessments found dense gel-bodies with embedded conidia, even after 14 days.

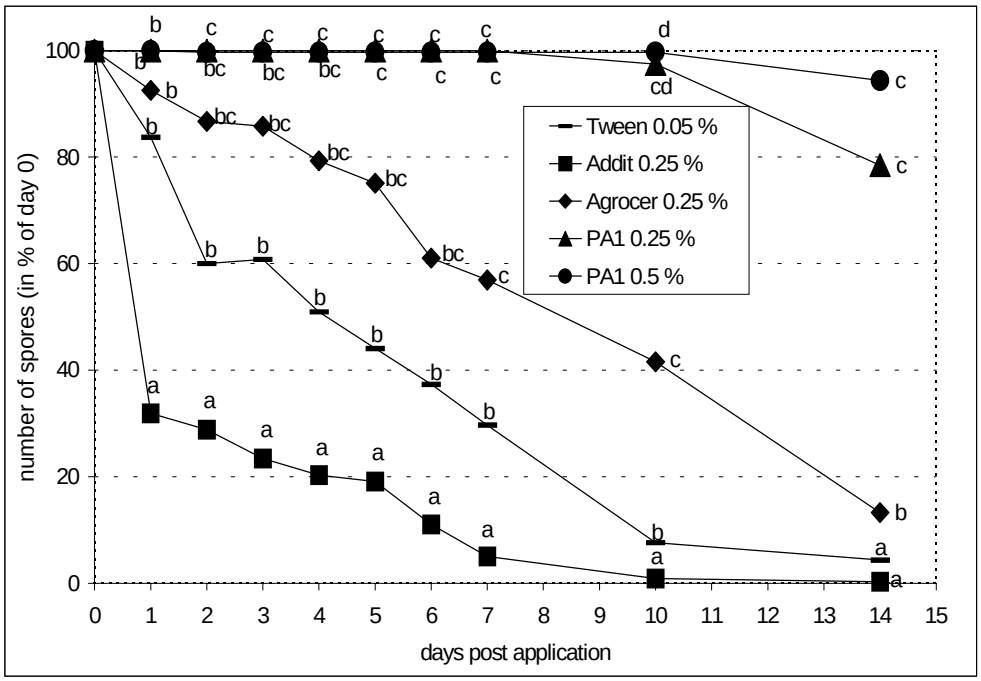

Figure 3-18. Effect of different formulations on the adhesion of spores of *M. anisopliae* var. *anisopliae* 43 on the abaxial leaf surface of tomato leaves under greenhouse conditions (10^6 spores / ml, different letters on the same day of evaluation indicate significant differences, DUNN'S, $p \leq 0.05$).

The viability of the fungal spores was assessed on day 0, 7 and 14 post application (Figure 3-19). On day 0 a significantly higher viability could be observed with the additive PA1 compared to the Tween® 80 formulation. The formulation with Addit® and Agrocer® resulted in a slightly but not significantly higher viability. On day 7 no significant differences could be found between Tween® 80, Addit® or Agrocer®, the spore viability decreased to about 40 %. With PA1 (0.25 %) about 60 % of the conidia were still viable but the difference to the Tween® 80, Addit® and Agrocer® formulations was not significant. The results on day 14 were corresponding except for the Agrocer® formulation where less than 10 % of the conidia were still viable. Increasing the concentration of PA1 to 0.5 % led to an increase in the viability of the spores. About 90 % and 80 % were found to be viable after seven and fourteen days.

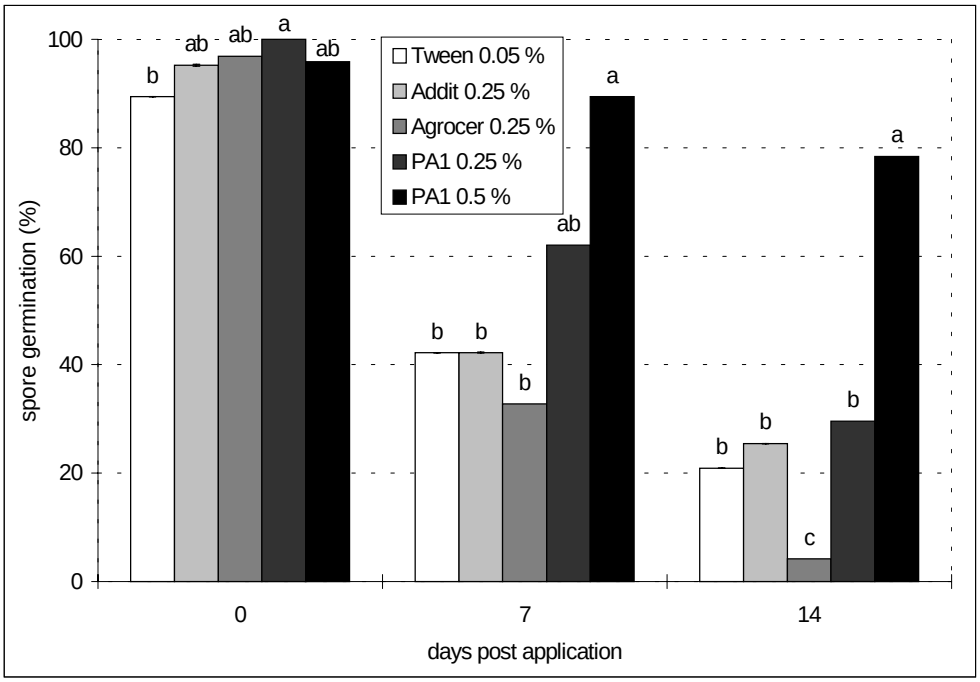

Figure 3-19. Viability of spores of *M. anisopliae* var. *anisopliae* 43 formulated with different additives on the abaxial leaf surface of tomato leaves under greenhouse conditions (10^6 spores / ml, different letters on the same day of evaluation indicate significant differences, DUNN'S, $p \leq 0.05$).

3.4.4 Spore distribution on whitefly larvae

The distribution of formulations – and hence of the spores – on the larvae is as important as the distribution on leaf surfaces. The distribution of the formulation on the insect, affected by the "wettability" of the host and the hydrophobic properties of the formulation, and the distribution of the spores within the formulation might have an effect on the control of the target. The effect of additives on the distribution of conidia on larvae of the target insect was investigated with a low temperature laser scanning microscope and a confocal laser scanning microscope (Figure 3-20, Figure 3-21).

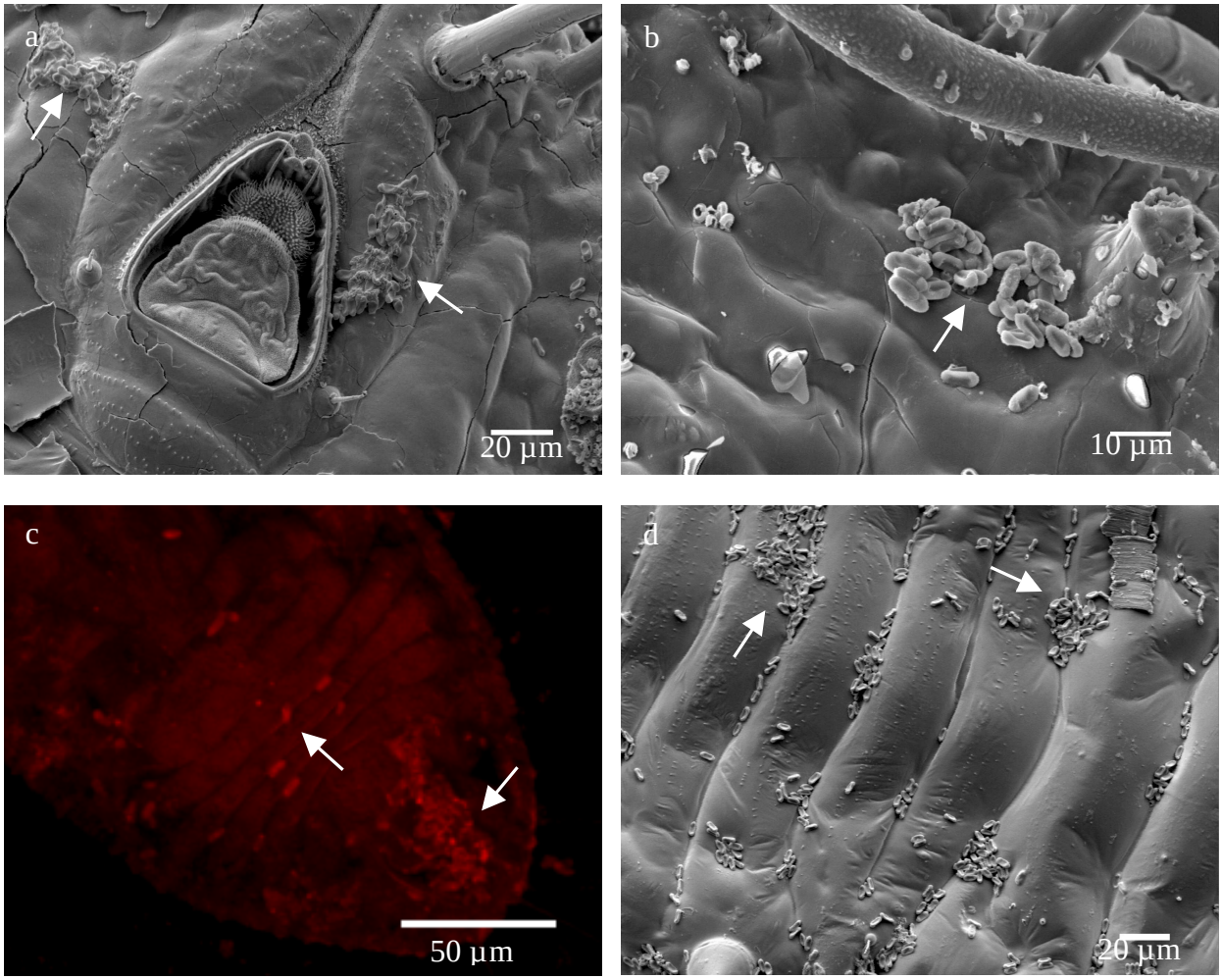

Figure 3-20. Effect of different additives on the spore distribution of *M. anisopliae* on the cuticle of 4th instar larvae of *T. vaporariorum*: Tween® 80 (a) and (b), Addit® (c) and (d) (images (a), (b) and (d) by LTSEM, (c) by CLSM).

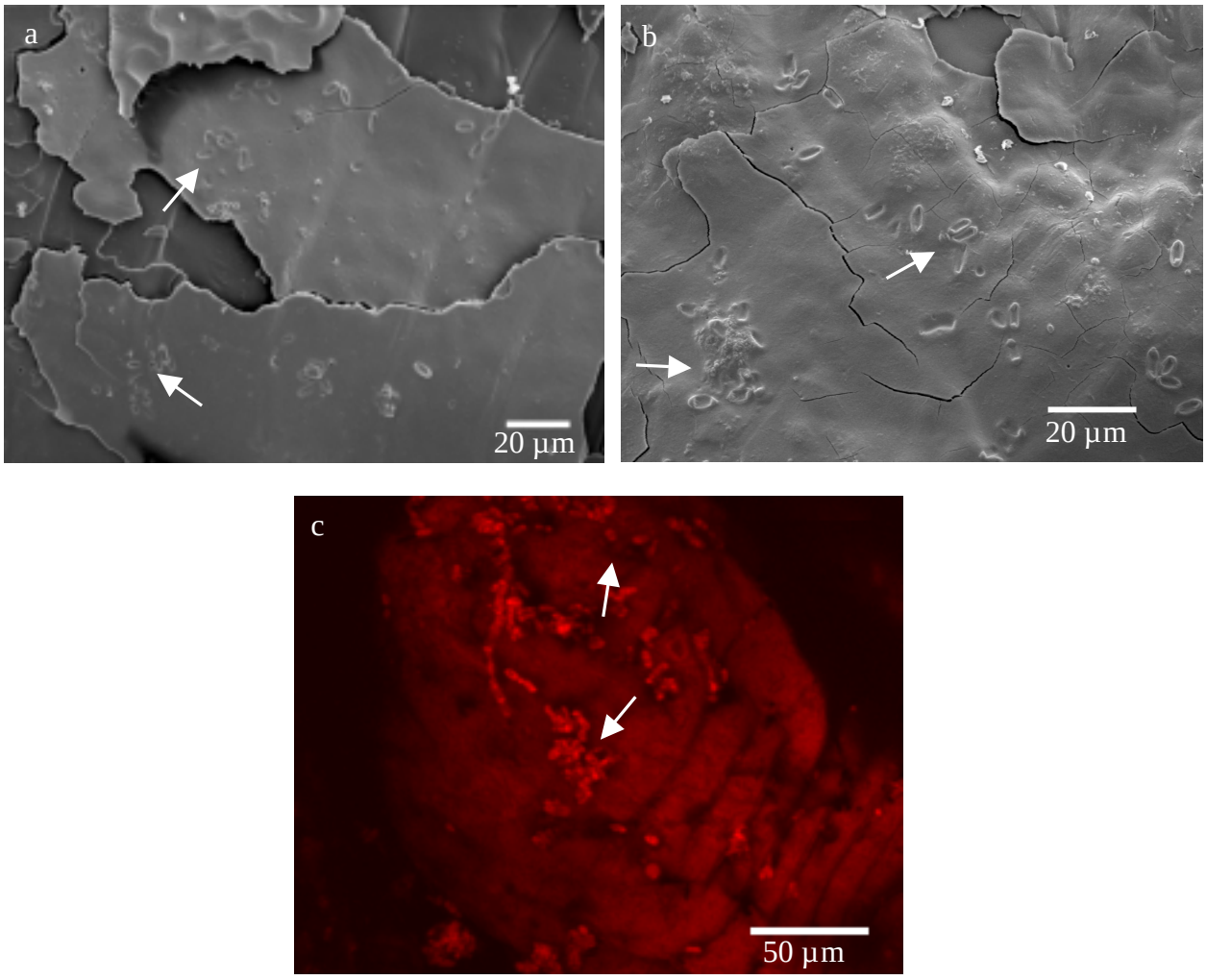

Figure 3-21. Effect of different additives on the spore distribution of *M. anisopliae* on the cuticle of 4th instar larvae of *T. vaporariorum*: Agrocer® (a) and (b), PA1 (c) (images (a) and (b) by LTSEM, (c) by CLSM).

