Environmental-Stress Tolerant Formulations of *Metarhizium anisopliae* var. *acridum* for Control of African Desert Locust (*Schistocerca gregaria*)

by

Jarrod Ethan Leland

Dissertation submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree

Of

Doctor of Philosophy

In

Entomology

Approved By:

_____	_____
Donald E. Mullins, Chairperson Professor of Entomology	Herman L. Warren, Co-Chairperson Professor of Plant Pathology, Physiology and Weed Science
_____	_____
Edwin E. Lewis Assistant Professor of Entomology	Orson K. Miller Professor of Biology and Curator of Fungi
_____	
Richard F. Helm Associate Professor of Wood Science and Forest Products	

October 3, 2001
Blacksburg, Virginia 24061

Key Words: Microbial Biopesticide, Entomopathogenic Fungi, Formulation, Solar Radiation, African Desert Locust

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(ABSTRACT)

Entomopathogenic fungi are highly susceptible to the damaging effects of solar radiation. Attempts to protect entomopathogenic fungi from solar radiation have been, for the most part, unsuccessful. A new strategy for formulating entomopathogenic fungi for protection from solar radiation and desiccation has been tested using the acridid entomopathogen *Metarhizium anisopliae* var *acridum* (IMI 330189). This strategy involves coating spores with water-soluble materials that provide protection from solar radiation and enhance spore survival during drying. Development of this formulation involved the following: 1) production of an infective spore-type in liquid culture that could survive drying; 2) coating spores during an air-drying process; 3) reducing formulation particle size for oil suspension; 4) testing the effects spore coating on spore-tolerance simulated sunlight; and 6) testing the effects of spore coating infectivity to *Schistocerca americana*.

Aerial conidia, submerged conidia, and blastospores produced in a high-osmolality liquid medium all had high desiccation tolerance relative to blastospores produced in Adamek's media. Blastospores produced in high osmolality medium were the most infective to *S. americana* in an aqueous 20% molasses solution followed by submerged conidia and aerial conidia, with LT_{50} values (95% C.I.) at 1 x 10^6 spores/insect of 7.8 d (6.7 to 9.0 d), 10.5 d (9.5 to 11.6 d), 14.6 d (11.9 to 18.0 d), respectively. Comparisons were made among cell-wall characteristics of these spore-types, including cell-wall thickness, lectin-binding, charge, and hydrophobicity.

An optimal spore-coating formulation was selected on the basis of spore survival and germination over time after air-drying, particle-size reduction, and storage at 28 C. This spore-coating formulation, consisting of skim milk, Kraft lignin (Curan 100®) and

glycerol, greatly improved the tolerance of aerial conidia and submerged conidia to simulated sunlight, increasing the LT_{50} (95% C.I.) of aerial conidia from 4.0 hr (3.1-5.1) to 17.0 hr (12.5-23.0). The spore coating formulation decreased the infectivity of spores in oil to adult *S. americana*; reducing the LT_{50} values of aerial conidia at a dose of 1×10^5 spores / insect from 5.8 d (4.9-6.9 d) to 8.2 d (7.3-9.3 d).

Dedication

This dissertation is dedicated to my family. My mother and father, Laurence and Deborah Leland, have been a wellspring of strong values and towering examples of the potential for human good. They have taught me how to nurture by example, how to love by love, and how to grow by patience. During the most difficult times of this work, I would come home to Myra and the troubled and turbulent world would melt away. Hand in hand we have made this journey. I cannot imagine a stronger or softer hand to hold.

Acknowledgments

This research was funded by a USAID/Africa Bureau Project Grant No. AOT-G-00-97-00386-00 entitled Development of Biopesticides for Grasshopper and Locust Control in Sub-Saharan Africa managed by the Office of International Research and Development at Virginia Tech.

I cannot fully express in words my sincere gratitude for the friendship and guidance that my major advisor Dr. Donald Mullins has extended throughout my graduate education. His concern for my growth as a professional and as an individual has always transcended the boundaries of the workplace. His approach to mentoring provided for me the freedom to explore combined with the solid foundation of thoughtful advice. I have known few individuals in my life that have been so willing to go so far to help others. Despite the many lessons that he has taught me in relation to my research, his greatest lessons have been fundamental values and principles that guide our human relations and our contributions to society.

I would also like to thank my co-advisor, Dr. Herman Warren and the members of my committee, Dr. Edwin Lewis, Dr. Richard Helm, and Dr. Orson Miller for their support and guidance. Their doors were always open and they always provided kind and insightful advice. Dr. Larry Vaughan in the Office of International Research and Development, was for all practical purposes a sixth committee member on this dissertation. He was instrumental in the foundation and development of this research.

I would like to thank Dr. S. K. De Datta for his support throughout the course of this dissertation. He had tremendous faith in my ability to overcome what seemed to be an insurmountable task.

This research was conducted with the assistance of many generous individuals. The research team at INRA, Dr. Nathalie Smits, Dr. Jacques Fargues, Dr. Marc Rougier, Herve de Conchard, Jean-Marc Thuiller, and Bruno Serate and EBCL/USDA-ARS Dr. Charles Quimby and Guy Mercadier treated me like family during my visit to their research centers, and continued this warm hospitality throughout our collaborative research effort. Sandra Gabbert was instrumental in the analysis of spore carbohydrate

concentrations and put in a tremendous amount of extra effort to help me answer those last few critical questions. June Mullins prepared spores for transmission electron microscopy with artistic precision and attention to detail. Bob Wright and Dr. Wolfgang Glasser of the Department of Wood Science and Forest Products provided excellent advice related to lignin chemistry. In addition to extensive use of the laboratories of Dr. Herman Warren, the following faculty members provided laboratory facilities and related expertise Dr. Orson Miller in the Department of Biology , Dr. Foster Agblevor in Department of Biological Systems Engineering, Dr. Joseph Falkinham in the Fralin Biotechnology Center, and Dr. L. T. Kok in the Department of Entomology. Several undergraduate students assisted with various aspects of this research throughout the course of this study including Neal Gwaltney, Heather Hardcastle, Josh Herr, Anne Nielsen, Mike Reidy, Shimah Wheatley, and Elizabeth Wiegand.

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Chapter 1. Introduction, Review of Literature, and Objectives

3.5. Introduction

Locusts and grasshoppers (Orthoptera : Acrididiae) are highly conspicuous and unpredictable agricultural pests that can severely disrupt local economies and cause severe food shortages in subsistence farming systems. Under favorable climatic conditions, primarily related to moisture availability for egg eclosion and plant growth, populations may increase rapidly, resulting in gregarious and migratory behavior and swarm outbreaks in some species. National authorities are generally able to control outbreaks at an early stage by preventative control measures. However, when these control measures fail due to inadequate early control, political limitations, or natural disasters, plagues may develop. This has occurred most recently with desert locust *Schistocerca gregaria* (Forskål) in the Red Sea basin in 1986 and 1992, with the migratory locust *Locusta migratoria capito* (Sauss) in Madagascar in 1995, and with the Italian locust *Calliptamus italicus* L. in Kazakhstan in 1997 (Lomer et al., 2001). Early control efforts of locusts first used the organochlorine dieldrin in barrier strips against hopper bands and in aerial application against swarms. In an effort to reduce environmental impact of chemical pesticides, control campaigns in the early 1980's against the desert, migratory and red (*Nomadacris septemfasciata* Serville) locusts turned to less persistent organophosphates, such as fenitrothion and malathion. Currently, modern locust control relies on organophosphates (e.g. fenitrothion), carbamates (e.g. bendiocarb), pyrethroids (deltamethrin and lamda-cyhalothrin), fipronil, and insect growth regulators (e.g. dimilin and triflumeron).

Concerns over environmental issues related to chemical pesticide use such as; impact on humans, livestock and wildlife including natural enemies of locusts and grasshoppers, water contamination and pesticide disposal issues have led to changes in chemical strategies and development of Integrated Pest Management (IPM) programs. Development of an IPM approach to control grasshoppers and locusts includes: 1)

exploring environmentally-sound locust control-technologies; 2) evaluating novel and existing technologies for efficacy and environmental impact; and 3) integrating novel and existing technologies in combination with improved forecasting and monitoring for potential outbreaks (Lomer et al., 2001).

Biological control with pathogens may play an important role in this integrated pest management strategy. Extensive screening of virulence and spore stability has lead to the selection of *Metarhizium anisopliae* var. *acridum* (Metschnikoff) Sorokin(IMI 330189) for commercial development as a microbial biopesticide of grasshoppers and locusts. Industrial development of this microbial biopesticide faces many challenges; in addition to maximizing spore production, spores must be dried to ensure adequate shelf-life and spore inactivation in the field due to solar radiation continues to be a serious problem despite extensive research efforts. The research in this dissertation examines a novel approach to microbial pesticide formulation for improved solar-radiation tolerance. Development of this formulation must integrate information on spore biology, spore-drying processes and coating formulations particularly as they relate to spore-survival during formulation, infectivity and spore-tolerance to UV-radiation. This approach relies on information already available in the scientific and patent literature related to the biology of *Metarhizium* spp., stress-tolerance in biological systems, and approaches to formulating microbial biopesticides as well as the development of new information necessary for implementation of this formulation strategy. Formulation of microbial biopesticides has been a little-studied area of research, which may provide an opportunity to significantly enhance the utility of entomopathogenic microorganisms for insect control. However, efforts to develop a better understanding of spore biology, particularly in relation to stress tolerance, integrated with cultural conditions, formulation, storage and application are needed. Research described in this dissertation is provided in an effort to expand our knowledge on a specific entomopathogenic fungus, *Metarhizium anisopliae* var. *acridum* and examine a novel approach to microbial biopesticide formulation.

1.2. General Biology of *Metarhizium*

The first species of the genus *Metarhizium* (Subdivision Deteromycotina; Class Hyphomycetes; Order Moniliales), *Metarhizium anisopliae,* was isolated from the Coleopteran species *Anisopliae austriaca* by Metchnikoff in 1878. *Metarhizium* spp. occur ubiquitously around the world in alternating life stages between a soil saprophytic stage and an insect pathogen stage. *Metarhizium* spp (including, *M. anisopliae*, *M. flavoviride*, *M. album* and *M. brunneum*) generally have wide host ranges; however, there is host specificity among isolates of these species.

Under natural conditions, *Metarhizium spp.* produce two spore-types. Aerial conidia, which are produced on phialides during the saprophytic life stage or on host cadaver, and are defined as asexual spores produced on specialized sporogenous hyphae known as phialides. A second spore-type is produced in the insect hemolymph that is commonly referred to as a "blastospore", which are characterized on the basis of possessing similar cell-wall characteristics to hyphae and are referred to as "blastospores" because of propagation by production through a pore ("blastic spore production"). However, it has been shown for *B. bassiana* that the propagation stage in the insect hemolymph may shed surface carbohydrates and structural components (e.g. chitin) resulting in a protoplast-like spore form (Pendland et al., 1993).

Metarhizium spp. produce three spore-types *in vitro*. Aerial conidia and blastospores described in the previous paragraph can be produced in solid culture and liquid culture, respectively. In addition, submerged conidia, which are produced in liquid culture containing limited nitrogen and excess carbon, have been characterized on the basis of similar size characteristics and coloration to aerial conidia and spore production on phialide-like sporogenous cells (Jenkins and Prior, 1993). The practical advantages of producing submerged conidia in liquid culture that have similar characteristics to aerial conidia are the ease of production in liquid culture combined with the added environmental stability that "true conidia" are thought to possess over blastospores (Jenkins and Goettel, 1997). Cultural conditions in liquid fermentation can be closely

monitored and controlled and the technology is well established in industry. Aerial conidia possess a coating of monoamines known as hydrophobins that may offer some environmental protection and increase spore hydrophobicity. Hydophobins have also been shown to be present on the surface of submerged conidia of the closely related entomopathogenic fungus *Beauveria bassiana* (Bidochka et al., 1995). However, *M. anisopliae* var *acridum* submerged conidia are hydrophilic and cannot be formulated easily in oils (Jenkins and Thomas, 1996).

Comparisons of cell-wall surface characteristics, virulence and stability have been made among the three spore-types of *B. bassiana*; however similar comparisons have not been made for the three spore-types of *Metarhizium* spp. Blastospores, submerged conidia, and aerial conidia of *B. bassiana* may be differentiated by spore morphology, sporogenous-cell morphology, spore-surface characteristics, infectivity, and shelf-life. Scanning electron microscopy of the spore surface (Thomas et al., 1987) and polyacrylamide gel electrophoresis of proteins from the spore surface (Boucias et al., 1988) have shown differences among these three spore-types. The infectivity, shelf-life and cell-wall-surface characteristics (surface carbohydrates and hydrophobicity) have been compared among these spore-types (Hegedus et al., 1992). Blastospores are the least hydrophobic, possess relatively few surface carbohydrates, and do not survive storage well, but are the most virulent spore form. Submerged conidia show similarities to aerial conidia with respect to hydrophobicity, surface carbohydrates, and virulence; however they have a shorter shelf-life than aerial conidia at sub-freezing temperatures.

1.3. The Need for More Persistent Entomopathogenic Fungal Biopesticides

Secondary pick-up of *M. anisopliae* var *acridum* either by persistent spores on vegetation or horizontal transmission through sporulating cadavers may contribute significantly to control of African acridid pests (Jenkins and Thomas, 1996; Lomer et al., 1993; Thomas et al., 1997). The horizontal transmission of spores from infected cadavers is only significant in environments where humidity is high enough to allow sporulation of

infected cadavers, although *M. anisopliae* may infect at much lower humidity (Lomer et al., 1993; Thomas et al., 1997; Bateman et al., 1993). In arid climates where epizootics cannot be established, insect control is limited to direct contact with the spray residue or secondary pick up from vegetation, increasing the needed for formulations that enhance spore persistence in the environment.

1.4. Effects of Environmental Stress on Spore Viability

When developing a fungal biopesticide, spore viability must be evaluated at each of the stages in the process, these include; initial viability following spore production, survival during the formulating process, shelf-life of the formulated spores, and persistence of the formulated spores in the environment. Measures may be taken at each of these stages to enhance field persistence of the final product. Spore physiology and morphology are affected by culturing conditions during spore production, which influence spore-stress tolerance. Protective materials may be added to spores during the formulation process to enhance tolerance to heat, desiccation and UV. Spores can be stored under conditions that maximize shelf life. Timing spray applications can minimize the effects of environmental stress. Spores may be killed during storage and in the environment by water-stress; including osmotic stress and extreme desiccation, thermal-stress, and sunlight. Of these factors, thermal stress is the most significant for formulated spores prior to application. However, the effects of thermal-stress on spores may be mitigated by drying spores to 4-6% moisture and keeping them dry in the formulated material (Hedgecock et al., 1995; McClatchie et al., 1994; Moore et al., 1997; Hong et al., 1997; 1998). The most damaging factor to spores after field application is exposure to ultraviolet radiation from sunlight (Ignoffo, 1992).

1.4.1. Ultraviolet Light

Ultraviolet-radiation from sunlight (particularly UVB, 280-320 nm) causes direct structural effects on DNA or indirect damage caused by the formation of reactive oxygen molecules, with the indirect action by reactive oxygen molecules likely being the most significant damaging factor (Ignoffo and Garcia, 1978, 1994; Ignoffo, 1992). The half-life of most entomopathogenic fungal conidia ranges from 1 to 4 hr in simulated sunlight and 4 to 400 hours in natural sunlight on foliage; the difference likely being the effects of shielding provided by the plant or sampling time in field studies (Ignoffo, 1992). Fargues et al. (1996) compared the susceptibility of aerial conidia from 65 isolates of *B. bassiana*, 23 isolates of *M. anisopliae*, 14 isolates of *M. flavoviride* (including the isolate IMI 330189 reclassified as *M. anisopliae* var. *acridum*), and 33 isolates of *Paecilomyces fumosoroseus*. Overall, isolates of *M. flavoviride* were the most tolerant to simulated sunlight followed by *B. bassiana* and *M. anisopliae*, *with P. fumosoroseus* being the least tolerant. Following the longest exposure time (8 hr) survival of *M. flavoviride* and *M. anisopliae* isolates ranged from 0.1 to 11%, and from 0 to 1%, respectively (Fargues et al., 1996). Survival of *M. anisopliae* var *acridum* (IMI 330189) aerial conidia was 46, 23, 8, and 5% following 1, 2, 4, and 8 hours of exposure to simulated sunlight, respectively.

In addition to causing spore mortality, exposure to UV inhibits the germination rate of *Metarhizium* spp. (Alves et al., 1998; Hunt et al., 1994; Moore et al., 1993). The delay in germination following exposure to simulated sunlight may be the result of damage to proteins and nucleic acids leading to slower growth rates, a defense response of the conidia, or the result of time and energy being devoted to repair mechanisms (Moore et al., 1993). Strategies for protecting spores from sunlight will be discussed in Section 1.5 Formulation of Entomopathogenic Fungi.

1.4.2. Reactive Oxygen Molecules

As mentioned in the previous paragraph the primary damaging effects of ultraviolet light are hypothesized to occur through indirect damage caused by the formation of reactive oxygen molecules (Ignoffo and Garcia, 1978, 1994; Ignoffo, 1992).

In addition hydrogen peroxide (H_2O_2) may accumulate in cells due to the partial inactivation of catalase, resulting from the combined effects of heat and NaCl (Bucker and Martin, 1992). Hydrogen peroxide H_2O_2 inactivates certain superoxide dismutases. The inactivation of these enzymes leads to accumulation of superoxide ($O_2^{\cdot}$) within the cell. Hydrogen peroxide may then combine with superoxide to form the most potent oxidant, hydroxyl free radical ($OH^{\cdot}$). Antioxidants and oxidative enzymes have been used to reduce the effects of reactive oxygen molecules on *Bacillus thuringiensis* and *Baculovirus heliothis* caused by exposure to UV-radiation (Ignoffo and Garcia, 1978; 1994), and to enhance the shelf life of *M. anisopliae* var *acridum* in oil (Moore et al., 1995).

It is possible that the spore physiology of *M. anisopliae* var *acridum* could be manipulated to reduce the effects of reactive oxygen molecules. Mannitol is a scavenger of superoxide radical (Chaturvedi et al., 1996) and is also the primary polyol produced in *M. anisopliae* (Hallsworth and Magan, 1994 a, b; 1996). Chaturvedi et al. (1996) demonstrated that a mutant of the fungus *Cryptococcus neoformans*, which produced lower concentrations of mannitol than the wild type, was more susceptible to polymophonuclear neutriphils which kill *C. neoformans* by oxidative mechanisms. Mannitol concentrations in *M. anisopliae* may be manipulated by altering carbohydrate type and carbohydrate concentration (Hallsworth and Magan, 1994a); water activity (Hallsworth and Magan, 1994b); and temperature and pH (Hallsworth and Magan, 1996) in solid culture.

1.4.3. Water Stress

Water relationships are critical to the proper functioning of entomopathogenic fungi. Spore germination and hyphal growth are optimal at 95 - 100% r.h. and these functions cease at 93% r.h. (Burgess, 1998). However, relative humidity of the microenvironment must be considered when evaluating germination potential, and spores may be able to obtain enough moisture from insect intersegmental membranes to

germinate in dry climates (Bateman et al., 1993; Burgess, 1998). The shelf-life of entomopathogenic fungi is greatly affected by moisture content. Shelf-life of *M. anisopliae* var *acridum* is optimal at either very high r.h. (96-98%), by maintaining a metabolic state but not allowing germination (Daoust and Roberts; 1983); or very low moisture content (4-6%) by reducing spore metabolism (Hedgecock et al., 1995; McClatchie et al., 1994; Moore et al., 1997; Hong et al., 1997; 1998). In addition to reduced spore metabolism, at low moisture contents (4 to 6%) it is likely that non-freezable water has been removed from the spores, suggesting a potential role for disaccharides in stabilizing membranes and protiens as described by the "water replacement hypothesis", which will be explained in Section 1.3.3.2 (Crowe et al., 1990; Beker et al., 1984). Reduction in metabolism at higher moisture contents may also be obtained by removing oxygen from the formulated material (Jin et al., 1999). Membrane damage by imbibition may also occur during spore rehydration resulting in loss of cytoplasmic material. These damaging effects may be reduced by incubating spores in a high r.h. environment for at least 1 hr prior to suspension in water (Moore et al., 1997) or by soaking spores in a surfactant mixture, such as ethoxylated alcohol, with a hydrophile-lipophile balance (HLB) number of 10 (Jin et al., 1999).

The effects of water-stress on cells may be divided into two broad categories: 1) effects at higher moisture contents where "free water" is still available within the cell and 2) effects at lower moisture content where "bound water" that surrounds and stabilizes biomolecules has been removed (Crowe et al., 1990). We will first consider damaging effects at higher moisture contents including osmotolerance, effects of imbibition during rehydration, and freeze-thaw damage. Next we will look at the effects of removing bound water from the cell on the stability of biomolecules.

1.4.3.1. Water Balance : Role of Compatible Solutes

At higher moisture contents, compatible solutes function to stabilize biomolecules within cells. Compatible solutes; including electrolytes such as inorganic salts and

nitrogenous compounds and non-electrolytes such as carbohydrates and polyols, may accumulate in high concentration without inhibiting or destabilizing enzymes (Brown, 1976). The hypothesized mechanism by which compatible solutes function to stabilize biomolecules (e.g, proteins and membranes) involves preferential exclusion of the compatible solutes from the surface of the biomolecules resulting in a thermodynamically disfavorable situation. The presence of compatible solutes is hypothesized to reduce biomolecule denaturation because denaturing of the biomolecule would result in increased surface area of the biomolecule and, therefore, an increase in the thermodynamically disfavorable situation (Crowe et al., 1990).

Polyols are also used by spores in osmotic regulation and polyol concentrations in aerial conidia have been shown to influence both germination at reduced water availability (Hallsworth and Magan, 1995) and lag phase of germination of *M. anisopliae* and *V. lecanii* on melon cotton aphids, *Aphis gossypii* (Andersen et al., 1999). The concentrations of various molecular weight polyols (glycerol, aribitol, erythritol and mannitol) in *M. anisopliae* may be manipulated by altering carbohydrate type and carbohydrate concentration (Hallsworth and Magan, 1994a); water activity (Hallsworth and Magan, 1994b); and temperature and pH (Hallsworth and Magan, 1996) in solid culture.