Applying the Tween® 80 formulation (Figure 3-20 a, b) resulted mainly in clusters of spores that were found all over the insect. Formulating the conidia in Addit® (Figure 3-20 c, d) or PA1 (Figure 3-21 c) led mainly to a distribution of solitary conidia and a few small clusters. The formulation with Agrocer® (Figure 3-21 a, b) developed a "film" in which the conidia were embedded and spread solitarily all over the insect cuticle.

3.4.5 Spore germination of the antagonists

Under greenhouse conditions the relative humidity on the leaf surfaces varies and is found to be less than 100 %. Reduced relative humidity is supposed to be a limiting factor for the growth of the entomopathogenic fungi. Therefore, the spore germination of two strains of *M. anisopliae* var. *anisopliae* and one strain of *Paecilomyces fumosoroseus* was examined at reduced relative humidity and the effect of different additives was investigated *in vitro* (Figure 3-22, Figure 3-24, Figure 3-24). Additives can be able to extract substances from the insect cuticle that can stimulate spore germination or act fungistatically (IBRAHIM *et al.*, 1999; SOSA-GOMEZ *et al.*, 1997). Germination of conidia was therefore also examined on insect cuticle at reduced relative humidity (Figure 3-25).

For *M. anisopliae* var. *anisopliae* V245 the time required for a spore germination rate of 50 % (GT_{50}) at 98 % RH was significantly reduced by PA1 (1 %) compared to the other formulations. In contrast, Agrocer ® or Stockosorb ®Agro (0.05 %) increased the GT_{50} (Figure 3-22). At 96 % RH it could be observed as well that the germination time was longer for the conidia formulated with Agrocer ® and Stockosorb ®Agro (0.05 %) compared to the other formulations. The formulation with PA1 (1 %) and Addit ® led to a significantly shorter germination time. The results for *P. fumosoroseus* 2 at 96 % RH were found to be similar (Figure 3-23).

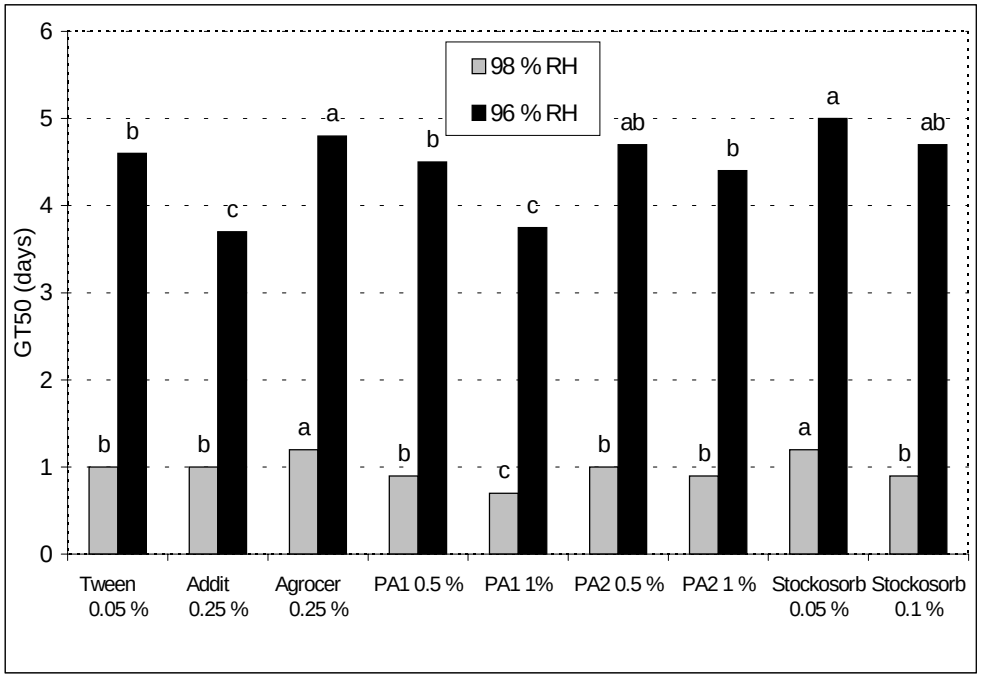

Figure 3-22. Effect of different additives on the time required for 50 % spore germination (GT_{50}) of *M. anisopliae* var. *anisopliae* V245 at 96 % and 98 % RH (26 °C, darkness, different letters indicate significant differences between data evaluated under the same conditions, TUKEY, $p \leq 0.05$).

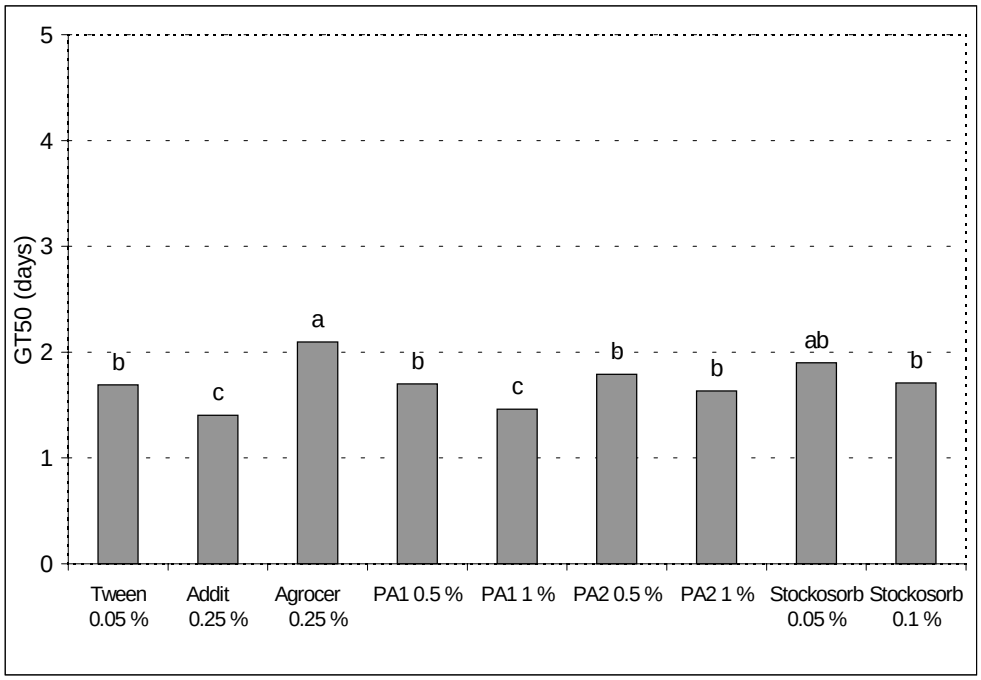

Figure 3-23. Effect of different additives on the time required for 50 % spore germination (GT_{50}) of *P. fumosoroseus* 2 at 96 % RH (26 °C, darkness, different letters indicate significant differences between additives, TUKEY, $p \leq 0.05$).

The strain 97 of *M. anisopliae* var. *anisopliae* was more tolerant to low humidities than the other strains investigated (compare 3.2.2). Spore germination could still be observed at 94 % RH in all formulations (Figure 3-24). The results corresponded with those for the other isolates. The GT_{50} was reduced significantly by Addit® and PA1 (1 %), while Agrocer® and Stockosorb®Agro (0.05 %) increased the time required for spore germination.

Investigations with *M. anisopliae* var. *anisopliae* V245 on elytra of *B. discoidalis* led to slightly different results (Figure 3-25). No differences were observed at 98 % RH, the time taken for spore germination was one day with either formulation. For 96 % RH a significant reduction of the GT_{50} was found with PA1 in a concentration of 0.5 % and 1 %. Contrary to the investigations on agar medium the conidia were still germinating at 94 % RH. The effects of the additives were comparable to those *in vitro*. The formulation with Addit® and PA1 (1 %) led to a decrease in the time taken for germination while the formulation with Agrocer® resulted in a significant increase.

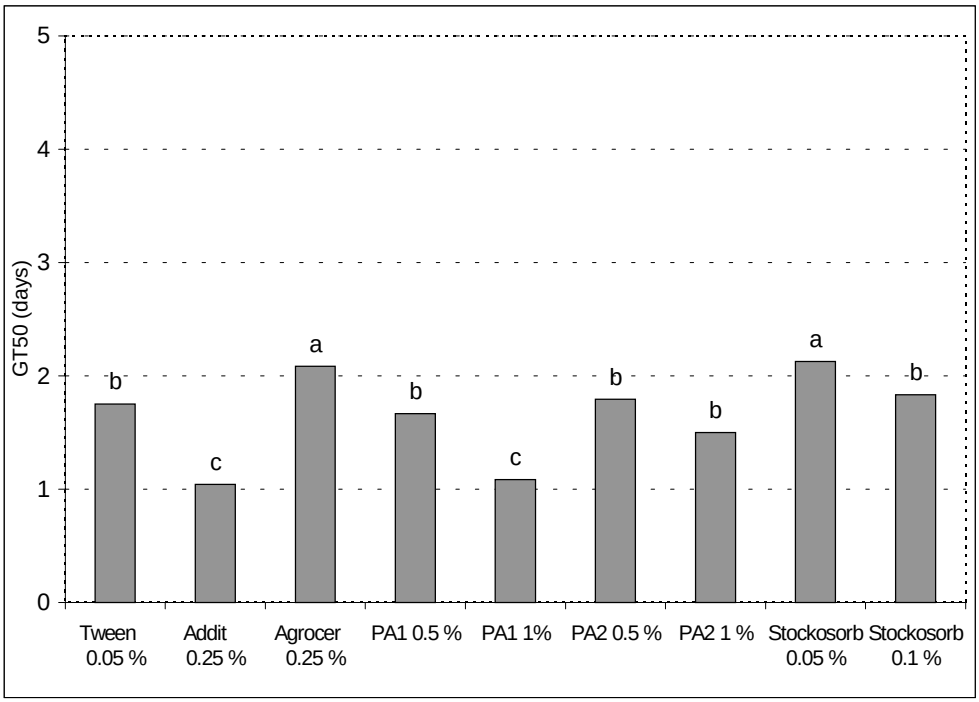

Figure 3-24. Effect of different additives on the time required for 50 % spore germination (GT_{50}) of *M. anisopliae* var. *anisopliae* 97 at 94 % RH (26 °C, darkness, different letters indicate significant differences between additives, TUKEY, $p \leq 0.05$).

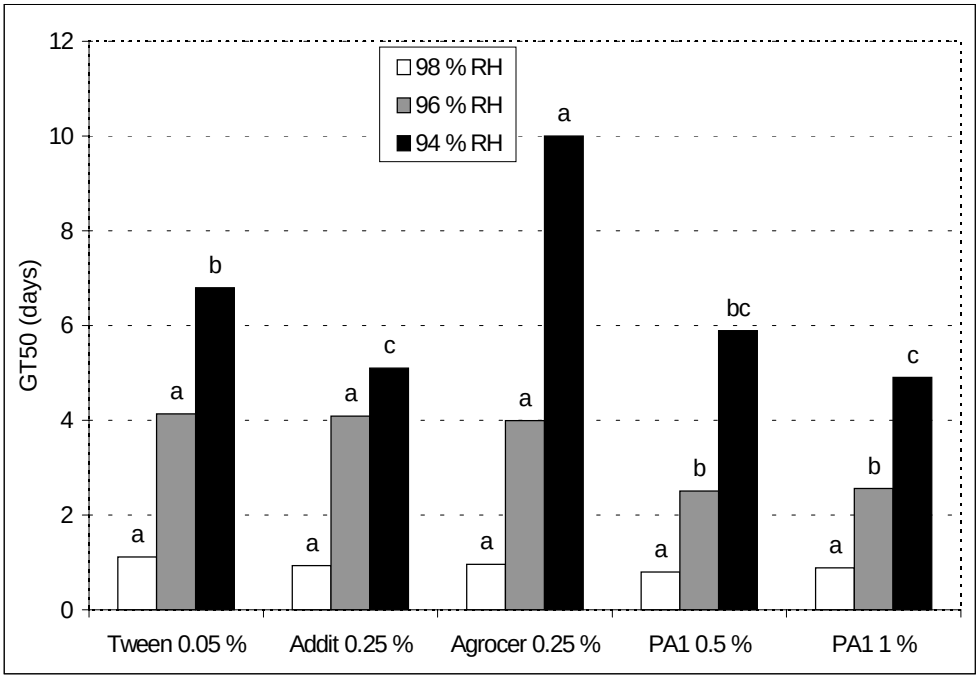

Figure 3-25. Effect of different additives on the time taken for 50 % spore germination (GT_{50}) of *M. anisopliae* var. *anisopliae* V245 at 94 %, 96 % and 98 % RH on the cuticle of *Blaberus discoidalis* (26 °C, darkness, different letters indicate significant differences between data evaluated under the same conditions, TUKEY, $p \leq 0.05$).