1.4.3.2. Extreme Dryness: Role of Non-reducing Disaccharides in Extreme Dryness

Biomolecules (e.g. membranes and protiens) are surrounded by a molecular layer of water that acts to stabilize the biomolecules by hydrogen bonding ("crystaligraphically bound") (Crowe et al., 1990). Under conditions of extreme dryness this bound water is removed resulting in destabilization of the biomolecules. Nonreducing disaccharides (particularly trehalose) have the ability to stabilize membranes (Crowe et al., 1984; 1988; 1990) and enzymes (Carpenter and Crowe, 1988 a, b; Colaco et al., 1992) by preserving them in a "glass state" in conditions of extreme desiccation when molecular "bound" water is removed. This property appears to be unique to disaccharides and larger

molecular weight sugars and is not a property of other cryoprotectants, such as "compatible solutes" described above, which only provide stability in the presence of free water (Crowe et al., 1990). Disaccharides provide increased desiccation protection when present on both sides of the phospholipid bilayer, which requires either the production of endogenous sugar or uptake from the environment (Crowe et al., 1986). However, exogenous disaccharides have also been shown to enhance the ability of microorganisms to survive desiccation (Leslie et al., 1995). Aerial conidia of *M. anisopliae* are able to survive drying to moisture contents of less than 5% moisture suggestion a role of trehalose in the since bound water has likely been removed at this low moisture content. Aerial conidia of *Metarhizium anisopliae* have been shown to produce trehalose in low concentrations (Hallsworth and Magan, 1994). Several researchers have demonstrated the beneficial effects of various non-reducing disaccharides, particularly trehalose, on survival of yeast during freeze-drying (Berny et al., 1991; Diniz-Mendez et al., 1999; Lodato et al., 1999; Tan et al., 1995). In addition, maltose has been used to protect mycelia of *M. anisopliae* during air-drying (Krueger et al., 1992).

1.4.4. Heat Stress

The shelf life of fungal biopesticides generally decreases with increasing temperature (Burgess, 1998). Drying *M. anisopliae* aerial conidia improves their thermal-stress tolerance (Hedgecock et al., 1995; McClatchie et al., 1994; Moore et al., 1997; Hong et al., 1997; 1998). The additional thermal-stress tolerance provided by preserving *M. anisopliae* var. *acridum* aerial conidia in a state of anabiosis may be particularly important in its field application where temperatures may exceed 50 C and storage facilities may not provide adequate temperature regulation (McClatchie et al., 1994). Hong et al., (1997) demonstrated that there was a negative logarithmic relationship between the moisture content of *M. anisopliae* var. *acridum* aerial conidia and their ability to survive thermal stress (50 C). The lower limit of this relationship was 4.6% moisture and the upper limit was between 21.2% and 31.8% moisture (Hong et al., 1998).

Between these lower an upper limits the survival of aerial conidia at 50 C was improved with decreasing moisture content. Below the lower limit, additional drying did not further improve thermal-stress tolerance, and above the upper limit, additional moisture did not further reduce thermal-stress tolerance.

The method of drying *M. anisopliae* var *acridum* aerial conidia influences the thermal-stress tolerance of dried spores (Hong et al., 2000). Although the faster convective air-drying process may remove water more efficiently; aerial conidia of *M. anisopliae* var *acridum* survived better in storage at high temperatures (50 C) when initially dried more slowly at 50 to 60% r.h (Hong et al., 2000). It is likely that *M. anisopliae* undergoes some physiological changes in preparation for anabiosis at the slower drying rates; however, the nature of these changes are not known. In nature entomopathogenic fungi survive dry conditions either as mycelium or hyphal bodies in insect cadavers, or as aerial conidia. Drying of aerial conidia in the environment may be rapid but drying of mycelium and hyphal bodies in cadavers would be expected to be relatively slow (Burgess, 1998).

As discussed in the previous section, at the low spore moisture content (4.6%) that is optimal for thermal-stress tolerance, bound water has likely been removed from the cell suggesting a role for disaccharides. In addition to the potential role of endogenously-produced trehalose to enhance desiccation tolerance, addition of exogenous sugar prior to drying may enhance desiccation tolerance (Leslie et al., 1995). The physical properties of this exogenous sugar (i.e. hydrogen bonding potential and glass transition temperature) may affect the thermal-stress tolerance of the dry spores (Tan et al., 1995). As storage temperature begins to exceed the onset of the glass transition temperature of the sugar (T_{on}), activity of molecular water increased resulting in deterioration of the dry spore.

1.5. Manipulating the Physiology of Fungal Biopesticides to Improve Persistence and Virulence

In addition to affecting the type of spore that is produced (aerial conidia, submerged conidia, or blastospore) as described in Section 1.1, cultural condition may be used to improve spore physiology for improved persistence and virulence. Some examples of manipulation of culturing conditions leading to improvements in physiological characteristics and corresponding efficacy of fungal biopesticides include the following:

1) Water activity in solid culture - the water activity of solid media was correlated with polyol concentrations, germination rates for four entomopathogenic fungi (*Metarhizium anisopliae*, *Beauveria bassiana*, *Verticillium lecanii*, and *Paecilomyces farinosus*), and lag phase of germination of *M. anisopliae* and *V. lecanii* on melon cotton aphids, *Aphis gossypii* (Andersen et al., 1999);
2) Osmolality in liquid culture - osmolality of liquid media was correlated with spore trehalose concentrations, desiccation tolerance, and disease protection in the plant disease protectant, *Trichoderma harzianum* (Harman et al., 1991);
3) Carbon to nitrogen ratios - carbon to nitrogen (CN) ratios in liquid media were correlated with spore-carbohydrate concentrations, shelf-life at 25 C and adherance to insect cuticle in the entomopathogen *B. bassiana* (Lane et al., 1991 a, b); with desiccation tolerance in the entomopathogenic fungus, *Paecilomyces fumosoroseus* (Jackson et al., 1997); and with gross morphology, germination rates, and infectivity of the bioherbicide *Colletotrichum truncatum*; (Jackson, 1997; Schisler et al., 1990)
4) Temperature - culture temperature has been correlated with cell wall thickness, germ tube formation, and infectivity of the mycoherbicide *Alternaria helianthi* (Abbas et al., 1995).

The potential beneficial effects of polyols and trehalose, including scavenging of superoxides, water balance and stability at high moisture content, and desiccation tolerance, are described in the previous sections (Sections 1.3.2, 1.3.3.1, and 1.3.3.2, respectively). Spore polyol and trehalose concentrations have been manipulated in *M. anisopliae* solid culture by alterations in carbohydrate source, carbohydrate concentration, water activity, pH and temperature, but parallel work has not been

conducted in liquid culture for *Metarhizium* spp. (Hallsworth and Magan, 1994 a, b, 1996).

1.6. Formulation of Entomopathogenic Fungi

1.6.1. Granular Formulations

Strategies for application of granular formulations of entomopathogenic fungi include the application of dry mycelium or spores formulated as contact baits. Strategies that use application of dry mycelium rely on sporulation in the field to produce the infective aerial conidia and are, therefore, impractical for control in arid climates (Knudsen et al., 1991; Krueger et al., 1992; Pereira and Roberts, 1991; Burgess et al., 1998). However, contact baits that use maize-starch extrusion technology or formulation with wheat bran have shown promise in the control of *S. gregaria* and *Melanoplus sanguinipes* (Caudwell and Gatehouse, 1996 a,b, Inglis et al., 1996). The contact baits protect spores from sunlight and attract grasshoppers and locusts, thereby, increasing contact with the spores. The spores do not infect through the alimentary canal since S. *gregaria* is known to produce gut antifungal toxins (Dillon and Charnley, 1986, 1995), rather the insects become surface contaminated while ingesting the bait substrate (Inglis et al., 1996).

1.6.2. Sprayable Formulation

Strategies for protecting microbial biopesticides in liquid formulations from solar degradation have used the following general approaches: 1) the use of oil-soluble sunscreens with oil-carriers (Hunt et al., 1994; Moore et al., 1993; Shah et al., 1998); 2) the use of oil-water emulsions (Alves et al., 1998); 3) the use of water-soluble or suspendable absorbers or blockers with water carriers (Cohen et al., 1991, 2001; Ignoffo

et al., 1997; Shapiro 1989, 1992, Shapiro and Robertson, 1990; Shasha et al., 1998) or 4) encapsulation (e.g. non-soluble starch) with a water carrier (Behle, et al., 1997; Ignoffo et al., 1991; McGuire and Shasha, 1995; McGuire et al., 1990, 1994, 1996; Shasha and Dunkle, 1989; Shasha and McGuire, 1989; Tamez-Guerra, et al., 1996). The first three of these strategies have been used for *Metarhizium* spp.

1.6.2.1. Aqueous Formulations

Aqueous formulations form a large contact angle with hydrophobic surfaces; therefore, when sunscreens are added to water-based formulations a large amount of sunscreen is sited over the spore protecting it from sunlight (Burgess, 1998). In addition, the water droplet evaporates concentrating the UV-blocker or absorber and increasing protection to the spore (Burgess, 1998). Inglis et al. (1995) tested the effects of 11 sunscreens (10 absorbers and a UV blocker) with water based formulation of *B. bassiana* in laboratory tests on leaves using simulated sunlight and in the field. Five of the formulations that provided the greatest protection (195 to 454 fold increase) in the laboratory following exposure to 3 hr of simulated sunlight were tested in the field. Only two of these provided significant protection following 8 hr of exposure to natural sunlight. The UV-absorber Tinopal LPW increased survival by 6.6 times and the UV-blocker, clay increased survival by 8.0 times (Inglis et al., 1995; Burgess, 1998). Researchers at USDA-ARS have received a patent for a lignin-based formulation that improves UV-protection and rainfastness (Shasha et al., 1998). Water-soluble lignins are combined with a multivalent salt in an aqueous suspension, cross-links between the cation on a multivalent salt and the anionic cinnamyl alcohol residues of the lignin molecules occur as the water droplet evaporates. This reaction coats the microorganism with a lignin material that has reduced water solubility. Examples in the patent include the use of *Bacillus thuringiensis*, baculovirus, and a chemical herbicide, but claims also include the use of entomopathogenic fungi. (Shasha et al., 1998)

1.6.2.2. Oil-Based Formulations

The use of oil-based formulations appear to provide three important advantages over water-based formulations, these include: 1) *Metarhizium anisopliae* var. *acridum* is more infective at low humidity in oil-based formulations (Bateman et al., 1993; Prior et al., 1988); 2) oil-based formulations allow the fungal biopesticide to be applied in a desiccated state, which reduces effects of thermal stress (McClatchie et al., 1994; Hedgecock et al., 1995; Hong et al., 1997, 1998); and 3) oil-based formulations are compatible with ultra-low volume application, which is important for reducing spray volume when controlling Desert Locusts in remote areas. These advantages may be of particular important when using entomopathogenic fungi to control insect pests in environments of high heat, low humidity, and high insolation, such as Desert Locust control in the Sahel.