Relative humidity is easy to measure in the greenhouse and outside. Nevertheless, relative humidity in the air is not always corresponding to the microclimate on the leaves or around the insects (MILNER *et al.*, 1997). Therefore, the relative humidity required for fungal germination *in vitro* might not be similar to the humidity required in the greenhouse. Microscopical assessments of spore germination on larvae of *T. vaporariorum* were performed to evaluate the effect of the additives under greenhouse conditions (Figure 3-26). Specimens were taken from bioassays and fixated two days after inoculation. A germination rate of almost 100 % could be observed for the conidia formulated in Addit ® (Figure 3-26 b). Spore germination was lower for the PA1 formulation at a concentration of 0.5 % and only a few spores germinated when formulated in Tween ® 80 (Figure 3-26 a, d). No spore germination could be observed for the conidia formulated in Agrocer ® (Figure 3-26 c). Addit ® and PA1 showed a high potential for enhancing spore germination of *M. anisopliae* on *T. vaporariorum* under greenhouse conditions.

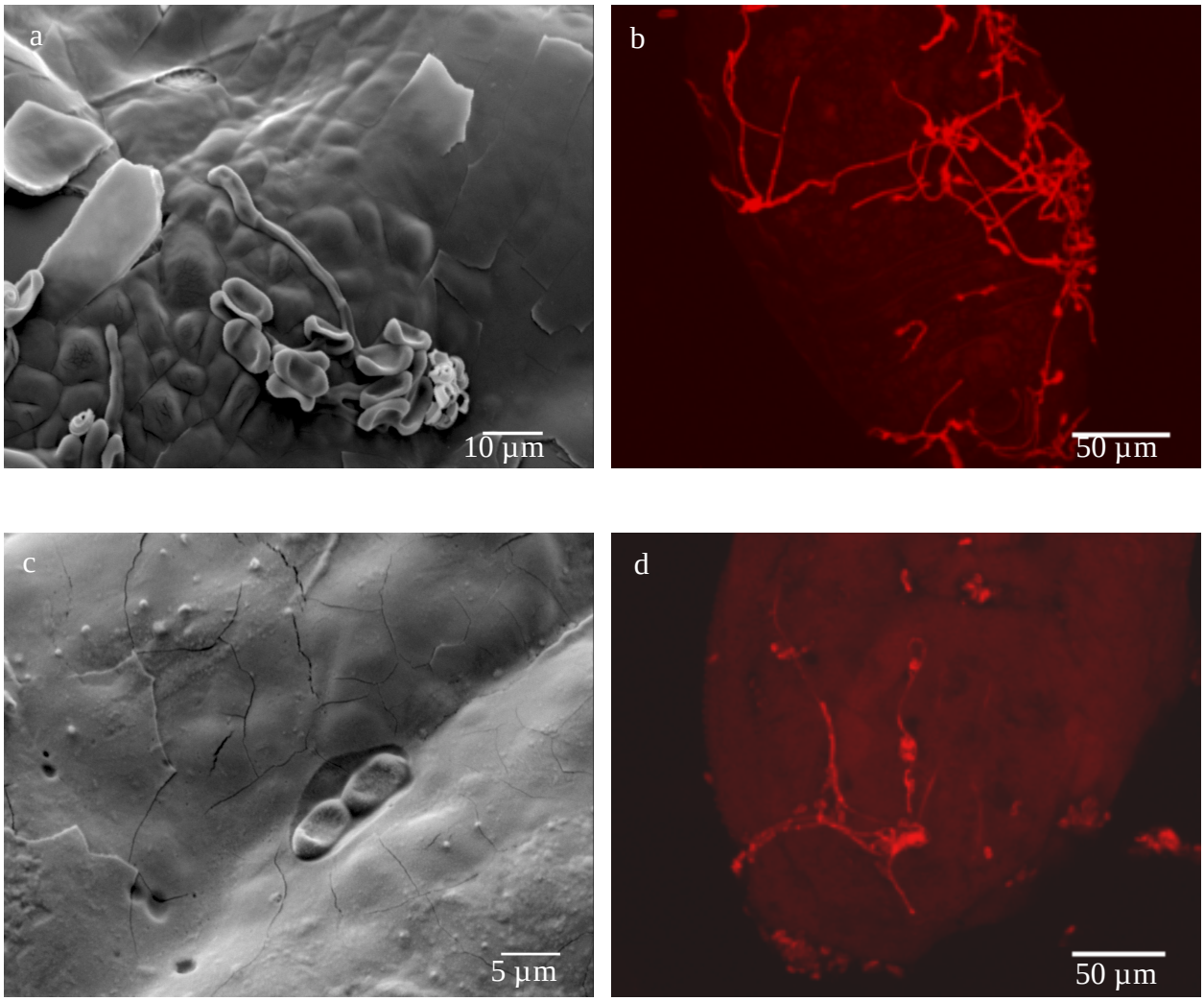

Figure 3-26. Effect of different additives on the spore germination of *M. anisopliae* on 4th instar larvae of *T. vaporariorum* under greenhouse conditions: Tween® 80 (a), Addit® (b), Agrocer® (c) and PA1 (d) (evaluation 2 days after inoculation, images (a) and (c) by LTSEM, (b) and (d) by CLSM).

3.4.6 Control of the target insect

All results so far can only give hints on the performance of the antagonists for the control of the target insect in the greenhouse. Thus, the subject of the final investigations was the effect of the additives on the efficacy of entomopathogenic fungi against whiteflies. Experiments were carried out with different isolates of *M. anisopliae* var. *anisopliae* and *P. fumosoroseus* against the 1st and the 2nd instar of *B. argentifolii* and *T. vaporariorum*. Furthermore, the potential of additives in a prophylactic treatment was investigated on *T. vaporariorum*.

3.4.6.1 Efficacy of entomopathogens in a curative treatment

The potential of Addit®, Agrocer® and PA1 to enhance the efficacy of *M. anisopliae* var. *anisopliae* V245 and *P. fumosoroseus* 1 against *B. argentifolii* was investigated on cotton (Figure 3-27). Differences between the effect of the additives were observed in the speed of kill and the total mortality. While the addition of Agrocer® or PA1 in a concentration of 0.5 % did not cause any difference in the mortality of the larvae of the silverleaf whitefly compared to the formulation with Tween® 80, a significant increase in the mortality could be observed with Addit®. More than 80 % of the larvae were already dead after two days compared to 30 to 40 % with the other formulations. After three days about 100 % mortality were monitored with the Addit® formulation but not more than 50 % when Tween® 80, Agrocer® or PA1 had been added to the spore suspension.

Similar investigations with *P. fumosoroseus* 1 led to different results (Table 3-12). No enhancing effect could be observed for either formulation compared to Tween® 80. On the 8th day after application the larval mortality didn't exceed 65 % and no significant differences were found between a formulation with Tween® 80, Addit® or PA1.

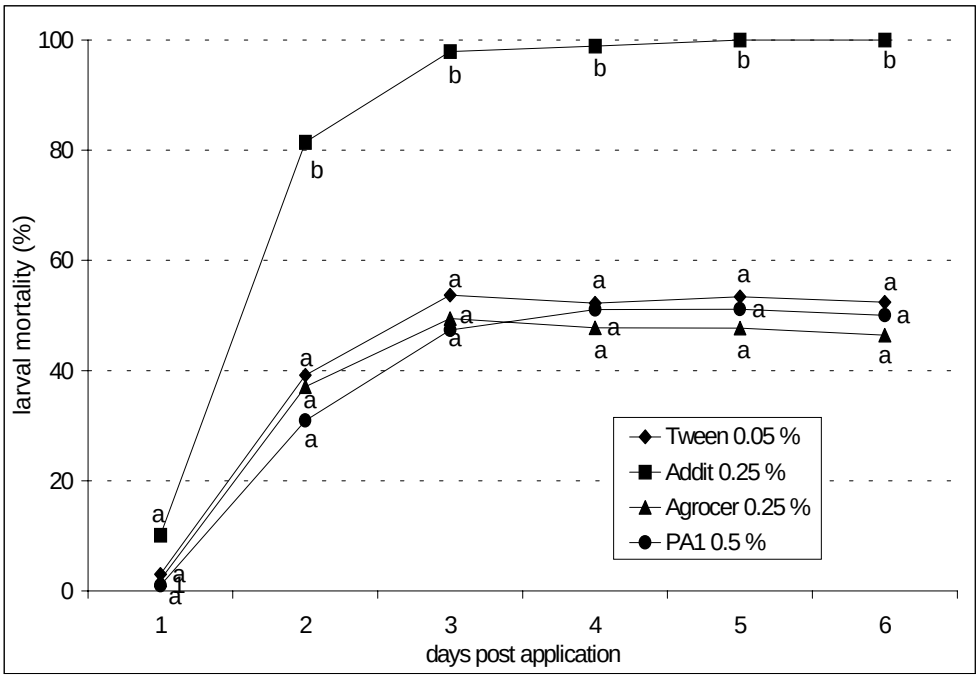

Figure 3-27. Effect of different additives on the efficacy of *M. anisopliae* var. *anisopliae* V245 for the control of 1st stage larvae of *B. argentifolii* under greenhouse conditions (cotton, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters on the same day of evaluation indicate significant differences, DUNN'S, $p \leq 0.05$).

Table 3-12. Effect of different additives on the efficacy of *P. fumosoroseus* 1 against 1st instar larvae of *B. argentifolii* under greenhouse conditions, 8 days post application (cotton, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925)).

additive	larval mortality (%)
Tween® 80 (0.05 %)	52
Addit® (0.25 %)	65
PA1 (0.25 %)	58
PA1 (0.5 %)	65

no significant differences between the treatments (DUNN'S, $p \leq 0.05$)

Corresponding investigations were performed with *T. vaporariorum* on tomato. When *M. anisopliae* var. *anisopliae* V245 was applied to 1st instar larvae the mortality rates were generally lower than those for the silverleaf whitefly (Figure 3-28).

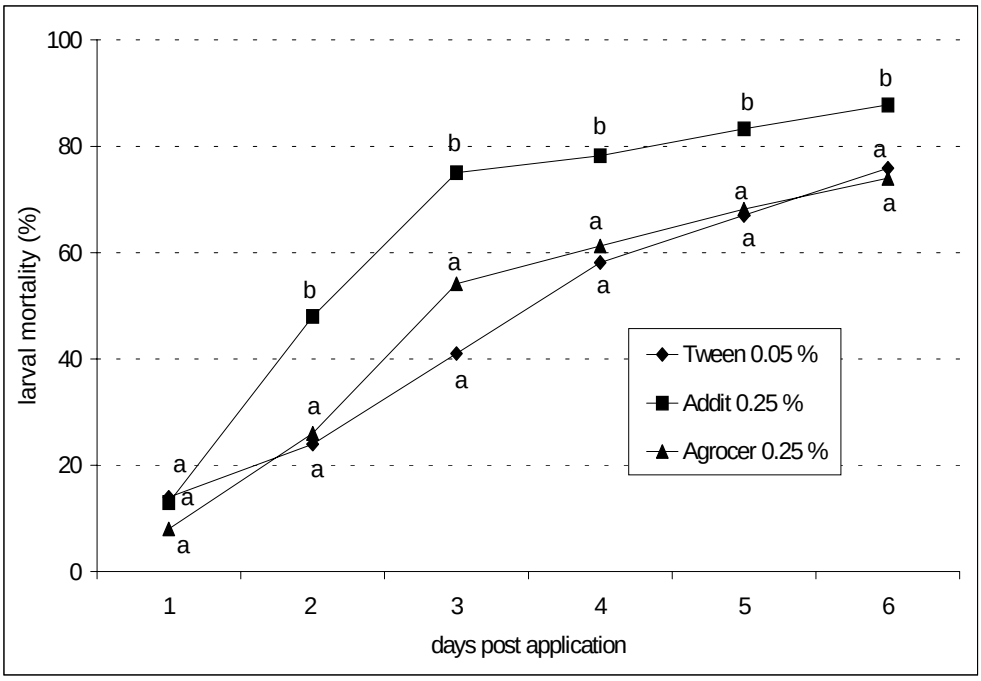

Figure 3-28. Effect of different additives on the efficacy of *M. anisopliae* var. *anisopliae* V245 against 1st instar larvae of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters on the same day of evaluation indicate significant differences, DUNN'S, $p \leq 0.05$).

The effects of the additives on the efficacy of the fungus were similar to those against the silverleaf whitefly and significant differences could be observed in the speed of kill and the total mortality. Formulating the spores in Agrocer® did not result in differences compared to the Tween® 80 formulation where about 25 % of the larvae were dead after two days and about 75 % mortality were monitored after six days, but a significant increase was found with Addit®, respectively. After two days about 50 % mortality could be observed while 88 % of the larvae were dead after six days.

Different larval stages were found to be not equally susceptible to entomopathogenic fungi, susceptibility decreased for the older stages (compare chapter 3.1.3.2). Therefore, investigations were carried out on 2nd instar larvae of *T. vaporariorum* using *M. anisopliae* var. *anisopliae* 27 together with PA1 (Figure 3-29, Figure 3-30), *M. anisopliae* var. *anisopliae* V245 together with Addit® (Table 3-13) and *P. fumosoroseus* 2 formulated in Addit® or PA1 as biological control agents (Figure 3-31).

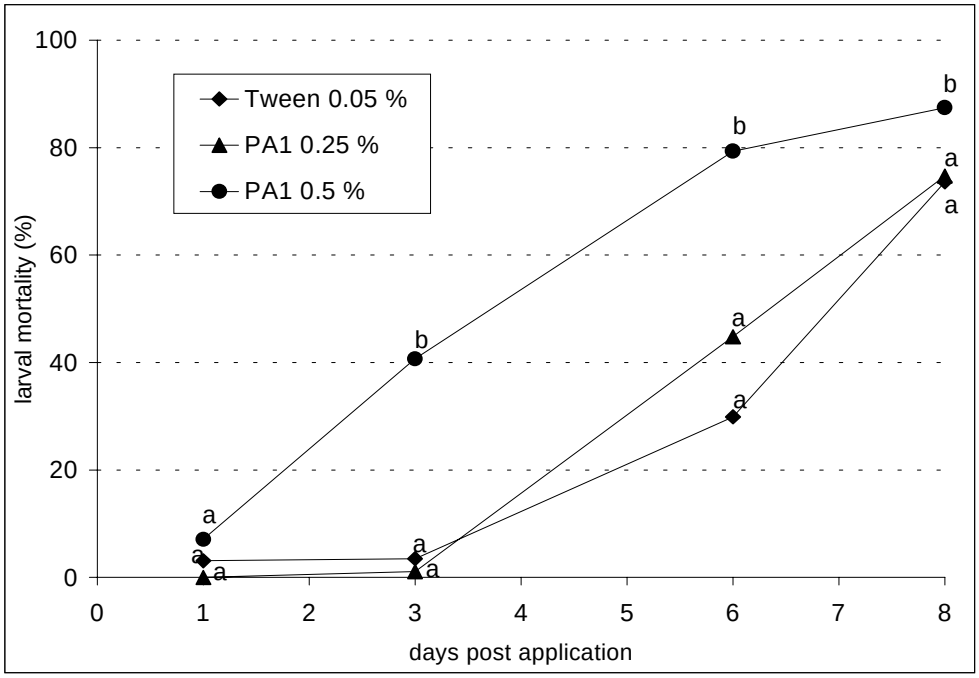

Figure 3-29. Effect of PA1 on the efficacy of *M. anisopliae* var. *anisopliae* 27 for the control of 2nd instar larvae of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters on the same day of evaluation indicate significant differences, TUKEY, $p \leq 0.05$).

Formulating the conidia in PA1 at a concentration of 0.25 % did not result in significant differences compared to the Tween ® 80 formulation (Figure 3-29). Increasing the concentration to 0.5 % culminated in a significantly higher mortality and a higher speed of kill of the target insect. After three days about 40 % mortality were observed with 0.5 % of PA1 compared to about 8 % with 0.25 % or Tween ® 80. The total mortality was about 85 % for the higher concentration of PA1 but only 76 % with the other formulations.

After eight days under greenhouse conditions detached tomato leaves were transferred into moist chambers to bring about fungal emergence from the dead insect larvae (Figure 3-30). Significant differences were found in the fungal colonisation of the insects for the different formulations. *M. anisopliae* var. *anisopliae* 27 was found to emerge out of less than 20 % of the dead larvae that had been treated with a spore suspension with Tween ® 80 beforehand. Significantly more fungal growth was found on the larvae when PA1 had been applied.

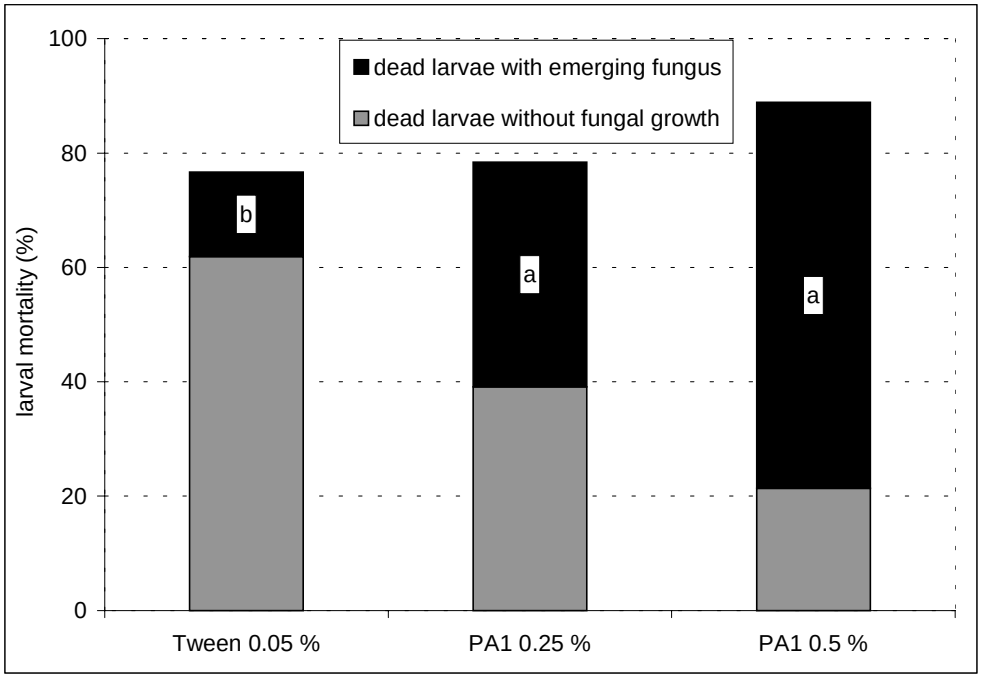

Figure 3-30. Effect of different additives on the control efficacy and fungal emergence of *M. anisopliae* var. *anisopliae* 27 from larvae of *T. vaporariorum* (greenhouse conditions from day 0 to day 8 post application, moist chamber from day 9 to day 11 pa, tomato, 10^7 spores/ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), different letters indicate significant differences, TUKEY, $p \leq 0.05$).

Alike experiments were carried out with formulating the spores of *M. anisopliae* var. *anisopliae* V245 with Addit®. No differences compared to the formulation with Tween® 80 could be found concerning total mortality or speed of kill but fungal colonisation was found to be different (Table 3-13). After 10 days not more than 66 % of the larvae were dead. Examining fungal emergence after three days of incubation in a moist chamber led to a significantly higher number of larvae with emerging fungus in the Addit® treatment than in the Tween® 80 treatment.

The effect of Addit® and PA1 on *P. fumosoroseus* was also investigated on 2nd instar larvae of *T. vaporariorum*. No significant differences could be observed when formulating *P. fumosoroseus* 2 with Tween® 80 or PA1, neither concerning total mortality nor fungal colonisation (Figure 3-31). Comparing Addit® to the other treatments this formulation caused a significantly higher mortality than the others and the number of colonised dead larvae was also significantly higher compared to the other formulations.

Table 3-13. Effect of Tween® 80 and Addit® on the control efficacy and fungal emergence of *M. anisopliae* var. *anisopliae* V245 against 2nd instar larvae of *T. vaporariorum* (greenhouse conditions from day 0 to day 10 post application, moist chamber from day 11 to day 13 pa, tomato, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925)).

additive	**larval mortality (%)**				**fungal emergence**
	2 dpa	4 dpa	8 dpa	10 dpa	**(in % of dead larvae)**
Tween® 80 (0.05 %)	3 a	25 a	58 a	66 a	20 b
Addit® (0.25 %)	8 a	25 a	44 a	66 a	45 a

significant letters indicate significant differences within the columns (t-Test, $p \leq 0.05$)

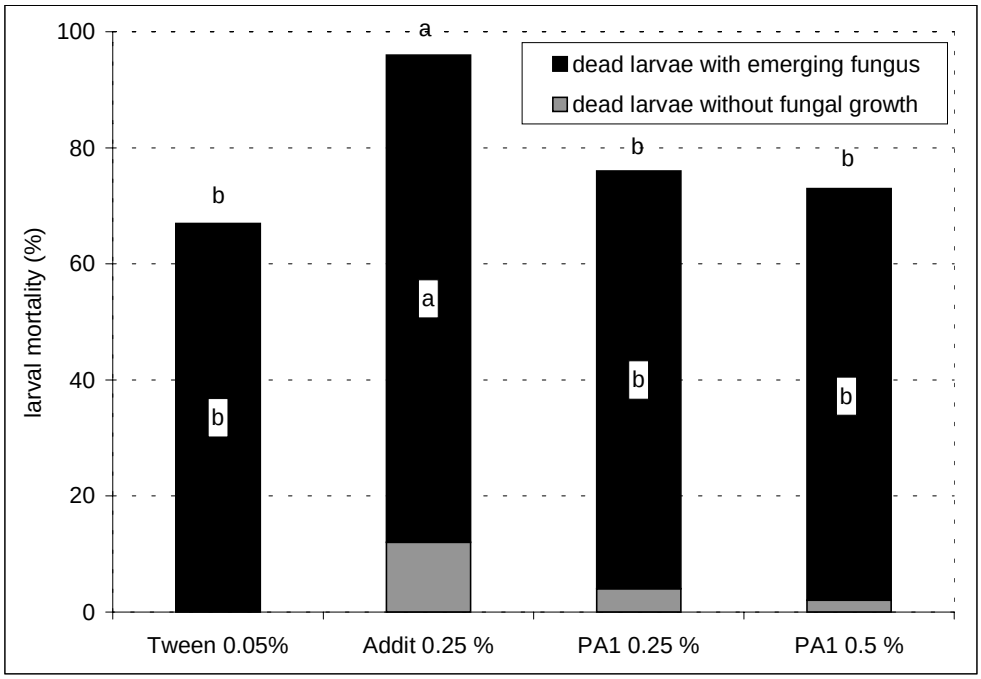

Figure 3-31. Effect of different additives on the control efficacy and fungal emergence of *P. fumosoroseus* 2 against 2nd instar larvae of *T. vaporariorum* (greenhouse conditions from day 0 to day 8 post application, moist chamber from day 9 to day 11 pa, tomato, 10^7 spores / ml, values corrected for controls by Abbott's formula (ABBOTT, 1925), significant letters indicate significant differences, DUNN'S, $p \leq 0.05$).

Comprehensively, Agrocer ® had no effect on the efficacy of either fungus against whiteflies. PA1 at a concentration of 0.25 % enhanced fungal colonisation of *M. anisopliae* of 2nd instar larvae of *T. vaporariorum*, at a concentration of 0.5 % not only colonisation but also total mortality and speed of kill of the fungus were increased. Addit ® had no effect on *P. fumosoroseus* against *B. argentifolii* but increased speed of kill, total mortality and fungal emergence of *M. anisopliae* against the silverleaf whitefly and of both fungi against *T. vaporariorum*.

3.4.6.2 Efficacy of the antagonist in a prophylactic control

One problem of entomopathogenic fungi is a decreasing efficacy when being exposed to high temperature, low humidity and UV-light (IGNOFFO, 1992). Spores loose their viability and therefore the ability to infect insect larvae (compare chapter 3.4.3). The potential of different additives to preserve the efficacy of the antagonist over time was investigated in prophylactic treatments.

M. anisopliae var. *anisopliae* 43 was applied in a formulation with Tween® 80 or Addit® before infestation and oviposition, egg hatch and larval mortality were determined (Table 3-14). Fungal emergence from the dead larvae was examined after 72 hours of incubation in a moist chamber (Figure 3-32). No significant differences were found in oviposition, egg hatch or larval mortality after treatments with a Tween® 80 or an Addit® formulation, no matter how many days after application the infestation had taken place. Applying the antagonist formulated with Tween® 80 immediately before or one week before the infestation led to a higher rate of dead larvae with emerging fungus than with the Addit® formulation.