Oil-soluble sunscreens and sunblockers have failed to enhance efficacy *Metarhizium anisopliae* var. *acridum* in field trials, although some of these materials enhance UV-protection in laboratory experiments on glass (Hunt et al., 1994, Moore et al., 1993; Shah et al., 1998). Oil-soluble sunscreens provide protection in laboratory studies on glass where they are applied in 100 µm films on Petri plates (Hunt et al., 1994; Moore et al., 1993) but not when applied on leave surfaces (Inglis et al., 1995). On leaf surfaces the sunscreens spread with the oil carrier over hydrophobic (plant) surfaces and may be absorbed by plant mesophyll leaving the spores exposed to direct sunlight (Inglis et al., 1995; Burgess, 1998). The interaction of water-based and oil-based formulations with hydrophobic surfaces, and a description of a novel approach to incorporating sunscreens into oil-based microbial formulations is depicted in Figure 1-1.

Spore suspension in emulsifiable-oil formulations has also been shown to significantly enhance the tolerance of *Metarhizium anisopliae* to simulated sunlight in laboratory experiments on glass (Alves et al., 1998). Spore germination values in the best

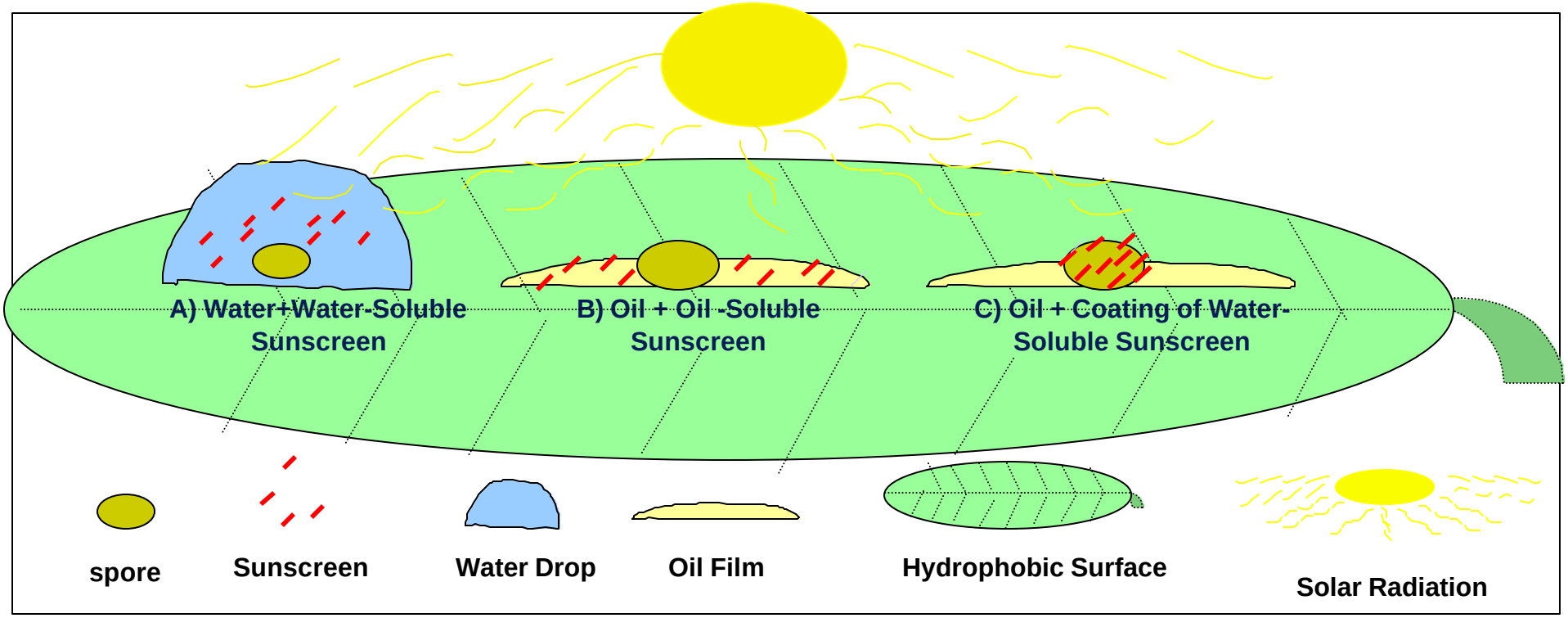

Figure 1-1. The interaction of water-based and oil-based formulations with hydrophobic surfaces, and a description of a new approach to incorporating sunscreens into oil-based microbial formulations.

A. The water droplet forms a large contact angle on the hydrophobic surface. As the droplet dries the water-soluble sunscreen concentrations around the spore (Burgess, 1998; Inglis et al., 1995; Shasha et al. 1998).

B. The oil droplet forms a thin film on the hydrophobic surface. As the oil droplet spreads over the surface, the oil-soluble sunscreen spreads with the oil exposing the spore (Burgess, 1998; Hunt et al., 1994; Inglis et al., 1995).

C. As the oil droplet spreads over the surface the water-soluble sunscreen coating continues to protect the spore. The coating may dissolve in the presence of water to allow infection. (The approach taken in this study)

oil formulation, oil emulsion, and in water were 73 ± 13%, 50 ± 12%, and 0.5 ± 0.2% following 6 hr of exposure to simulated sunlight. Oil-emulsion may be particularly useful for formulated *M. anisopliae* submerged conidia that cannot be suspended directly in oil.

An oil emulsion was also tested in field trials of *M. anisopliae* var *acridum* submerged conidia against *S. gregaria* and *Z. variegatus* (Jenkins and Thomas, 1996). Application of submerged conidia in an oil-emulsion resulted in significantly shorter mean survival times of *S. gregaria* than aerial conidia in oil or submerged conidia in water plus adjuvants or water alone. Spray residues of submerged conidia were sufficiently persistent to cause mortality by secondary pick-up from vegetation. (Jenkins and Thomas, 1996)

1.7. The Infection Process

Understanding the mechanisms that entomopathogenic fungi use to infect insects will aid in the development of more infective fungal biopesticides by enhancing characteristics of spore physiology and physical/chemical properties of the spore formulation. For a spore to successfully infect an insect it must overcome the following obstacles: 1) adherance to the insect cuticle; 2) germination on the cuticle; 3) formation of infective structures; 4) penetration of the insect cuticle and 5) evasion of the host immune system.

1.7.1. Adherence to the Insect Cuticle

The adhesion of the infectious stage may be divided into three stages: 1) adsorption, which is a passive phenomenon that is less host specific and is mediated by primarily by hydrophobic and to a lesser extent electrostatic interactions; 2) consolidation of the attachment, which is more host specific and is an active process involving metabolic activity, and 3) germination and growth of the fungus on the cuticle until

penetration; which is also an active process and is more host-specific (Boucias and Pendland, 1991). Aerial conidia of *M. anisopliae* are covered with a rodlet layer, which is highly hydrophobic and is likely responsible for the adherence of aerial conidia to the insect cuticle (Sosa-Gomez et al., 1997). The higher infectivity of *M. anisopliae* var *acridum* aerial conidia suspended in oil over water at low humidity is hypothesized to be a result of increased adhesion to the cuticle (Bateman et al., 1993; Prior et al., 1988). Host specificity and consolidation of the attachment may involve lectins and various enzymes present on the spore-surface and level of these spore-surface antigens have been correlated with virulence and host specificity (Boucias and Pendland, 1991; Rath et al., 1995). The distribution of conidia on the insect cuticle may be related to both the chemistry, particularly cuticular lipids, of the cuticle and the topography, particularly setae and epicuticle folds (Sosa-Gomez et al., 1997)

1.7.2. Requirements of *Metarhizium* for Germination

Optimum humidity for spore germination and mycelial growth is between 95 and 100% humidity and ceases at 92% relative humidity (Burgess, 1998). However, spores are able to obtain adequate from humid microenvironment of insect insersegmental membranes and thereby infect in arid climates (Bateman et al., 1993; Burgess, 1998). Germination triggers related to the presence or absence and concentration of exogenous nutrient correlate with surface characteristics of the host insect species from which the isolate was obtained (St. Leger et al., 1992; 1994). For example, Homoptera are more likely to produce sugar-rich secretions, and isolates of *M. anisopliae* from Homoptera, which germinate better in glucose media. Host specificity may also be related to the presence of fungistatic compounds (Butt et al., 1995; Sosa-Gomez et al., 1997). Blastospores are generally thought to have faster germination rates than conidia (Thomas et al., 1987). Spores with faster germination rates may have a greater potential for infection by reducing potential for desiccation, effects of other microorganisms, or loss

during molting while on the insect cuticle (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al, 1983).

1.7.3. Formation of the Appressorium

For most insects, penetration of the host cuticle requires the formation of a specific series of infection structures. Of these infection structures, the first and most important is the appressorium (St Leger et al., 1994). St Leger et al., (1991) proposed a model for the triggering of appressorium formation that involved tactile cues from the topography of the insect cuticle. This model is supported by studies that have demonstrated *M. anisopliae* producing appressoria *in vitro* on hard hydrophobic surfaces (St Leger et al., 1989). Appressorium formation *in vivo* is also influenced by surface topography (St Leger et al., 1991). In contrast, Butt et al. (1995) found no obvious indication of thigmotrophic or thigmotactic behavior on aphid and beetle cuticles. Differentiation *in vitro* is stimulated by low concentrations of complex nutrients (St Leger et al., 1989). However, differentiation is catabolite repressed in some isolates by readily available carbon. The pattern of catabolite repression appears to be related to the nutrient conditions of the host from which the isolate was obtained, similar to germination triggers (St Leger et al., 1992, 1994).

1.7.4. Production of Cuticle Degrading Enzymes

Penetration of the insect cuticle by entomopathogenic fungi requires mechanical pressure and cuticle degrading enzymes, including; proteases, chitinases and lipases. Cuticle-degrading enzymes likely work in concert to degrade the highly refractory insect cuticle. It appears that some of the enzyme systems may be redundant to ensure infectivity in the event that one system is inhibited (St Leger et al., 1994). Adding glucose or alanine to conidia germinating *in vitro* represses both appressorium formation

and PR1 expression, suggesting that there is a coordinated regulation of catabolite repression (Clarkson and Charnley, 1996). However, this catabolite repression mechanism may depend on host-specificity (St Leger et al., 1994).

1.7.5. Life Stages within the Locust and Toxin Production

Growth of fungal pathogens in the hemolymph may be as yeast-like blastospores, hyphal bodies or protoplasts, rather than in the form of a mycelium. These growth forms are designed to aid in dispersal through the insect hemolymph and evade the host immune system. Avoiding phagocytosis by the host immune system may explain the absence of certain carbohydrates and cell-wall structural components (e.g. chitin) in protoplast-like spores of *B. bassiana* that are produced *in vivo*, which are present on blastospores that are produced *in vitro* (Pendland et al., 1993).

Metarhizium anisopliae produces a group of cyclic depsipeptides known as destruxins (DTXs) (Clarkson and Charnley, 1996). In *M. anisopliae* 19 variants have been described, and 17 have been described from *M. flavoviride* . DTXs have been shown to be insecticidal by injection and, in some cases, when ingested by mouth. DTXs have diverse modes of action including depolarization of muscle $^{2+}$ membrane Ca^{2+} channels, inhibition hemocyte (blood cell) function, and inhibition of vacuolar-type ATPase.