Table 3-14. Effect of Tween® 80 and Addit® on the efficacy of *M. anisopliae* var. *anisopliae* 43 in a prophylactic treatment on oviposition, egg hatch and larval mortality of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores/ml, infestation on day 0, 7 and 14 post application, oviposition expressed in percent of the control treated with Tween® 80 only, values for egg hatch and larval mortality corrected for controls by Abbot's formula (ABBOTT, 1925)).

additive	**oviposition, 2 dpi (%) infestation (dpa)**			**egg hatch, 10 dpi (%) infestation (dpa)**			**larval mortality, 20 dpi (%) infestation (dpa)**		
	0	**7**	**14**	**0**	**7**	**14**	**0**	**7**	**14**
Tween® 80 (0.05 %)	116	136	93	76	8	3	19	7	14
Addit® (0.25 %)	113	112	63	97	5	1	33	2	19

no significant differences between the treatments (t-Test, $p \leq 0.05$)

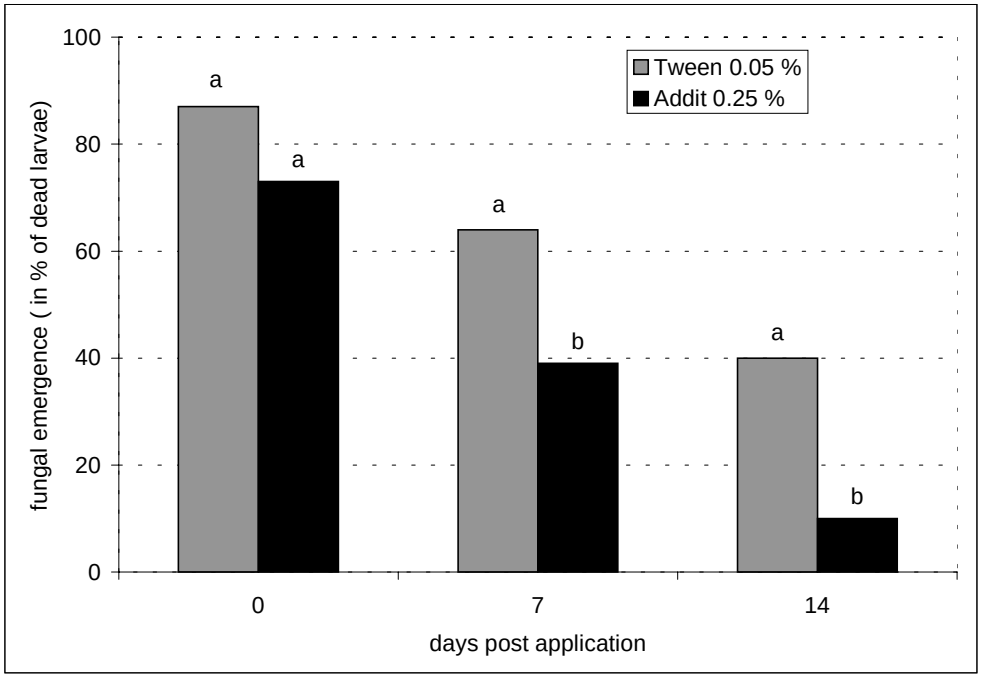

Figure 3-32. Effect of Tween® 80 and Addit® on the fungal colonisation of *M. anisopliae* var. *anisopliae* 43 of 3rd instar larvae of *T. vaporariorum* in a prophylactic treatment (evaluation after 3 days of incubation in a moist chamber, tomato, 10^7 spores/ml, infestation on day 0, 7 or 14 post application, significant differences between formulations on the same day of application, t-Test, $p \leq 0.05$).

The effect of PA1 on *M. anisopliae* var. *anisopliae* 27 was tested in a different experiment under the same conditions (Table 3-15). Corresponding to the investigations on Addit®, a prophylactic treatment with *M. anisopliae* var. *anisopliae* 27 formulated in PA1 or Tween® 80 had no effect on oviposition, egg hatch or larval mortality. Rates for fungal colonisation of dead larvae were yet found to be different (Figure 3-33). With PA1 (0.5 %) a significantly higher rate of colonised larvae was found compared to the Tween® 80 formulation when the infestation took place on the same day or seven days after application. For an infestation at 14 days after application, no effect of PA1 on the fungus could be observed.

Table 3-15. Effect of Tween® 80 and PA1 on the efficacy of *M. anisopliae* var. *anisopliae* 27 in a prophylactic treatment on oviposition, egg hatch and larval mortality of *T. vaporariorum* under greenhouse conditions (tomato, 10^7 spores/ml, infestation on day 0, 7 and 14 post application, oviposition expressed in percent of the control treated with Tween® 80 only, values for egg hatch and larval mortality corrected for controls by Abbot's formula (ABBOTT, 1925)).

additive	oviposition 2 dpi (%) infestation (dpa)			egg hatch 10 dpi (%) infestation (dpa)			larval mortality, 20 dpi (%) infestation (dpa)		
	0	7	14	0	7	14	0	7	14
Tween® 80 (0.05 %)	149	203	138	34	50	30	57	45	61
PA1 (0.25 %)	135	161	132	16	46	33	55	29	54
PA1 (0.5 %)	129	97	213	59	72	41	68	25	55

no significant differences between the formulations (TUKEY, $p \leq 0.05$)

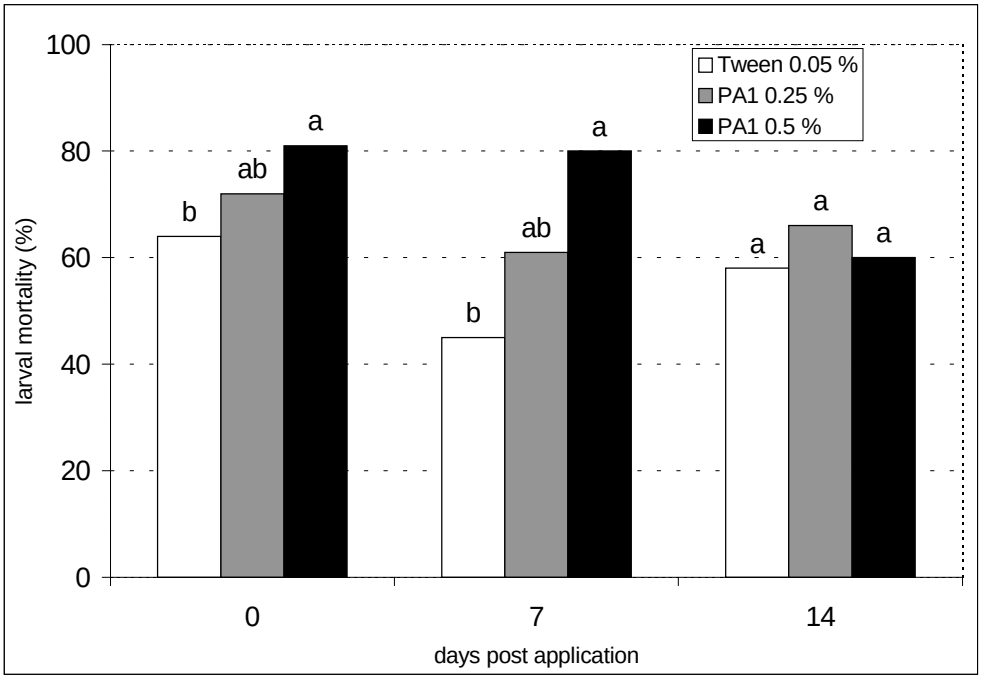

Figure 3-33. Effect of Tween® 80 and PA1 on the fungal colonisation of *M. anisopliae* var. *anisopliae* 27 of 3rd instar larvae of *T. vaporariorum* in a prophylactic treatment (evaluation after 3 days of incubation in a moist chamber, tomato, 10^7 spores/ml, infestation on day 0, 7 or 14 post application, significant differences between formulations on the same day of application, TUKEY, $p \leq 0.05$).

A prophylactic treatment was also carried out for investigating the effect of *M. anisopliae* var. *anisopliae* formulated with different additives adults of *T. vaporariorum*. The location of probing and oviposition were used as parameters in a choice and no-choice assay (Table 3-16). When formulated with Tween® 80 no effect of the antagonist on whitefly adults concerning their location of probing or oviposition was found (compare Table 3-2). The effect of different additives was therefore investigated. After applying spores formulated with PA1 at a concentration of 0.25 %, significantly less adults and eggs were found on these leaves compared to the ones treated with a formulation of *M. anisopliae* and Tween® 80 shortly before infestation. PA1 in a 0.5 % concentration had the same effect when the infestation took place one week after the application. No significant differences were found with Addit®.

While Addit® had no effect on the efficacy of *M. anisopliae* in a prophylactic treatment, PA1 enhanced fungal colonisation and decreased oviposition and the number of adults on the leaves in a preference experiment.

Table 3-16. Effect of *M. anisopliae* var. *anisopliae* 43 formulated with different additives in a prophylactic treatment on the preference of adults of *T. vaporariorum* in the location of probing and oviposition on detached tomato leaves (26 °C, 70 %RH, 10^7 spores / ml, infestation on day 0, 7 and 14 post application).

additive leaf 1/ additive leaf 2	**infestation 0 dpa**		**infestation 7 dpa**		**infestation 14 dpa**	
	no. of adults l 1/l 2 (%)	**no. of eggs l 1/l 2 (%)**	**no. of adults l 1/l 2 (%)**	**no. of eggs l 1/l 2 (%)**	**no. of adults l 1/l 2 (%)**	**no. of eggs l 1/l 2 (%)**
Tween® 80 (0.05 %) / Addit® (0.25 %)	41 / 29	49 / 51	28 / 41	42 / 59	49 / 33	41 / 59
Tween® 80 (0.05 %) / PA1 (0.25 %)	56 / 33*	69 / 31*	39 / 32	49 / 51	42 / 42	46 / 54
Tween® 80 (0.05 %) / PA1 (0.5 %)	38 / 48	38 / 62	63 / 26*	80 / 20*	48 / 28	58 / 42

* significant differences between the number of adults or eggs on the leaves (t-Test, $p \leq 0.05$)

4 Discussion

Naturally occurring entomopathogens play an important role in our ecosystem. Invertebrates, viruses, bacteria and fungi can be found as regulatory factors in insect populations. Hence, many species are used as biological control agents of insect pests in row and glasshouse crops, orchards, turf, stored products and forestry and for abatement of vector insects of veterinary and medical importance. However, while fungal insecticides have been employed widely in China and to a lesser extent in Eastern Europe as well as in parts of South America, fungi have been little used elsewhere (CHARNLEY, 1997).

The aim of this project was to optimise the efficacy of the entomopathogenic fungi *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* for the control of the silverleaf whitefly, *Bemisia argentifolii,* and the greenhouse whitefly, *Trialeurodes vaporariorum,* for protected crops in Israel and Germany. The virulence of the isolates towards both whitefly species was determined to identify the most promising candidate. The effect of the insects' developmental stage was considered and investigated as another factor affecting virulence. Investigations on the production conditions were performed in order to characterise the isolates. Emphasis was given to formulations, thus first elucidating the effect of additives on the shelf-life of conidia. The potential of different additives for enhancing the virulence of the microbial control agents in a curative and prophylactic treatment was then determined. In order to explain this enhancement the effect of the additives on different stages of application and pathogenesis was elucidated. Hence, the ability of different additives to improve inoculum targeting was evaluated as well as the performance of differently formulated conidia under challenging environmental conditions and their persistence on the leaf. Furthermore, the compatibility of the fungi with insect growth regulators and the potential of a combinatory treatment were investigated in order to integrate the microbial control agents with other control strategies.

Paecilomyces fumosoroseus strains occur in soils and insects world-wide. Their efficacy against *B. argentifolii* and *T. vaporariorum* has been described by FANG *et al.* (1985) and WRAIGHT *et al.* (1998). *Metarhizium anisopliae* has a very wide host range but was mostly employed as biological control agent against beetles and locusts (ZIMMERMANN, 1992). Its efficacy against thrips and whiteflies was first described by VESTERGAARD *et al.* (1995) and by MALSAM *et al.* (1998). Nevertheless, different

strains of the same species do not have equal potentials for the control of the same arthropod species (ALTRE *et al.*, 1999; VEY *et al.*, 1982). Pathogenicity of the antagonist towards an insect species is related to the ability of the fungus to germinate on the insects' cuticle and to penetrate it, to its production of secondary metabolites and to the defence mechanisms of the host to prevent fungal infection and growth (KAIJIANG & ROBERTS, 1986; RATH *et al.*, 1996; CLARKSON *et al.*, 1998). The isolates tested differed in their pathogenicity as well as in their LT_{50} (lethal time $_{50}$), the time required for 50 % mortality of the target insect. Strains with a high control potential for the silverleaf whitefly had an equally high potential for the control of the greenhouse whitefly, and strains that were non-pathogenic towards *B. argentifolii* were non-pathogenic towards *T. vaporariorum* either. The strains tested were isolated from different insects, only one of them being a homopteran species, and from locations in different climatic zones. HALL (1982) and CHANDLER (1992) observed that isolates of *M. anisopliae* were more efficient when isolated from the same or a closely related insect species. In this case, no correlation could be determined between host or climatic zone and virulence of the isolates tested against greenhouse and silverleaf whitefly from Germany and Israel, confirming the results of BUTT (1992) and VESTERGAARD *et al.* (1995).