1.8. Experimental Objectives and Rational

The two main hypotheses that this study was designed to test were: 1) water-soluble coatings enhance the survival of *M. anisopliae* var *acridum* spores in oil following exposure to simulated sunlight and 2) water-soluble coatings do not reduce the infectivity of *M. anisopliae* var *acridum* spores in oil. Before these hypotheses could be tested a series of experiments were required to develop this formulation that were related to changes in spore biology during spore production, and stages in the formulation process including coating during drying, particle size reduction, and storage. The overall

strategy used in achieving these objectives that includes integration of the stages of development and testing is described in Figure 1-2. The specific objectives in this development process were as follows:

5. To compare the three spore-types of *M. anisopliae* var *acridum* (aerial conidia, submerged conidia, and blastospores) with respect to cell-wall characteristics, germination rate and drying stability. (Chapter 3)
6. To investigate the influence of media osmolality in liquid culture on cell-wall surface characteristics, drying stability, infectivity, and spore-carbohydrate concentrations. (Chapter 3)
7. To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival in aqueous suspension. (Chapter 4)
8. To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival following an air-drying process. (Chapter 4)
9. To determine if there is an effect of spore-coating formulation on spore survival after reducing particle size of the dry coated-spore formulation. (Chapter 4)
10. To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival after storing the dry coated-spore formulation at 28 C. (Chapter 4)
11. To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival following exposure to simulated sunlight. (Chapter 5)
12. To determine if there is an effect of spore-coating formulation on infectivity to *Schistocerca americana*. (Chapter 5).

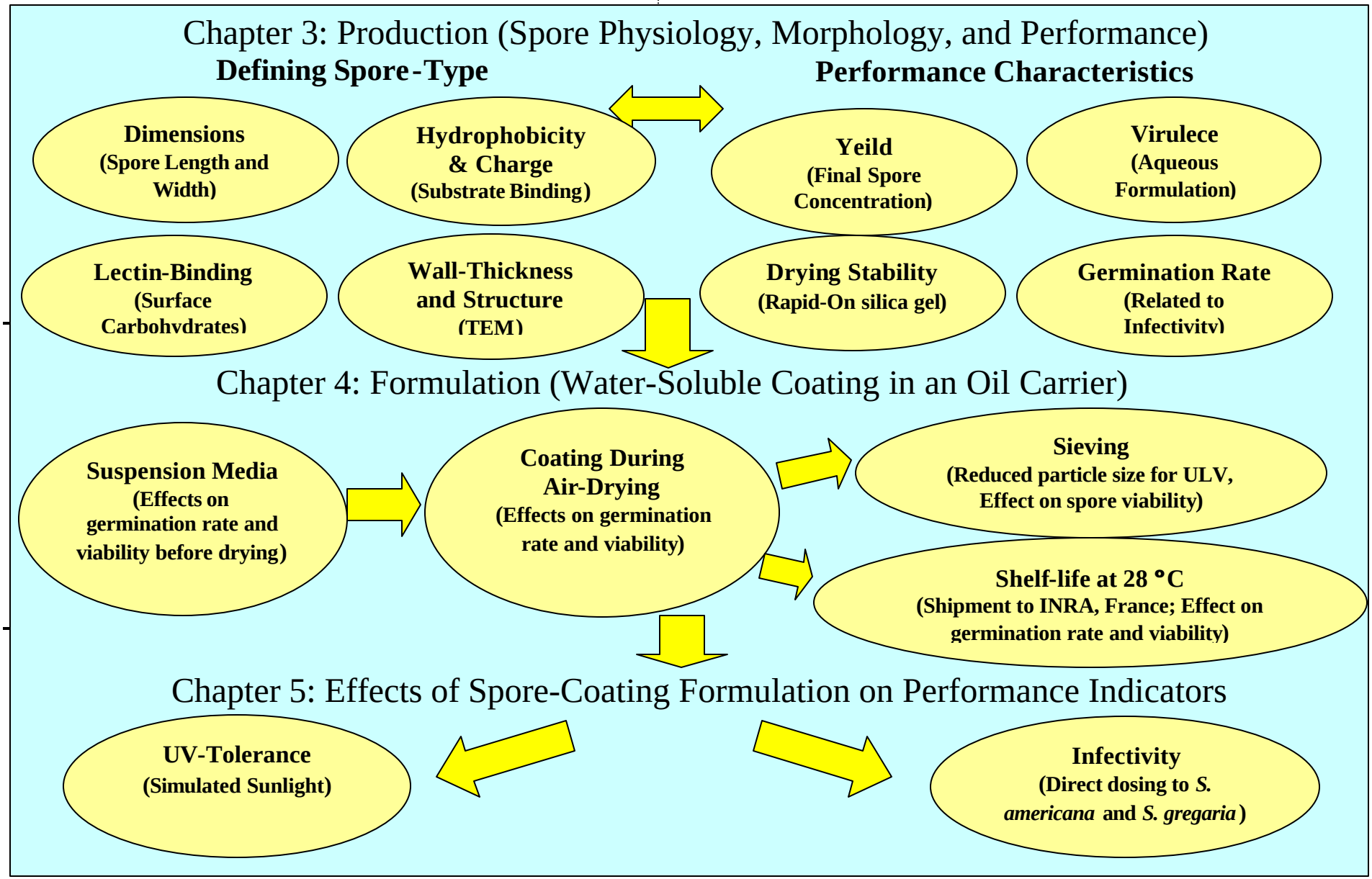

Figure 1-2. Schematic describing the strategy for developing and testing a dry, stabile formulation of submerged conidia that are coated with a water-soluble lignin for UV-protection and suspended in an oil carrier.

Chapter 2. General Materials and Methods

2.1. Collaboration of Laboratories and Institutions

Many of the procedures described in this dissertation required facilities, equipment, and expertise of collaborating laboratories and institutions. This study was conducted as part of a collaborative effort to develop and improve biopesticides for control of grasshopppers and locusts in Africa. The facilities of these various collaborating laboratories and institutions were used as a part of this collaborative effort. This required coordinating experiments conducted in this study with these project collaborators, which was particularly important for testing the effects of simulated sunlight and infectivity to *Schistocerca gregaria*.

Several laboratories were used on the Virginia Tech campus for this research. The majority of work was conducted in the laboratory of Dr. Herman Warren in the Department of Plant Pathology Physiology and Weed Science: including production of *M. anisopliae* var. *acridum* (IMI 330189) in Petri plate culture and shake flask culture and characterizing cell-wall surface characterisitics and drying stability. Some of the initial culturing work with various *Metarhizium* spp. isolates was conducted in the laboratory of Dr. Orson Miller in the Biology Department and representative samples of the original isolates were included in the Virginia Tech Type Culture Collection (*M. flavoviride* 200696 was classified ATCC 3590; *M. flavoviride* ATCC 200695 was classified VTCC 3591; *M. anisopliae* var. *acridum* IMI 330189 was classified ATCC 3592). Early production of *M. anisopliae* var *acridum* (IMI 330189) in a 1-L fermentation system was conducted in the laboratory of Dr. Joseph Falkinham in the Fralin Biotechnology Center, however all of the work described in this dissertation using a 1-L fermentation system was conducted in the laboratory of Dr. Foster Agblevor in Biological Systems Engineering. Transmission electron microscopy was conducted with the technical assistance of June Mullins using facilities in the College of Veterinary Medicine. Analysis of spore carbohydrates and freeze-drying was conducted with the technical assistance of Sandra Gabbert in the laboratory facilities (Pesticide Residue

Analysis Laboratory) of Roderick Young in the Department of Biochemistry. A colony of *Schistocerca americana* was initially maintained at the Quarantine Laboratory in the Department of Entomology and then was moved to the Insectary in the Department of Entomology. Bioassays on *S. americana* were conducted at the Urban Pest Control research Laboratory in the Department of Entomology.

In addition to laboratories on the Virginia Tech Campus, international collaborating institutions provided expertise and facilities for some of the work described in this dissertation. All of the simulated sunlight experiments and bioassays with *Schistocerca gregaria* were conducted at the Centre de Biologie et de Gestation des Populations (CBGP) of the Institut National de la Recherche Agronomique (INRA), Montferrier-sur-Lez, France under the direction of Dr. Jacques Fargues in collaboration with Dr. Nathalie Smits and with the assistance of Dr. Marc Rougier, Herve de Conchard, Jean-Marc Thuiller, and Bruno Serate. I traveled to INRA to conduct preliminary simulated sunlight experiments with freeze-dried spore-coating formulations and bioassays with *S. gregaria*. Subsequent simulated sunlight experiments were conducted under the direction of Dr. Nathalie Smits. Air-dried spore-coating formulations were prepared at Virginia Tech and shipped along with Petri plates for determining percent germination to INRA. After exposure to simulated sunlight, Petri plates were shipped back to Virginia Tech and percent germination counts were conducted at Virginia Tech. Colony forming unit counts (CFU) were conducted at INRA. Sieving of freeze-dried formulated spores in preliminary experiments and partial planning of simulated sunlight experiments and *S. gregaria* bioassays was conducted at the European Biological Control Laboratory (EBCL), United States Department of Agriculture (USDA), Agricultural Research Service (ARS); Montferrier-sur-Lez, France under the direction of Dr. Charles Quimby and the assistance of Guy Mercadier. Additional *S. gregaria* bioassays were conducted for submerged conidia and aerial conidia in air-dried coated-spore formulations at the International Center of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya by Nguya K. Maniania and Elizabeth Ouna. These bioassays have not been included in the dissertation because of high control mortality.

2.2. Fungal Culture

All experiments were conducted with the *M. anisopliae* var. *acridum* isolate IMI 330189, which was isolated from the grasshopper species *Ornithacris cavroisi* (Finot) *(Orthopera : Acrididae)* in Niger. This isolate was reclassified *M. anisoplaie* var *acridum* from its original classification *M. flavoviride* by Driver et al. (2000) on the basis of phylogenetic analysis of ribosomal DNA sequence data. The initial cultures were received from the LUBULOSA project. These cultures were transferred to Sabouraud dextrose agar (SDA), grown at 24 C and then conidia from these plates were harvested and stored with in 1 mL aliquots in 20% glycerol at -80 C as frozen stock culture. Aerial conidia that were either used directly in experiments or an inoculum for producing spores in liquid culture in all experiments described in this dissertation were produced on SDA at 24 C from this original frozen stock culture. The initial culture was transferred potato dextrose agar, grown in slant culture and stored with the Virginia Type Culture Collection (VTCC 3592).

2.3. *Schistocerca americana* Colony

Schistocerca americana eggs and nymphs were initially obtained from the Department of Entomology and Nematology at the University of Florida, Gainesville, and a laboratory colony of *S. americana* was established at Virginia Tech using similar rearing conditions as those used at the University of Florida. The colony was maintained at 32° C (14/12 h L:D photoperiod) at ambient relative humidity. Nymphs and adults were reared on a diet of Romaine lettuce and a dry diet consisting of wheat bran, whole wheat flour, soy flour and dry fish food (2:1:1:0.01). Depending upon the health of the colony an antibiotic regime consisting of 0.3% sulfamethazine : 0.6 % sulfathiozole, and 0.4% sulfapyridine was used. Three weeks prior to the bioassay, grasshoppers were

removed from this antibiotic regime. Ovipostition cages were made from 1-quart plastic containers filled with moist vermiculite.

2.4. General Methods

Spore concentrations were determined with a Neubauer hemacytometer using methods described by Tuite (1969). Concentration was based on a minimum of 200 spores resulting in estimates of concentrations within 10 to 15% of the actual value.