A number of factors influence the efficacy of entomogenous fungi, such as the developmental stage of the insect. Not all stages in an insects' life are equally susceptible to infection by entomopathogens. Egg hatch of *B. argentifolii* was reduced by *M. anisopliae* at high relative humidity. Additionally, mortality of the hatched larvae was significantly higher than in the control. Under greenhouse conditions the susceptibility of whitefly larvae to the antagonist decreased for the older larval stages. Neither their location of probing nor the reproduction of adults were affected. Correspondingly, FRANSEN *et al.* (1987) found that older instars of *T. vaporariorum* were less susceptible to the fungus *Aschersonia aleyrodis*, while adults were seldom infected. LACEY *et al.* (1999) detected low but significant mortality of eggs and hatched 1st instar larvae of *B. argentifolii* after the application of *P. fumosoroseus*.

MALSAM (1999) reported no differences in the susceptibility of different larval stages to entomopathogens, an increased adult mortality and a decrease in the reproduction caused by *M. anisopliae*. VAN DE VEIRE *et al.* (1996) observed similar susceptibility to

P. fumosoroseus in all larval stages and adults except for the 2nd stage, which was less susceptible. The insect cuticle acts as a barrier for fungal penetration and its thickness increases with every moulting so that differences in the susceptibility of different larval instars to entomopathogenic fungi can be explained by their cuticle properties (BOUCIAS & PENDLAND, 1991). The length of the intermoult period depends upon the environmental conditions and the shorter it gets the less time remains for the fungus to germinate and penetrate. If moulting occurs shortly after inoculation the penetrating fungus may be removed prior to the colonisation of the insect (VEY & FARGUES, 1977; FARGUES & RODRIGUEZ-RUEDA, 1971). Hence, the differences between the results can be explained by the time of inoculation regarding the remaining period to ecdysis.

For an effective and economic production process of entomopathogens for biological control, rapid growth and a high sporulation rate are as essential as the stable production of viable, infectious conidia (JENKINS *et al.*, 1998; MOORE & PRIOR, 1993). Culture conditions can greatly influence the virulence, longevity and ecological fitness of the resultant propagules. Therefore, they can be manipulated to increase mycoinsecticide efficiency. LEE *et al.* (1996), IM *et al.* (1988) and VILAS-BOAS *et al.* (1996) observed differences in growth and sporulation of a variety of entomopathogens on different solid culture media. Herein, media that enhanced mycelial growth did not necessarily increase the sporulation rate. *P. fumosoroseus* is mostly mass-produced in liquid culture but differences in growth and sporulation with different media have been reported as well (DE LA TORRE *et al.*, 1996; VIDAL *et al.*, 1998). When various solid media were evaluated for their effect on growth, sporulation and conidial viability of different strains of *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *P. fumosoroseus* requirements differed for strains but not between species.

Apart from the growth and sporulation the culture media had an effect on the viability of the produced conidia of *M. anisopliae* but not on those of *P. fumosoroseus.* This antagonist is mostly mass-produced in liquid cultures but showed a high potential for production of conidia on solid media as well. High standard deviations were observed for all the isolates of *M. anisopliae.* The high variability might result from the heterogeneity of the cultures, which were not obtained from single spore isolates. Cultures of *P. fumosoroseus*, though not obtained from single spore isolates either, seemed to be less

heterogeneous concerning growth, sporulation and conidial viability on different culture media. Information on the requirements of strains is crucial for a successful mass-production.

Different species and strains can differ in their need of available water for growth and sporulation. Reduced water availability in the culture medium can increase the efficiency of entomopathogens at lower humidity (HALLSWORTH & MAGAN, 1998). Only one of the isolates of *M. anisopliae* was tolerant to relative humidities below 96 % RH, which was not surprising as this isolate has a high potential for locust control under dry conditions (STEPHAN & ZIMMERMANN, 1998). Growth of the other isolates was delayed at 96 % RH and low or zero rates of sporulation could be found. This confirmed the results of MILNER *et al.* (1997) who noted that germination of 21 isolates of *M. anisopliae* was delayed at 96 % RH and inhibited below this humidity.

Molecular studies using PCR sequencing have shown considerable genetic variation among different isolates (CURRAN *et al.*, 1994). It is therefore not surprising that intraspecific variability was found between fungal species in the tolerance to this limiting factor (DRUMMOND *et al.*, 1987; VIDAL & FARGUES, 1998). HALLSWORTH & MAGAN (1994) reported the correlation between the growth of *B. bassiana* , *P. farinosus* and *M. anisopliae* at reduced water activities and their polyol accumulation in the cells. Information on this correlation could help for the selection of the suitable isolate and the modification of strains or the production of mutants with the ability to germinate and infect at low humidity (HALLSWORTH & MAGAN, 1995, 1998; MATEWELE *et al.*, 1994).

Culture conditions can also influence thermal tolerance. McCLATCHIE *et al.* (1994) reduced the thermal tolerance of conidia of *M. flavoviride* by increasing the sucrose content of the growth medium. Nevertheless, the virulence of fungal propagules, produced on different culture media, needs to be determined as well. KMITOVA & POPOWSKA-NOWAK (1995) and MALSAM (1999) reported an effect of culture media on the pathogenicity of different entomopathogens. ST. LEGER *et al.* (1991) found higher levels of enzymes in conidia from infected *Manduca sexta* larvae than in those cultured on an artificial medium. Conidia of *Conidiobolus obscurus* produced *in vitro* were less virulent against aphids than those produced *in vivo* (PAPIEROK, 1982). Therefore, the appropri-

ate culture conditions have to be investigated thoroughly. Besides an effective, inexpensive production of spores, retention of virulence is essential and attention should be paid to the stability of the product.

Investigating growth and sporulation on solid medium with defined water availability in order to determine the strains' requirement of moisture has been questioned by MILNER *et al.* (1997). They stated that determining levels of reduced relative humidity on the surface and above the solid medium, where fungal growth takes place, is difficult even if the water availability within the medium had been adjusted correctly. It is equally problematical to determine the humidity conditions around insects or on leaf surfaces because the microclimate differs from the surrounding air. Therefore, information on the requirements of different isolates on artificial media serves more for characterisation of strains than for statements about their performance in row or glasshouse crops.

Fungal isolates that have been identified as promising candidates for pest control must have adequate storage properties and be efficacious under greenhouse and field conditions. Formulation is an important factor that can greatly influence these properties. To begin with, additives can increase storage time of conidia. During application, additives can enhance the field efficacy resulting in high mortality and short LT_{50} values of the target insect. For instance, formulations can protect fungal spores against unfavourable environmental conditions. Rapid germination and infection are vital for the virulence of an isolate. Some formulations can improve fungal virulence by stimulating germination. Targeting of the inoculum can be affected by the distribution of the formulation – and hence the spores- on leaves and insects as well as by the persistence of the conidia on the leaf. The development of an inexpensive formulation that enhances shelf-life, environmental persistence and efficacy as well as enables the ease of application of entomopathogenic fungi is crucial for the acceptance of this biological control method.

The retention of high viability and virulence of entomopathogenic conidia during storage and after application is essential for the effective biological control of insects (McCLATCHIE *et al.*, 1994). Insuring an adequate shelf-life of the inoculum is a fundamental aspect for biological control with micro-organisms and many additives have been tested for their potential to prolong the shelf-life of spores of entomogenous fungi

under different conditions. The polymeric additive PA1 was observed to enhance conidial germination levels of *M. anisopliae* after storage in a liquid formulation at 4 °C and 26 °C. Retention of viability was generally higher when the spores were stored at 4 °C than at the higher temperature. These results confirm those of ALVES *et al.* (1987) and STATHERS *et al.* (1993) who reported that the germination level of formulated spores could be enhanced by some additives when stored at room temperature and below. Better retention of viability was usually obtained at temperatures below 15 °C.

Conidial moisture content is an important factor for tolerance and viability. ZIMMERMANN (1982) noted that increasing desiccation of *M. anisopliae* conidia increased its tolerance for high temperatures. DAOUST & ROBERTS (1983) reported that unformulated, dry conidia of *M. anisopliae* survived longest when stored either at moderate temperatures and high relative humidity or at low temperatures and low humidity. According to MORLEY-DAVIES *et al.* (1996) dried conidia of *M. anisopliae* usually showed higher germination levels than those formulated in paraffin oil at different temperatures. In contrast, MOORE *et al.* (1996a) found that storage of dried spores in oil formulation with silica gel resulted in higher viability of the spores than dry storage at temperatures above 20 °C while conidia stored dry retained higher viability than those formulated in oil when stored at temperatures below 20 °C.

In order to increase the shelf-life conidia have been dried in the presence of desiccating agents like silica gel and $CaCl_2$, formulated and stored (MOORE *et al.*, 1995). No publications were found about the drying of formulated fungal spores prior to storage. Drying of formulated conidia could facilitate application for the concentrated, dry product would only need to be diluted with water and sprayed. MUGNIER and JUNG (1985) noted the possibilities of long-term storage at 28 °C of bacteria and fungi, which were entrapped in biopolymer gels. The polymeric additives tested in this project do not dissolve in water but absorb it to create an inert matrix so that a certain moisture level is maintained even after drying (SIEVERDING, Stockhausen, Germany, pers. comm.). Shelf-life of *M. anisopliae* conidia was greatly improved at room temperature but not at 4 °C. It can be concluded that moisture for the conidia was provided by the additives so that their tolerance to storage at high temperatures could be enhanced for at least four months. An prolonged shelf-life at room temperature is a great advantage for producer

and grower who do not need to provide low temperature conditions. Nevertheless, retention of virulence of stored conidia has to be investigated to make sure that they are not only viable but still infectious to the target insect.

So far, the surfactant Tween® was mostly used for formulating the hydrophobic conidia of *M. anisopliae* and *P. fumosoroseus* in an aqueous suspension. Prior *et al.* (1988) showed that certain hyphomycete fungi were more infectious when applied in oil rather than in water. BATEMAN *et al.* (1993) and MALSAM *et al.* (in press) reported increased efficacy of *Metarhizium* sp. against locusts or *T. vaporariorum* in oil formulations. Polymeric additives have only recently been reported to enhance the efficacy of biological control agents. PIGGOT *et al.* (2000) used polymeric additives for the successful control of foliar pests with nematodes. The efficacy of *Beauveria bassiana* and *P. fumosoroseus* against arthropod pests in orchards was increased by an acrylic polymer formulation (PUTERKA, 1999).

The additive Addit® was found to greatly enhance the efficacy of *M. anisopliae* and *P. fumosoroseus* against *B. argentifolii* and *T. vaporariorum*. Reduced LT_{50} values and an increase in the total mortality were observed. The polymeric additive PA1 showed a high potential for increasing the efficacy of *M. anisopliae* for the control of *T. vaporariorum* but mortality of *B. argentifolii* could not be enhanced. Similarly, no effect on the efficacy of *P. fumosoroseus* against either whitefly species was found. Discrepancies between total mortality, which was visibly determined, and percentage of colonised, dead larvae after incubation in a moist chamber occurred in some bioassays. They can be explained by difficulties in the evaluation of the death of larvae. The insects could already be dead before the death is visibly determinable. Changes in the outward appearance of larvae, which indicate death might only be visible after a considerable period following the event of death. After incubation in a moist chamber at appropriate temperatures the fungus will emerge from all the larvae that have been infected, thus verifying mycosis and serving as an additional parameter for measuring fungal efficacy against insects as recommended by BUTT & GOETTEL (2000).

Differences in the potential of formulations to increase the overall effectiveness of biological control agents can have different reasons. When spraying, the distribution of the droplets on the leaves is an important factor for successful control. Uniform coverage of

the leaf enhances the chance of contact between target pest and antagonist. In the case of whiteflies, larvae, except 1st stage crawlers, are sedentary and remain on the same spot of the leaf surface until the adult hatches from the pupa. Hence, they can not acquire conidia of entomopathogenic fungi by moving around on the plant as for example Coleoptera do. Thus, uniform coverage of leaves is essential so that as many larvae as possible come in contact with the inoculum. The droplet distribution on the leaves can be affected by the application method as well as by the type of formulation (BATEMAN & ALVES, 2000; BICKERS, 1997). Significant differences were found in the droplet size of the formulations tested. Leaf coverage in large droplets was achieved by formulations with polymeric additives.

The retention of spray droplets on plant leaves depends not only on external factors such as spray volume, droplet size and velocity or surface tension but also on the fixed characteristics of the plant surface (HALLAM & JUNIPER, 1971). Plant leaf surfaces are covered by an epicuticular wax layer. In the case of water hitting this hydrophobic material, the droplets retract after flattening to minimise their contact with the surface, this retraction may be so rapid that part of the drop is ejected and lost from the surface (KLEIN, 2000). In most cases, less than 50 % of the initial spray volume is retained by the plant (WIRTH *et al.*, 1991). BERGERON *et al.* (2000) reported that a small concentration of a long, flexible polymer added to the water slowed down the retraction rate of the flattened drop on a hydrophobic surface, the reason being an increased elongational viscosity provided by the polymer. Thus, a uniform distribution of big droplets could be achieved by adding polymers to the aqueous spore suspension and the spray volume could be reduced. Nevertheless, it has to be taken into account that different plants have different surface properties which can cause water repellency and reduction of contaminating particles (BARTHLOTT & NEINHUIS, 1997). Therefore the '"wettability"' of the host plant itself has to be considered when evaluating the effect of additives on the distribution of the formulation on the leaf surface.