Percent germination was determined for aqueous spore suspensions (30 µL of approximately 1 x 10^6 spores/mL) spread on the surface 30 mm Petri plates containing 2% malt agar plus 0.001% benomyl and 0.02% chloramphenicol. Details on the development of germination methods using benomyl are described in Appendix II. Dry coated-spore formulations were placed in a 100% relative humidity (r.h.) environment for 1 hr to reduce the stress of rapid rehydration before dilution to approximately 1 x 10^6 spores/mL with sterile distilled water (Moore et al., 1997). Germination plates were incubated at 24 C and germination was stopped with 20% formalin at specified times. Percent germination was determined for at least 200 spores at 400 x magnification. All spores within a field of view were counted while running transects across the Petri plate. Spores were considered to be germinated if the germ tube was more than half the diameter of the spore.

All spore samples were stored at -80 C with 20% glycerol, unless the samples were to be used for determining carbohydrate concentrations, in which case they were stored at -80 C without glycerol.

Chapter 3. Effects of Liquid Culturing Conditions on the Morphology and Physiology of *Metarhizium anisopliae* var. *acridum* (IMI 330189) and Corresponding Effects on Drying Stability and Virulence to *Schistocerca americana*

3.1 Abstract

Metarhizium anisopliae var. *acridum* (IMI 330189) produces three distinct spore types; aerial conidia, submerged conidia and blastospores. The cell-wall characteristics of spores from various liquid media were examined with respect to dimensions, cell-wall thickness, surface lectins, charge, and hydrophobicity to further define each of these spore types. Submerged conidia had similar dimensions, cell-wall thickness, and hydrophobicity to aerial conidia but showed differences in their lectin-binding characteristics and charge. Submerged conidia could be differentiated from blastospores on the basis of their dimensions, cell-wall thickness, surface lectins, charge, and hydrophobicity. The germination rates and drying stability of spores produced in three liquid media and aerial culture were also determined. Submerged conidia were found to have faster germination rates than aerial conidia but similar drying stability. Blastospores had faster germination rates but lower drying stability rates than both submerged and aerial conidia.

The effect of increasing the osmolality of a submerged conidia-producing medium (VT-medium) with polyethylene glycol on spore characteristics was determined. At increased osmolality, the growth form shifted from submerged conidia to blastospore production. Blastospores produced at higher osmolality shared the characteristics of dimensions, thin cell walls, surface carbohydrates, charge, and germination rate with blastospores grown in Adamek's medium. However, blastospores from the high-osmolality medium had similar hydrophobic properties to both aerial conidia and submerged conidia. In addition, these blastospores were found to have similar drying stability to aerial conidia and greater drying stability than submerged conidia. Spores from high-osmolality medium were more virulent (LT_{50} 7.8 d) to *Schistocerca americana* adults than aerial and submerged conidia (14.6 d and 10.5 d, respectively).

3.2. Introduction

Culturing conditions can have a dramatic effect on the morphology and physiology of fungi. Manipulating spore physiology by altering culture conditions has been shown to be an important means for improving characteristics of fungal biopesticides. Some examples of changes in physiological or morphological characteristics and corresponding efficacy of fungal biopesticides include the following: 1) the water activity of solid media was correlated with polyol concentrations, germination rates for four entomopathogenic fungi (*Metarhizium anisopliae, Beauveria bassiana, Verticillium lecanii,* and *Paecilomyces farinosus*), and lag phase of germination of *M. anisopliae* and *V. lecanii* on melon cotton aphids, *Aphis gossypii* (Andersen et al., 1999); 2) osmolality of liquid media was correlated with spore trehalose concentrations, desiccation tolerance, and disease protection in the plant disease protectant, *Trichoderma harzianum* (Harman et al., 1991); 3) carbon to nitrogen (CN) ratios in liquid media were correlated with desiccation tolerance in the entomopathogenic fungus, *Paecilomyces fumosoroseus* (Jackson et al., 1997); and with gross morphology, germination rates, and infectivity of the bioherbicide *Colletotrichum truncatum*; (Jackson, 1997; Schisler et al., 1990); and 4) culture temperature has been correlated with cell wall thickness, germ tube formation, and infectivity of the mycoherbicide *Alternaria helianthi* (Abbas et al., 1995).

Distinct spore types may be produced in submerged culture that can be differentiated by their gross morphology, morphology of the sporogenous cell, cell-wall-surface characteristics, virulence, and stability. For example, depending on growth conditions, the entomopathogenic fungus, *Beauveria bassiana*, may produce three distinct spore types: 1) blastospores, which are characterized as being thin-walled, single-cell hyphal bodies, produced in nutrient rich media (Bidochka et al., 1987, Thomas et al., 1987); 2) aerial conidia produced in solid culture; or 3) submerged conidia produced in liquid culture, which are characterized as having a similar size and shape to aerial conidia, and may arise on mycelia or directly from blastospores in a process known as

microcycle conidiation (Thomas et al., 1987). Scanning electron microscopy of the spore surface (Thomas et al., 1987) and polyacrylamide gel electrophoresis of proteins from the spore surface have shown differences among these three spore types (Boucias et al., 1988). The virulence, stability and cell-wall-surface characteristics among these spore types was compared by Hegedus et al., (1992). The three spore types could be distinguished on the basis of hydrophobicity, lectin-binding characteristics, stability and virulence. Blastospores were the least hydrophobic, possessed relatively few surface carbohydrates and did not survive storage well, but were the most virulent spore form. Submerged conidia showed similarities to aerial conidia with respect to each of these properties. More recently, liquid media for *M. anisopliae* that supplies limited nitrogen and excess carbon has been reported to produce sporogenous cells and spores that are morphologically indistinguishable from aerial phialides (specialized sporogenous cells) and conidia, respectively (Jenkins and Prior, 1993). The practical advantages of producing spores in liquid culture that have similar characteristics to aerial conidia are the ease of production in liquid culture combined with the added environmental stability that "true conidia" are thought to possess over blastospores (Jenkins and Goettel, 1997). Cultural conditions in liquid fermentation can be closely monitored and controlled and the technology is well established in industry. Virulence, cell-wall surface characteristics, and drying stability of the three spore types of *M. anisopliae* have not been compared. Spore-surface characteristics would be useful in differentiating these spore types and would have practical application towards the interaction of the various spore types with insect cuticle (Boucias et al., 1988). The relative virulence and drying stability of these three spore types is yet to be determined, both of which would have practical applications when producing a dry formulation of this organism for field application.

There has been a considerable amount of work on the influence of cultural conditions on the concentrations of carbohydrates in *M. anisopliae* aerial conidia. The cultural conditions that have been investigated include; carbohydrate type and concentration (Hallsworth and Magan, 1994a), water activity (Halsworth and Magan, 1994b); culture age, temperature, and pH (Hallsworth and Magan, 1996). However, the

effect of cultural conditions on carbohydrate concentrations in *M. anisopliae* spores from liquid culture has not been reported.

Various carbohydrates may function to improve the efficacy of *Metarhizium* as a biopesticide. Specific carbohydrates may influence germination rates, water balance, potential for biomolecules to denature, desiccation tolerance, and scavenging of reactive oxygen molecules. Polyols are described as "compatible solutes", which means they may accumulate in high concentration without inhibiting or destabilizing enzymes (Brown, 1976). Polyols are used by spores in osmotic regulation and polyol concentrations in aerial conidia have been shown to influence germination at reduced water availability (Hallsworth and Magan, 1995) and lag phase of germination of *M. anisopliae* and *V. lecanii* on melon cotton aphids (Andersen et al., 1999). Polyols may prevent denaturing of biomolecules by functioning as compatible solutes. Compatible solutes are preferentially excluded from interacting with the surface of biomolecules (e.g, proteins) resulting in a thermodynamically disfavorable situation (Crowe et al., 1990). Because denaturing of the biomolecule would result in increased surface area and a decrease in entropy, the presence of compatible solutes appear to reduce biomolecule denaturation. In addition, the largest molecular weight polyol shown to accumulate in *Metarhizium*, mannitol, is also known to scavenge hydroxyl radical ($OH^{\cdot}$) and has been shown to protect fungi from oxidative damage (Bucker and Martin, 1992; Chaturvedi et al., 1996). Nonreducing disaccharides (particularly trehalose) have the ability to stabilize membranes (Crowe et al., 1984; 1988; 1990) and enzymes (Carpenter and Crowe, 1988 a, b; Colaco et al., 1992) by preserving them in a "glass state" in conditions of extreme desiccation when molecular water is removed.

The objectives of the research reported in this chapter are: 1) to determine if a spore can be produced in submerged culture that is morphologically indistinguishable from aerial conidia and compare this spore type with blastospores and aerial conidia with respect to cell-wall characteristics, germination rate and drying stability; and 2) to investigate the influence of media osmolality in submerged culture on cell-wall characteristics, drying stability, virulence and spore-carbohydrate concentrations.

3.3. Materials and Methods

3.3.1. Culturing Conditions for Liquid Culture

All experiments were conducted with the *M. anisopliae* var. *acridum* isolate IMI 330189, which was isolated from the grasshopper species *Ornithacris cavroisi* (Finot) *(Orthoptera : Acrididae)* in Niger. The initial cultures were received with permission from the LUBULOSA project. These cultures were transferred to Sabouraud dextrose agar (SDA), grown at 24 C and then conidia from these plates were harvested and stored in 1 mL aliquots in 20% glycerol at -80 C to be used as a starting culture for all experiments. All spore samples were stored at -80 C with 20% glycerol, unless the samples were to be used for determining carbohydrate concentrations, in which case they were stored at -80 C without glycerol.

Spores were produced in shake flask culture using 50 mL of the various media described below in 250 mL Erlenmyer baffled flasks. Air in the headspace over the media was continuously replenished using aquarium pumps, which produced air flow rates of approximately 300 to 600 mL/min. The initial pH of all media was adjusted to between 6.5 and 7.5 with sterile 1 N, NaOH before autoclaving. Each flask was inoculated with 120,000 conidia/mL of medium using two- to three-week old spores from IMI 330189 grown on SDA agar at 24 °C. Flasks were agitated continuously at 150 RPM and maintained at 24 °C. Growth conditions were based on protocols described by Jenkins and Prior (1993) and Adamek (1963).

The production of spores over time was determined using the following media:

1) Blastospores were produced in Adamek's medium containing 3% corn steep liquor, 4% glucose, and 4% yeast extract (Adamek, 1963).

2) Submerged conidia were produced in 3% sucrose (Sigma Chemical Co.): 3% waste brewers' yeast (Stroh's Batch, NPC-Inc, Eden N.C.) (Jenkins and Prior, 1993) (Jenkins' medium)

3) Submerged conidia were also produced in 4% waste brewer's yeast; 4% fructose; 5% lecithin (L-α phosphatidylcholine, Type II-S from soybean, Sigma Chemical Co.) (VT medium).

Spores produced in Adamek's medium have been described as blastospores, which are characterized as thin-walled, single-celled, hyphal bodies (Adamek, 1963). Spores produced in Jenkins' medium have been described as submerged conidia based on green pigmentation of the spores, spore dimensions, characteristic "pip" shape, and production on phialides (Jenkins and Prior, 1993). I examined spores produced in each of the three liquid media and aerial culture in greater detail to more fully describe each of the spore types.

The osmolality of VT medium was increased by adding polyethylene glycol 200 at concentrations of 5, 10, 15, 20, 25 and 30%. The osmolality (milliOsmoles) of each medium type was measured using an Advanced Instruments Model 3 MO Plus Micro Osmometer.

3.3.2. Effects of Liquid Culture Conditions on Cell-Wall-Surface Characteristics

All glassware was soaked in Contrad 70 detergent (Polysciences, Inc.) for 24 hr, 1% HCl for 24 hr; then rinsed seven times with distilled water at 4^{o} C, and heated at 170^{o}C for 4 hr.

Spores from various liquid media (Adameks, VT medium, and VT medium + 10% PEG) and solid medium (SDA) were washed five times in distilled water with centrifugation at 2800 x G (Fisher Scientific Marathon 6K centrifuge). The spore concentrations were determined using a hemacytometer and adjusted to 1×10^{7} spores/mL for lectin binding experiments and $2.5\text{-}8.9 \times 10^{6}$ cells/mL for hydrophobicity and surface charge experiments.

3.3.2.1 Lectin-Binding Characteristics

The lectin-binding characteristics of spores were determined using methods described by Hegedus et al. (1992). Three lectins were selected based on their ability to bind *M. anisopliae* hyphae (Pendland and Boucias, 1986) or their utility in distinguishing spore types of *B. bassiana* (Hegedus et al., 1992). Concanavalin A (Con-A) from *Conavalia ensiformis* binds α-D-mannosyl and/or α-D-glucosyl residues. Wheat germ agglutinin (WGA) binds n-acetyl-*B*-D-glucosaminyl and n-acetyl-*B*-D-glucosaminyl oligimers. Phytohaemagglutinin (PHA-P) from *Phaseolus volgaris* binds oligossaccharides. All lectins were conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Co.).

A 20-µL sample of each spore suspension was placed on a glass microscope slide and left undisturbed for 5 min to allow spore adhesion to the glass surface. The spores were then fixed with two drops of ice cold (-20 C) acetone. Non-adhering spores were rinsed with 1 mL of distilled water followed by flushing for 30 seconds with distilled water. The adhering spores were then treated with 40 µL of lectin solution (200 µg protein/mL saline) in low-light conditions. The treated slides were then rinsed with distilled water, wet mounted with cover slips and observed immediately in low-light conditions using an epifluorescence microscope (450-490 nm). FITC lectins fluoresce at 90% of their maximum excitation at 490 nm (Sigma Chemical Co.) and, therefore, estimation of lectin binding may have been slightly underestimated.

3.3.2.2 Charge Determination

Two approaches related to selection of substrates were used to evaluate the charge of spores. The first approach employed the use of chitin (Poly-[1-4]-β-N—acetyl-D-glucosamine, Sigma Chemical Co.) as an anionic substrate and chitosan (deacetylated chitin; Poly-[1-4]-β-D-glucosamine, Sigma Chemical Co.) as a cationic substrate. Chitin and chitosan were prepared by coarse grinding with an electric coffee grinder and

micropulverizing with a Wig-L-Bug Amalgamator (Cresent Dental Mfg. Co. Model 3110-3A). The chitin and chitosan substrates were then separately combined with suspensions of spores produced in each of the experimental media (3 mL) at 1 mg of substrate/mL of spore suspension in separate 250 mL Erlenmeyer flasks. The flasks were shaken (200 RPM) in a shake bath for 1 hr at 26^{o} C. Samples from each of the flasks were observed microscopically at 400 x magnification to determine the number of spores adhered to individual chitin or chitosan particles. Randomly selected fields of view were selected by running transects across the slide and the number of spores adhering to each particle was determined for all of the particles in each field of view.

The second approach used for evaluating spore-surface charge employed the use of more uniformly sized and charged substrates, Q-sepharose (cationic) and CM-sepharose (anionic), for charge binding tests (Sigma Chemical Co.). Since these substrates were originally suspended in ethanol, they were washed three times by centrifugation (2150 x g) with HPLC-grade water to remove the ethanol before using in this assay. The concentration of each substrate was diluted to 1/3 of the original stock concentration. Each of the substrates (200 µL) were combined with 100 µL of each spore suspension and an additional 200 µL of sterile HPLC-grade water. This suspension was vortexed for 30 seconds and then shaken (200 RPM) for 24 hours. Samples from each of the flasks were observed microscopically at 400 x magnification to determine the number of spores adhered to individual beads. Randomly selected fields of view were selected by running transects across the slide and the number of spores adhering to each bead was determined for all of the beads in each field of view.

3.3.2.3 Hydrophobicity

The hydrophobic microsphere assay (HMA) (Hazen and Hazen; 1988), was used to determine the hydrophobicity of spores. A suspension of hydrophobic microspheres was made by combining 24 µL of blue-dyed low-sulfate latex microspheres (1 µm diameter, Polysciences, Inc.), with 2 mL of 0.05 M PUM buffer (26.8g K_2HPO_4* nH_2O,

7.3g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4$ * $7H_2O$ adjusted to a pH of 7.2 with HCl). The latex microspheres and the PUM buffer were initially at 4 °C and maintained in an ice bath while they were being combined. Each spore suspension (100 µL) was combined with the microsphere suspension (100 µL) in a 2-mL glass vial. The suspensions were equilibrated for 1-2 minutes at room temperature, vortexed for 30 seconds, and then immediately observed by bright field microscopy at 1000 x magnification. Photographs were taken of randomly selected spores of each type by counting beads on all spores in one field of view and then running transects across the slide. The number of latex beads adhering to each spore was determined for approximately 40 spores of each type from photographs taken at 1000 x magnification.

3.3.3 Effects of Liquid Culture Conditions on Spore Morphology

The morphology of spores from four liquid media and one aerial culture was examined using light microscopy to determine spore dimension (length and width) and transmission electron microscopy (TEM) to determine cell-wall thickness. Length and width were measured for 30 to 45 spores from each medium type at 1000 x magnification using an optical micrometer.

The cell-wall thickness of spores was measured from printed micrographs obtained from transmission electron microscopy (TEM). Undiluted spore samples of the final (6 d) product from the four liquid media and aerial conidia suspended in 0.05% Tween 80 were prepared for TEM by centrifuging (2200 x g) in a microcentrifuge (Fisher Scientific, Model 59A). The supernatant was removed and the spore pellets were re-suspended in modified Karnovsky's fixative (0.5% paraformaldehyde (Fisher Scientific) and 2.3% gluteraldehyde (Polysciences Inc.) in 0.1M Na cacodylate (Polysciences Inc.) at pH 7). Spores were allowed to fix overnight at 4 C. After fixation, samples were centrifuged (2200 x g) and the pellet was resuspended in 2% agar at 55 C, then centrifuged again (2200 x g). Spores, which were entrapped in the cooled, solidified agar, were removed from the tip of the microcentrifuge tube and diced into approximately

1-mm square pieces. These pieces were then rinsed with three changes (10 min each) of 0.1M Na cacodylate buffer. The entrapped spores were then post-fixed with 1% osmium tetroxide in 0.1M Na cacodylate for 1 hour, followed by rinsing twice in 0.1M Na cacodylate for 10 min each. Dehydration was accomplished with ethanol by incubating for 10 min each in the following concentrations; 50%, 70%, 95% (twice), 100% (twice); followed by propylene oxide (three times). The samples were then perfused with Epon 812 mixed 50:50 with propylene oxide for 12 hr, followed by a 6 hr incubation in 100% Epon 812 with continual rocking motion (rocking plate). Samples were then embedded in fresh Epon 812 and cured for 48 hr at 60 C. The embedded spores were sectioned using a microtome (Porter Blum MT2) and the sections were examined using a JOEL 100 CX-II transmission electron microscope.

Photographs of 11 to 22 spores from each spore suspension were taken at 19,440 x magnification. The cell wall thickness was measured from photographs using a fine-scale metric ruler for both walls that intersected the longest and shortest axes dividing the spore, for a total of 4 measurements per spore. Measurements of aerial conidia cell walls did not include the rodlet layer.

3.3.4. Effects of Liquid Culture Conditions on Drying Stability

Methods for evaluating the drying stability of spores produced in liquid media and aerial culture were derived from Cliquet and Jackson (1997). The initial germination rate and percent germination after drying was compared for three replicates of spores produced in Ademek's medium, VT medium, VT medium + 10%PEG, and aerial conidia. Spore samples ranging from 3.4×10^8 to 6.4×10^8 spores/mL were washed three times by centrifuging at 2000 x g in a microcentrifuge (Fisher Scientific 59A) and resuspended in either distilled water, or 5% polyethylene glycol (PEG) 600. Spores grown in VT medium + 10% PEG 200 were washed in either distilled water or 20% PEG 600.

Spores that were either not washed, washed in distilled water, or washed in an isoosmotic solution of PEG 600 were diluted to approximately 1×10^6 spores/mL with

sterile distilled water and spread on 2% malt agar + 0.001% benomyl to test for germination. These plates were incubated at 24 C and germination was stopped with 20% formalin for the first plate after 6 hr and for the second plate after 42 hr of incubation. Percent germination was determined for at least 200 spores at 400 X magnification. A spore was considered to be germinated if the germ tube was more than half the diameter of the spore.

For the drying test, non-indicating silica gel (Type II, Sigma Chemical Co.) was mixed with deionized water for 5 min and then placed in a drying oven at 100 C for 24 hr. This process was repeated until the silica gel had broken into particles of approximately 2 mm in diameter. The silica gel particles (1 g) were placed into 15 mL glass screw cap vials. Aliquots (100 µL) of each spore suspension that had been either not washed, washed with distilled water, or washed with PEG solution were added to each vial. Vials were immediately vortexed for 10 seconds and then immediately rehydrated with 10 mL of distilled water. These spore suspensions (30 µL) were plated onto a 30 mm Petri plate containing 2% malt agar + 0.001% benomyl. Petri plates were incubated at 24 C and germination was stopped with 20% formalin for the first plate after 6 hr and for the second plate after 42 hours of incubation. Percent germination was determined for at least 200 spores at 400 X magnification.

3.3.5. Effects of Osmolality during Liquid Culture on Virulence to *Schistocerca americana*

Methods for evaluating the virulence of *M. anisopliae* var. *acridum* to *S. americana* were adapted from Sieglaff et al., (1997; 1998). *Schistocerca americana* eggs and nymphs were initially obtained from the Department of Entomology and Nematology at the University of Florida, Gainesville, and a laboratory colony of *S. americana* was established at Virginia Tech using similar rearing conditions as those used at the University of Florida. The colony was maintained at 32° C (14/12 h L:D photoperiod). Nymphs and adults were reared on a diet of Romaine lettuce and a dry diet consisting of wheat bran, whole wheat flour, soy flour and dry fish food (2:1:1:0.01). Depending upon

the health of the colony an antibiotic regime consisting of 0.3% sulfamethazine : 0.6 % sulfathiozole, and 0.4% sulfapyridine was used. Three weeks prior to the bioassay, grasshoppers were removed from this antibiotic regime. Adult *S. americana* at 2- to 3-wk after their final molt were placed into individual 500 mL plastic screened cages 24 hr prior to inoculation.