Alone among insect pathogens, fungi have "contact activity" like many chemical insecticides. The relationship between inoculum density in the crop and the target insect is the decisive factor for the development of fungal-induced epizootics. The level of inoculum introduced into the crop and maintained within it has to exceed this epizootic threshold

for achieving successful pest control (BUTT *et al.*, 1994; INYANG *et al.*, 1998). Oil formulations have been reported to decrease the inoculum threshold of two mycoherbicides for development of epizootics (AMSELLEM *et al.*, 1990). Presumably, susceptibility of most insects to entomopathogens is related to spore dosage. The speed of kill is influenced by the number of infection propagules in contact with the cuticle. For most insect/pathogen combinations a positive correlation between the number of infective spores and mortality by mycosis has been established (LIU *et al.*, 1989; VESTERGAARD *et al.*, 1995). Additionally, not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi (BUTT & GOETTEL, 2000). The preferential sites of invasion by fungi are often the buccal cavity, the area under the elytra, the intersegmental folds or spiracles, where locally high humidity promotes germination and the cuticle is nonsclerotised and more easily penetrated (CHARNLEY, 1989; CLARKSON & CHARNLEY, 1996; HAJEK & ST. LEGER, 1994; SCHABEL, 1976).

Effective inoculum targeting is a crucial factor for successful pest control. Spreading of the conidia on the larvae themselves is another vital point. It is reported by WAGNER *et al.* (1996) that different surface sculptures of insects resulted in differences in "wettability" and contaminability. ST. LEGER *et al.* (1991) found differences in the germ tube lengths before appressoria formation on young and old fifth instar larvae of *Manduca sexta* with different surface structures, thus indicating an effect of host surface topography on the speed of kill. Similar responses to cuticle topography have been demonstrated during the infection of *Calliphora vomitoria* on sites where there is minimal microfolding of the epicuticle (ST. LEGER *et al.* 1987a). BUTT *et al.* (1995) and WRAIGHT *et al.* (1990) noted the response of *M. anisopliae* to cuticular cues of aphids and flea beetles resulting in adhesion and germination of the antagonist on specific sites on the host body with thinner cuticle and therefore higher mortalities and lower LT_{50} values. Although no literature was found on preferential penetration sites on whitefly scales it can be concluded that sites with thinner cuticle, like intersegmental membranes, are more suitable for penetration than others. Therefore, the distribution of conidia of entomopathogens on the whitefly larvae could have an effect on mortality and speed of kill.

Microscopical assessments of larvae, to which a droplet of inoculum had been applied, found an effect of the formulation on the distribution of spores. Conidia were either

spread evenly or in clusters on the insect cuticle surface. The formulations investigated had different hydrophobic properties and viscosities, which, together with the "wettability" of the larval cuticle, resulted in different distribution patterns. The formulation served as carrier for the conidia, which were also hydrophobic, and might have been transported with different velocities, depending on the formulation properties. Additionally, the distribution of the spores in the formulation itself could have been different, also depending on formulation properties. IBRAHIM *et al.* (1990) observed that conidia of *M. anisopliae* in oil formulations were flowing over the surface of insect and plant cuticle while aqueous spore suspensions remained as drops on the surface after application.

Presumably, formulations that contain a hydrophobic component could deposit conidia on sites which were conductive for germination and infection. Aggregations of spores were found to germinate faster than single conidia, probably because of mutual nutrient provision (BUTT, University of Wales, UK, pers. comm.). Conclusively, the increased efficacy of *M. anisopliae* with Addit ® and PA1 could have resulted from the aspect of effective inoculum targeting and accelerated spore germination.

Spore germination is an important period in the infection cycle of entomopathogenic fungi. It is influenced by a wide range of factors: water, ions, fatty acids, nutrients, host cuticle and physiological state of the host (BUTT, 1990). In general, moisture is a primary requirement for germination of conidia and survival of entomopathogenic fungi (FERRON, 1977; MILNER & LUTTON, 1986). Additives can have stimulatory or inhibitory effects on spore germination and many substances have already been tested, either for the possibilities of a combined treatment with entomopathogenic fungi and other control methods or for enhancing the efficacy of the fungi themselves. AGUDA *et al.* (1986) reported an inhibitory effect of neem oil on germination and sporulation of *M. anisopliae* and therefore recommended separate application when used in the same control programme. Different fatty, organic or phenolic acids were observed to have stimulatory and inhibitory effects on germination of *M. flavoviride* (BARNES & MOORE, 1997). In order to enhance the efficacy of *M. flavoviride* at low humidities, BATEMAN *et al.* (1993) successfully formulated the conidia in cotton seed oil. But not only germination is important. ALTRE *et al.* (1999) reported a correlation of germination speed with

virulence of *P. fumosoroseus* to the diamond back moth. The effect of different additives on the germination speed of *M. anisopliae* var. *anisopliae* and of *P. fumosoroseus* at low relative humidities was investigated on artificial medium. Germination levels of both species were increased at low relative humidity when Addit® or PA1 were added to the conidia.

Spore germination on artificial media can differ to a great extent from germination on insect cuticle. The insect cuticle is covered by a waxy layer containing fatty acids, lipids and sterols (HACKMAN, 1987). The cuticles of most insects contain fungistatic compounds that retard spore germination (LATGE *et al.* 1987). Cuticular fatty acids have a profound effect on spore germination and differentiation. They are either toxic, fungistatic, or, occasionally for pathogenic species, stimulatory (KERWIN, 1984; SMITH & GRULA, 1982). The ability of oils to extract substances from insect cuticle was noted by IBRAHIM *et al.* (1999). Those substances were found to have stimulatory or inhibitory effects on conidia of *M. anisopliae*. SMITH & GRULA (1982) reported a reduction in the rate of germination of *B. bassiana* conidia on the cuticle of the corn earworm, which was attributed to antifungal cuticle compounds. SOSA-GOMEZ *et al.* (1997) observed fungistatic effects of cuticle components of the southern green stink bug to conidia of *M. anisopliae*. Conidial germination of *V. lecanii* was delayed on whitefly scales compared with Czapek Dox complete medium, probably because of the lower availability of nutrients on the cuticle than in the medium (CHANDLER *et al.*, 1993). In contrast, 60 % of the spores of the entomophthoralean fungus, *Erynia radicans*, germinated within two hours of attachment on the potato leafhopper (WRAIGHT *et al.*, 1990).

M. anisopliae var. *anisopliae* strain V245, which did not germinate on artificial media below 96 % RH, germinated in a standard Tween® 80 formulation on the cuticle of *Blaberus discoidalis* at 94 % RH. It was indicated that stimulatory compounds for conidial germination were already present on or extracted from the insect cuticle by the additive. Formulating the spores in Addit® or PA1 resulted in a decrease in the time required for germination at low humidity which could be attributed to extraction of stimulatory compounds or provision of the necessary moisture. Despite this apparently invariable requirement for over 92 % RH *in vitro*, DRUMMOND *et al.* (1987) observed no correlation between the germination rate of *V. lecanii* strains on artificial medium at

low humidity and pathogenicity against whitefly scales. Similarly, CHANDLER *et al.* (1993) noted that a short period of high humidity would be sufficient for a small proportion of conidia to germinate on the host. MILNER *et al.* (1997) reported that low humidity was unlikely to adversely affect the efficacy of a mycoinsecticide in termite control which took place under dry climatic conditions. This might be explained by the fact that insects maintain a microclimate with high humidity around themselves so that enough humidity is provided for the fungus to germinate and penetrate. After the insects' death this microclimate is not maintained and therefore high relative humidity in the environment is required by the fungus to emerge from the cadaver and sporulate. Consequently, there might be no need to provide moisture for the germination of entomopathogenic fungi on insect cuticles by selected additives. However, the potential of additives to stimulate germination by extracting compounds from the cuticle and therefore to increase virulence is of great importance.

The success of biological control with entomopathogenic fungi partially depends on the persistence of applied inoculum on the leaf surfaces. A prophylactic treatment with conidia of *M. anisopliae* formulated in Addit® had no effect on the mortality of *T. vaporariorum*. After a prophylactic treatment with conidia formulated in PA1 mortality of the target insect could be increased, corresponding to the results for the curative treatments. Two weeks after inoculation mortalities of insects treated with either formulation of *M. anisopliae* were similar.

Plant pathogenic fungi and bacteria can be removed from leaf surfaces by rain splash (BUTTERWORTH & McCARTNEY, 1991; FITT *et al.*, 1989). Thus, inoculum of entomopathogenic fungi may also be washed off by rain splash or supplemental irrigation. Conidia, especially those on the upper leaf surface, can be inactivated by UV radiation (MOORE *et al.*, 1996b). INYANG *et al.* (1998) noted that inoculum is also diluted as a result of leaf expansion. These problems might be solved by improving the formulation. For instance, oil formulations are supposed to prolong conidial survival and decrease sensitivity to UV radiation compared with aqueous suspensions (ALVES *et al.*, 1998; INGLIS *et al.*, 1995; MOORE *et al.*, 1993).

Although plants in the greenhouse are not exposed to rain the amount of inoculum applied in a Tween® 80 formulation decreased quickly few days after application. Formu-

lating the spores with Addit® led to an even faster decline in the amount of inoculum, contradicting the results of INYANG *et al.* (2000) who found that a mixture of sunflower oil and 'Shellsol T' improved adhesion of *M. anisopliae* conidia to the surface of oilseed rape. Differences in the results might be caused by the type or concentration of oil or the different plant species. Addit® was formulated as an oil-in-water emulsion (0.25 %) while INYANG *et al.* (2000) used pure oil. In contrast to oilseed rape, which has a smooth surface, tomato leaves are very hairy and may have different "wettability" properties. When formulating the conidia of *M. anisopliae* in Addit® it was not surprising, that the rapid decline in the number of viable spores attached to the leaf was also visible in the decreased larval mortality.

Spore persistence on the leaves was best in the polymer formulations. Dry polymer left no visible residue on the leaf but microscopic assessment of the leaf impression on water agar found dense gel bodies with embedded conidia even two weeks after application. These gel bodies formed a sticky "film" and enabled conidial adhesion. Apparently conidia were also protected by the gel bodies, resulting in 80 % viable conidia two weeks after application while conidial viability decreased to about 20 % in all the other formulations tested. The gel-water matrix enhanced adhesion and protected the spores, the effect increased with higher concentration of the additive. Depending on the formulation in which conidia were applied amount and viability of inoculum on the leaves decreased over time. Larval mortality after a prophylactic treatment was decreased or increased, respectively. The polymeric additive PA1 was found to enhance persistence and viability of the inoculum under greenhouse conditions and should be tested in the field.

Choice and no-choice assays on the effect of formulated spores on adults concerning location of probing and oviposition indicated that PA1 had a repellent effect on adults. Less adults were found on the leaves treated with the PA1 formulation compared to Tween® 80 and oviposition was lower. LIU & STANSLY (1995) studied the effect of insecticide residues on oviposition of *B. argentifolii* and observed a high repelling potential of mineral oil. Considering that no differences were detected when applying Tween® 80 with or without conidia of *M. anisopliae* it could be concluded that the additive PA1 was responsible for the repelling of adults. As whiteflies usually prefer the

uppermost leaves, this potential could be used for manipulating whitefly distribution within the canopy in the framework of a push-pull strategy.

Talking about integration of entomopathogenic fungi into crop protection systems a lot of work has been done on the compatibility of entomopathogenic fungi with pesticides (MAJCHROWICZ & POPRAWSKI, 1993; MOHAMED *et al.*, 1987; MOORHOUSE *et al.*, 1992; TEDDERS, 1981; VANNINEN & HOKKANEN, 1988). Lesser publications can be found on the integration into an application programme with selective insecticides. Due to high population densities and short generation times, whitefly populations resistant to commonly used insecticides have developed (ISHAAYA & HOROWITZ, 2000).

An integrated approach in which sublethal doses of chemicals interact with microbial control agents might prevent the development of resistances. Synergisms between different species of entomopathogenic fungi and a variety of insecticides have been reported by different authors. QUINTELA & McCOY (1997), RAMAKRISHNAN *et al.* (1999) and ZECK & MONKE (1992) noted synergistic effects of entomopathogenic fungi and 'imidacloprid', which affects the nervous system of insects, against Isoptera and Coleoptera. Synergisms between entomopathogens and chitin synthesis inhibitors have been cited in some cases. The efficacy of *M. anisopliae* for the control of locusts was enhanced by 'teflubenzuron' (JOSHI *et al.*, 1992). HASSAN & CHARNLEY (1983, 1989) found synergistic effects of *M. anisopliae* and 'diflubenzuron' on *Manduca sexta*. In contrast, 'diflubenzuron' or 'teflubenzuron' failed to increase the efficacy of *M. anisopliae* for the control of the tobacco hornworm or of *B. bassiana* against an orthopteran species (DELGADO *et al.*, 1999; GUTIERREZ *et al.*, 1995).

A potential synergism between entomogenous fungi and two chitin synthesis inhibitors, 'buprofezin' and 'novaluron', was investigated in order to reduce pesticide input and prevent development of resistances by using sublethal doses of the insecticides. *M. anisopliae* and *P. fumosoroseus* proved to be compatible with the insect growth regulators, demonstrated by uninhibited spore germination and mycelial growth on media containing the insecticides. SAPIEHA & MIETKIEWSKI (1992) reported corresponding results for other chitin synthesis inhibitors. After the conidia had been soaked in aqueous formulations of the insecticides for 24 hours, germination rates on agar were observed to be generally higher than for shorter soaking periods. Pre-soaking of conidia of

M. anisopliae led to enhanced germination upon addition of nutrients (DILLON & CHARNLEY, 1990). The increased germination rate supposedly resulted from the water, activating an initial pre-swelling phase and thus facilitating nutrient uptake from the artificial media.

'Buprofezin' did not increase the efficacy of the antagonist in assays with optimum conditions for the fungus. Therefore, only 'novaluron' was tested under greenhouse conditions. 'Novaluron' had no effect in a combined treatment on *B. argentifolii* and only a slight increase in mortality was found for the 1st larval stage of *T. vaporariorum*. Chitin synthesis inhibitors act on the chitin formation. Chitin is built up successively during the arthropod life cycle. When the cuticle is shed by moulting and a new one is secreted, some layers of the chitin from the former instar remain and new layers are added. When treated with chitin synthesis inhibitors formation of new chitin layers is inhibited. Therefore, the larval stage after treatment has a lower level of chitin compared to an untreated control (KER, 1978).

Thus, the potential of a successive treatment with 'novaluron' and *M. anisopliae* was evaluated. A significant increase in whitefly mortality was observed for applying the fungus two days after the insecticide. Despite this synergism, total mortality was too low for the successful control of 2nd instar larvae of *T. vaporariorum* in protected crops.

Insect cuticle is a complex tissue, which consists of an epicuticle, containing phenol-stabilised proteins and a procuticle that comprises chitin fibrils embedded in a proteinaceous matrix (GILLESPIE *et al.* 2000). ST. LEGER *et al.* (1987b) reported that proteases play the dominant role in the penetration process of entomopathogenic fungi through insect cuticle rather than chitinases. Mutants deficient in protease synthesis showed reduced virulence as do normal isolates with inhibited protease activity (ST. LEGER *et al.* 1988). The synthesis of chitinases is substrate-induced and occurs after the proteases have degraded the protein that masks the chitin in the cuticle. Therefore, chitin is not the only main barrier for fungal penetration and a synergism between chitin synthesis inhibitors and entomopathogens might only work to a low extent.

The mode of action of chitin synthesis inhibitors on chitin formation is not yet elucidated completely. MAUCHAMP & PERRINEAU (1987) indicated that protein and chi-

tin microfibrils were not associated after treatment with benzoylphenyl ureas, a group of chitin synthesis inhibitors. HASSAN & CHARNLEY (1989) found that chitin layers of *Manduca sexta* cuticle, which are normally aligned horizontally, were replaced by vertical layers after treatment with 'diflubenzuron'. Another hypothesis also suggested that 'diflubenzuron' affected the assembling of chitin microfibrils (ZIMOWSKA *et al*., 1994).

'Novaluron' acts both by ingestion and contact (ISHAAYA *et al*., 1996). In the case of whiteflies, it acts on contact exclusively. A possible synergism between the insecticide and entomopathogenic fungi was evaluated for the control of the lepidopteran species *Spodoptera littoralis.* The Egyptian cotton leafworm is another important pest of the tropical and subtropical region. 'Novaluron', acting by ingestion in this case, had considerable potential for the control of *S. littoralis* (ISHAAYA *et al*., 1996). Not many publications could be found on the successful control of the Egyptian cotton leafworm by entomopathogenic fungi.

Although HASSANI *et al.* (1998) reported high virulence of different strains of *M. anisopliae* and *P. fumosoroseus,* higher mortality than 15 % could not be achieved with the isolates of the two species tested. A successive treatment with 'novaluron' led to an increase in the speed of kill and the total mortality compared to the application of the insecticide alone. It can be concluded that *M. anisopliae* and 'novaluron' can act synergistically against *S. littoralis* when applied with a time interval. The combination of 'novaluron' with the antagonist showed a high potential for reducing the amount of pesticide while controlling the pest successfully. The differences to *T. vaporariorum* might result in the different mode of uptake of the insecticide. Biochemical assessments on the chitin and protein content of *S. littoralis* cuticle did not elucidate the mode of action of 'novaluron' or the combinatory treatment with *M. anisopliae.*

Metarhizium anisopliae and *Paecilomyces fumosoroseus* have a high potential for the control of *Bemisia argentifolii* and *T. vaporariorum*. Isolates with high efficacy against both species could be found despite the different climatic regions in which the pests naturally occur. The developmental stage of the insect affected fungal virulence but all larval stages could be controlled. Intraspecific variation was high in both fungal species and production conditions need to be adjusted accordingly for an effective mass-production.

Additives influence many aspects of the application process and the pathogenesis. PA1 and Stockosorb®Agro prolonged the shelf-life of *M. anisopliae* conidia at room temperature and the method of drying spores in the formulation enabled ease of application.

Addit® and PA1 enhanced the efficacy of fungi against the target insects. Reasons could be found in improved inoculum targeting and decreased germination time due to the ability of the additives to extract stimulatory compounds from the insect cuticle. Additionally, PA1 increased the efficacy of *M. anisopliae* against *T. vaporariorum* in a prophylactic treatment because of its ability to enhance the persistence of viable conidia on the leaf surface. This work has shown that polymeric additives, which have only recently been discovered for the formulation of biological control agents, have a high potential for increasing the effectiveness of entomopathogenic fungi for crop protection. Further research needs to be done to optimise formulations which minimise the need for expensive procedures in storage and application of entomopathogens, while maintaining their efficacy.

Effective pest control in row and glasshouse crops with microbial control agents alone is still difficult because of their dependency on environmental conditions. On the other hand the augmented use of chemical insecticides increases the pesticide input into the environment, thus affecting non-target organisms, and can be dangerous for grower and consumer. Selective insecticides have the advantage of only affecting the target insect but especially in the case of whiteflies resistant populations develop rapidly due to short generation times and high population densities. Thus, the combination of the entomopathogenic fungus *M. anisopliae* with selective insecticides such as chitin synthesis inhibitors was supposed to offer good possibilities for enhancing the antagonists efficacy and decreasing the probability of resistance development by reducing the amount of pesticides.

In the case of *Spodoptera littoralis* this approach offered a good possibility for efficient pest control with a reduced amount of pesticide. In contrast, with whiteflies the integrated approach did not control the pest effectively. Synergistic effects were only found against the 1st instar of the greenhouse whitefly but efficacy was yet too low. Further research is needed to optimise the existing potential of a combination of entomopathogenic fungi and chitin synthesis inhibitors for whitefly control. Longer time intervals or

application of conidia formulated with additives might improve the effectiveness of this integrated pest control.

Despite of its many advantages microbial pest control is not yet fully accepted. From the point of view of growers utilisation of entomopathogens is still costly and less effective compared to chemical insecticides. For the consumer biologically produced food is often too expensive and present quality standards can sometimes not be achieved. However, the reduction of pesticide input for food and environmental health is becoming more and more important. With the increasing public awareness of the importance of nature conservation and the reduction of pesticide residues in food and environment, the appreciation for the advantages of biological control might increase and synergistic combinations of microbial control agents with other technologies might be considered.

Important advances have been achieved in the field of microbial control in the last years. Formulations for effective pest control have been developed, which facilitate storage and application. Combinations of different entomopathogenic fungi have been discovered for the overcome of temperature constraints (INGLIS *et al.*, 1997). Synergistic effects of *Bacillus thuringiensis* and *M. anisopliae* on a lepidopteran species indicated another approach for integrated pest control (BROUSSEAU *et al.*, 1998). Biological control organisms have been found to be harmless to beneficial insects so inoculum targeting - and therefore pest control - can be improved by honey and bumble bees as vectors of micro-organisms e.g. bacteria for the control of fire blight and *M. anisopliae* for the control of the Coleoptera *Meligethes aeneus* (BUTT *et al.*, 1998; VANNESTE, 1996).

Biological control should not be seen as a possible complete replacement of chemical control. It is a powerful option and can be applied over a much larger area than at present. An integrated approach together with chemical control and different biological control measures should be envisioned. This approach is mutually beneficial. For chemical control it may result in extended use of products because of slower resistance development. The excellent potential of entomopathogenic fungi and other biological control organisms for the use in IPM programs has already been demonstrated and this project can contribute a further step to the full exploitation of biological control agents. However, with further advances more questions arise and problems need to be addressed. In order to serve agriculture as well as the environment and human health, we

should concentrate on the development of effective IPM methods. Designing such environmentally safer and less costly IPM strategies is a challenge for our profession.

5 Summary

The entomopathogenic fungi *Paecilomyces fumosoroseus* and *Metarhizium anisopliae* are soil-borne organisms with a very broad host-range. While *P. fumosoroseus* is well-known for being pathogenic to homopteran pests, the efficacy of *M. anisopliae* against thrips and whiteflies was described only recently. The aim of this project was to select strains of the microbial control agents with a high control potential for the whitefly species *Bemisia argentifolii*, which occurs in tropical and subtropical areas, and *Trialeurodes vaporariorum*, the dominant species in Northern Europe. While looking at different aspects of the production, formulation and application process, emphasis was given both to the integration of this biological control measure into crop protection programmes and to the optimisation of cultivation and formulation of the antagonists in order to implement a practical approach for the control of both insect species in protected crops.

- Among the isolates tested two of *P. fumosoroseus* and three of *M. anisopliae* were found with an equally high potential for the control of both whitefly species although the fungi derived from different climatic zones and had been isolated from different insects. *M. anisopliae* had an effect on egg hatch under high relative humidity and on all larval stages of *T. vaporariorum* under greenhouse conditions, but efficacy was decreasing for the older larval stages. The fungus was neither repelling the insects from the leaf nor decreasing oviposition.

- Differences in fungal requirement concerning cultivation media and conditions were found to be intra- but not interspecific. *P. fumosoroseus*, which is so far mainly produced in liquid media, was satisfactorily growing and sporulating on solid media. *M. anisopliae* var. *anisopliae* 97 proved to be the most tolerant isolate to low humidity.

- For the integration of the entomopathogenic fungi into existing crop protection programmes the effect of a combined treatment of the entomopathogens and two chitin synthesis inhibitors, 'novaluron' and 'buprofezin', was investigated. Both insecticides had no effect on the fungi and synergistic effects were found for *M. anisopliae* and 'novaluron'. When applied with a time interval of two days the combined treatment increased mortality of 1st instar larvae of *T. vaporariorum* as well as the speed of kill and the mortality of larvae of *Spodoptera littoralis*, the Egyptian cotton

leafworm. Control of the greenhouse whitefly was not sufficient for an effective pest control but a combination of the antagonist with 'novaluron' offered high potential for reducing the pesticide input for the control of *S. littoralis* distinctly.

- Shelf-life is a critical factor in the production of entomopathogens. When different polymeric additives were evaluated for their potential to enhance the storage potential of conidia of *M. anisopliae*, PA1 was found to increase the shelf-life in a liquid formulation at room temperature and at 4 °C. When the conidia were dried in the formulation this additive and Stockosorb®Agro increased the viability of stored conidia at room temperature, thus offering a good possibility for storage under ambient temperatures.

- The success of biological control with entomopathogenic fungi partially depends on the persistence of applied inoculum on the leaf surfaces. The polymeric additive PA1 promoted the adhesion of spores on the abaxial leaf surface of tomato plants and preserved their viability for two weeks, while conidia formulated in Addit® did not persist on the leaf and lost their viability rapidly. Microscopical assessments of the polymer found dense gel-bodies, which enhanced adhesion and protected the embedded conidia from environmental influences.

- Distribution of formulations –and hence of conidia- on the leaves and insect surfaces was affected by the type of additive. Droplet sizes and coverage of leaf surfaces were found to be different in the formulations investigated and so was the aggregation of conidia on insect larvae. Average droplet diameters ranged from 200 to 900 µm and spores were spread evenly or clustered together. The lowest droplet sizes and clusters of conidia were found for a formulation with Tween® 80, the biggest droplets and clusters as well as single conidia were observed for the polymeric additive PA1, which almost covered the leaf completely.

- Spore germination was affected by different humidities but additives were found to increase germination at low humidity. Investigations on artificial medium, on insect cuticle pieces *in vitro* and on whitefly larvae under greenhouse conditions indicated faster germination of *M. anisopliae* when formulated in Addit® or in the polymeric additive PA1.

- Pest control of whiteflies with entomopathogenic fungi could be enhanced by Addit® and by the polymer PA1. While Addit® was found to increase the efficacy of *M. anisopliae* and *P. fumosoroseus* against *Bemisia argentifolii* as well as against *Trialeurodes vaporariorum*, PA1 enhanced the efficiency of *M. anisopliae* for the control of *T. vaporariorum*. PA1 was also found to have a repellent effect for at least one week on adult probing and oviposition in a prophylactic treatment.

These investigations demonstrate that polymeric additives, which have only recently been discovered for the formulation of biological control agents, and Addit®, developed as additive for *Verticillium lecanii,* have a high potential for increasing the effectiveness of *M. anisopliae* and *P. fumosoroseus* for crop protection. The prospect for integrating the microbial control agents into IPM-programs for the control of *B. argentifolii* and *T. vaporariorum* is discussed.

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RESUME

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