Submerged spores *of M. anisopliae* var. *acridum* isolate IMI 330189 were produced in VT medium and VT medium +10% PEG 200 and were stored at -80 C in 20% glycerol as described previously (Section 3.3.1. Culturing Conditions for Liquid Culture). Aerial conidia were harvested from two- to three-week old plate cultures on SDA grown at 24 C. Four replicates of spores from each of the various media were combined. All spores were formulated in 20% molasses in sterile distilled water (Stephan et al., 1997). Controls were exposed to 20% molasses in sterile distilled water. The percent germination of each spore suspension was checked for 200 spores at a concentration of approximately 1 x 10^6 spores/mL of 20% molasses in sterile distilled water on 2% malt agar after 24 hr of incubation at 24 °C. Four concentrations were made for each spore-type: 5 x 10^8, 5 x 10^7, 5 x 10^6, and 5 x 10^5 spores/mL. Adults were removed from the cages and treated by topical application of 2 µL of formulated inocula directly under the pronotal shield with a Hamilton microsyringe (Sieglaff et al., 1997, 1998). Therefore, the four doses for each formulation were 1 x 10^6, 1 x 10^5, 1 x 10^4, and 1 x 10^3 spores/insect. Controls were treated with 2 µL of 20% molasses in sterile distilled water. This entire bioassay was repeated three times, with 6, 9, and 10 insects per dose to provide a total of 25 insects per dose. The experimental design was a randomized complete block design using the individual times that the entire bioassay was conducted as a blocking factor.

For the bioassay, individually-caged *S. americana* were maintained at 32 °C, and a photoperiod of 14:10 (L:D) in a controlled environment. The humidity in the chamber was maintained at 50 ± 5%, with a humidity regulating system based on saturated K_2CO_3 solutions. The system allowed forced air exchange over a cheesecloth wicking system saturated with K_2CO_3. Relative humidity was monitored with a Fisher Scientific traceable printing hygrometer/thermometer (Model # 11-661-17A). Mortality was checked and a fresh 5-cm diameter Romaine lettuce disk was provided daily. Mortality was compared

among spores from the various media by determining the 95% confidence intervals for LD_{50}'s and LT_{50}'s at the highest dose for individual replicates of the bioassay over time and for the combined three experiments using probit analysis. Dead grasshoppers were removed and surface sterilized using a method described by Lacey (1997). Dead grasshoppers were soaked in 70% ethanol for a few seconds and then rinsed in sterile distilled water. The cadavers were then placed into 0.5% sodium hypochlorite for two minutes, followed by two rinses with sterile distilled water. They were then incubated in a Petri dish with moist filter paper under sterile conditions until sporulation occurred.

3.3.6. Statistical Analysis

Comparisons among cell-wall surface characteristics were made using by analysis of variance. Normality was tested using Wilk-Shapiro test (Shapiro and Wilk, 1965). Comparisons that were made for small sample sizes were made using a nonparametric test (Wilcoxon two sample test).

3.4. Results and Discussion

3.4.1. Culturing Conditions for Liquid Culture

3.4.1.1. Production of *M. anisopliae* var. *acridum* in Submerged Culture

The concentration of spores produced in three liquid media after 6 d of incubation are presented in Table 3-1. In each medium type spore production reached its maximum concentration within 6 d of incubation and samples of spores at this incubation time were used for characterizing surface characteristics, morphology, drying stability and virulence in the remainder of this chapter.

Hyphae formed mycellial pellets in Jenkins' medium. Lecithin has been shown to reduce mycellial pellet formation in Adamek's medium (Kleespies and Zimmermann, 1998) and was added to medium adapted from Jenkins and Prior (1993), (VT medium), to attempt to reduce mycellial pellet formation. In addition, the carbon source (sucrose) from Jenkins' medium was replaced with fructose with the idea that high fructose corn syrup would be a less expensive medium component. Although VT medium did not increase spore production over the Jenkins' medium, it decreased the appearance of mycellial pellets. The spores produced in both Jenkins' medium and VT medium had gross morphology similar to aerial conidia (size and shape); whereas those produced in Ademek's medium had gross morphologies similar to blastospores. This will be discussed further in Section 3.4.3. (Effects of Liquid Culture Conditions on Spore Morphology).

Table 3-1. Final Concentration of Spores in Three Liquid Media after 6 d of Incubation

Media	Spores Concentration (spores x 10^8 / mL)[1]
Adameks medium	2.9 ± 1.2 a[2]
Jenkins' medium	5.6 ± 1.7 b
VT medium	5.6 ± 0.2 b

[1] Values presented as means ± standard deviations

[2] Values followed by different letters are significantly different at the $\alpha = 0.05$ level n= 4 replicates per medium type (Wilcoxon two-sample test for non-paired ranked observations).

3.4.1.2. Spore Production in Media of Varying Osmolality

Spores were produced in VT medium and VT medium supplemented with six levels of PEG 200. The osmolality of these media and spore production over time are presented in Table 3-2. Spore production was increased by the addition of up to 15% PEG 200 (0.75M), which had an osmolality approximately 3.5 times greater than the osmolality of the VT medium ($\alpha = 0.05$). Spore production rapidly declined at PEG 200 concentrations of 20% or higher, which represented osmolalites greater than 5 times the osmolality of VT medium ($\alpha = 0.05$). At increased osmolality there appeared to be a

reduction in the formation of mycelial aggregates. This effect was also observed by Humphreys et al. (1989) and Kleespies and Zimmerman (1992); both working with *M. anisopliae* in liquid culture; and by Inch and Trinci (1987) when working with *Paecilomyces farinosus* in liquid culture. Humphreys et al. (1989) found that blastospore production in medium containing 10 g/L glucose and 20 g/L yeast extract was increased by the addition of PEG 200 from 0.3 M to0.7M. Kleespies and Zimmerman (1992) found that supplementing Adamek's medium with 5% PEG 200 resulted in an increase in blastospore production in three strains of *M. anisopliae*, but higher levels of PEG 200 (10 and 15%) resulted in a decrease in blastospore production and an increase in vacuolization.

Table 3-2. Osmolality and Spore Production Over Time for VT medium Supplemented with Increasing Concentration of Polyethylene Glycol 200 (PEG)

Media	Osmolality (mOsm)	Spore Concentration[1] (spores x 10^8 / mL)[2]		
		2 Days	4 Days	6 Days
VT medium	432	0.3 ± 0.06 abc[3]	6.4 ± 0.8 a	7.6 ± 0.8 a
VT medium + 5% PEG	804	0.5 ± 0.2 a	8.4 ± 2.1 a	10.2 ± 1.7 b
VT medium + 10% PEG	1222	0.3 ± 0.01 b	5.4 ± 2.4 a	9.5 ± 1.3 b
VT medium + 15% PEG	1454	0.2 ± 0.1 c	6.9 ± 2.2 a	12.3 ± 2.8 b
VT medium + 20% PEG	2154	0.04 ± 0.02 d	0.2 ± 0.2 b	1.6 ± 0.3 c
VT medium + 25% PEG	not measured	0.04 ± 0.001 d	0.1 ± 0.05 b	0.2 ± 0.04 d
VT medium + 30% PEG	off scale	0.2 ± 0.1 c	0.5 ± 0.6 b	0.6 ± 0.6 cd

[1] Initial spore concentration was 1.5 x 10^5 spores/mL
[2] Values presented as means ± standard deviations
[3] Values followed by different are significantly different than values within the same column at the $\alpha = 0.05$ level n = 3 replicates per media type (Wilcoxon two-sample test for non-paired ranked observations).

3.4.2. Effects of Liquid Culture Conditions on Spore Morphology

3.4.2.1. Dimensions of Spores

Gross morphology, such as spore dimensions, are often used to characterize spore types (Kleespies and Zimmerman, 1992; Bidochka et al., 1987; Thomas et al., 1987; Jackson and Schisler, 1992) and may correlate with other cell-wall properties and spore-performance indicators such as stability and virulence. The dimensions of spores produced in aerial culture and various liquid media are presented in Table 3.3. Spores produced in VT medium or Jenkins' medium could not be distinguished on the basis of length from aerial conidia, but were slightly narrower than aerial conidia ($\alpha = 0.05$). Spores produced in Adamek's medium were significantly longer than both aerial conidia and spores produced in VT medium ($\alpha = 0.05$), and appeared to be more variable in length. Spores produced in VT medium + 10% PEG had similar length to spores from Adamek's medium, but could not be statistically separated from spores from VT medium ($\alpha = 0.05$).

Table 3-3. Spore Dimensions and Cell Wall Thickness of Spores Produced in Four Types of Liquid Media and Aerial Culture

	Spore Dimensions[1]		Cell Wall Thickness
Media	Length (µm)	Width (µm)	(µm)
SDA (Aerial)	5.7 ± 0.9 a[2]	4.2 ± 0.5 a	0.17 ± 0.05 a
Adamek's medium	6.5 ± 1.7 b	4.2 ± 0.6 a	0.10 ± 0.05 b
VT medium	5.8 ± 0.7 ac	3.2 ± 0.3 b	0.15 ± 0.03 a
VT medium +10% PEG	6.4 ± 0.9 bc	3.4 ± 0.4 b	0.07 ± 0.03 c
Jenkins' medium	5.7 ± 0.7 a	3.5 ± 0.6 b	0.17 ± 0.02 a

[1] Values presented as means ± standard deviations

[2]Numbers followed by different letters within the same column are significantly different at the $\alpha = 0.05$ level (Tukeys HSD). General linear model was used for spore dimensions (n = between 30 and 45 spores) and for cell wall thickness (n = between 11 and 22 spores).

3.4.2.2. Cell-Wall Thickness

In liquid media *Metarhizium anisopliae* typically produces hyphal bodies, known as blastospores, which have cell walls that are indistinguishable from hyphae and are formed by hyphal constriction, separation at the septa, or yeast-like budding (Jenkins and Goettel, 1997). However, in specific cultural conditions certain isolates of *M. anisopliae* can also form conidia in liquid culture, which were defined based on their gross morphology and morphology of the sporogenous cell (Jenkins and Prior, 1993). By examining thin sections of spores with transmission electron microscopy, we were able to determine that there was stratification within the cell wall and cell wall thickness. Aerial conidia had cells walls composed of two less-electron dense layers separated by an electron dense layer (Figure 3-1a). The electron-dense layer appeared to surround the entire cell. Spores produced in either Jenkins' medium or VT medium generally had cells walls similar to aerial conidia, which were composed of two less-electron dense layers separated by an electron dense layer (Figures 3-1b and 3-1c, respectively). The electron dense layer, however, generally did not surround the entire cell. The electron dense layer was more typically observed at the tip of the spore along it's longest axis giving the spore its characteristic 'pip' shape. Spores produced in Adamek's medium or VT medium + 10% PEG had cell walls that were composed of a single less-electron dense layer (Figures 3-1d and 3-1e, respectively).

The cell-wall thickness of spores from various media are presented in Table 3-3 along with spore dimensions. The mean cell wall thickness and variance may be slightly higher when measuring from thin sections of TEM micrographs than the actual thickness and variance of the population. This is because the cross section may not represent the precise middle cross section of the spore. However, because the variance would be higher from TEM micrographs than the variance of the actual population, statistical differences between cell-wall thickness measurements among spores from various media still should reflect differences in the actual population. Based on this method of determining cell-wall thickness, aerial conidia, spores from Jenkins' medium, and spores from VT medium all had similar cell wall thickness, which were thicker than cell walls of

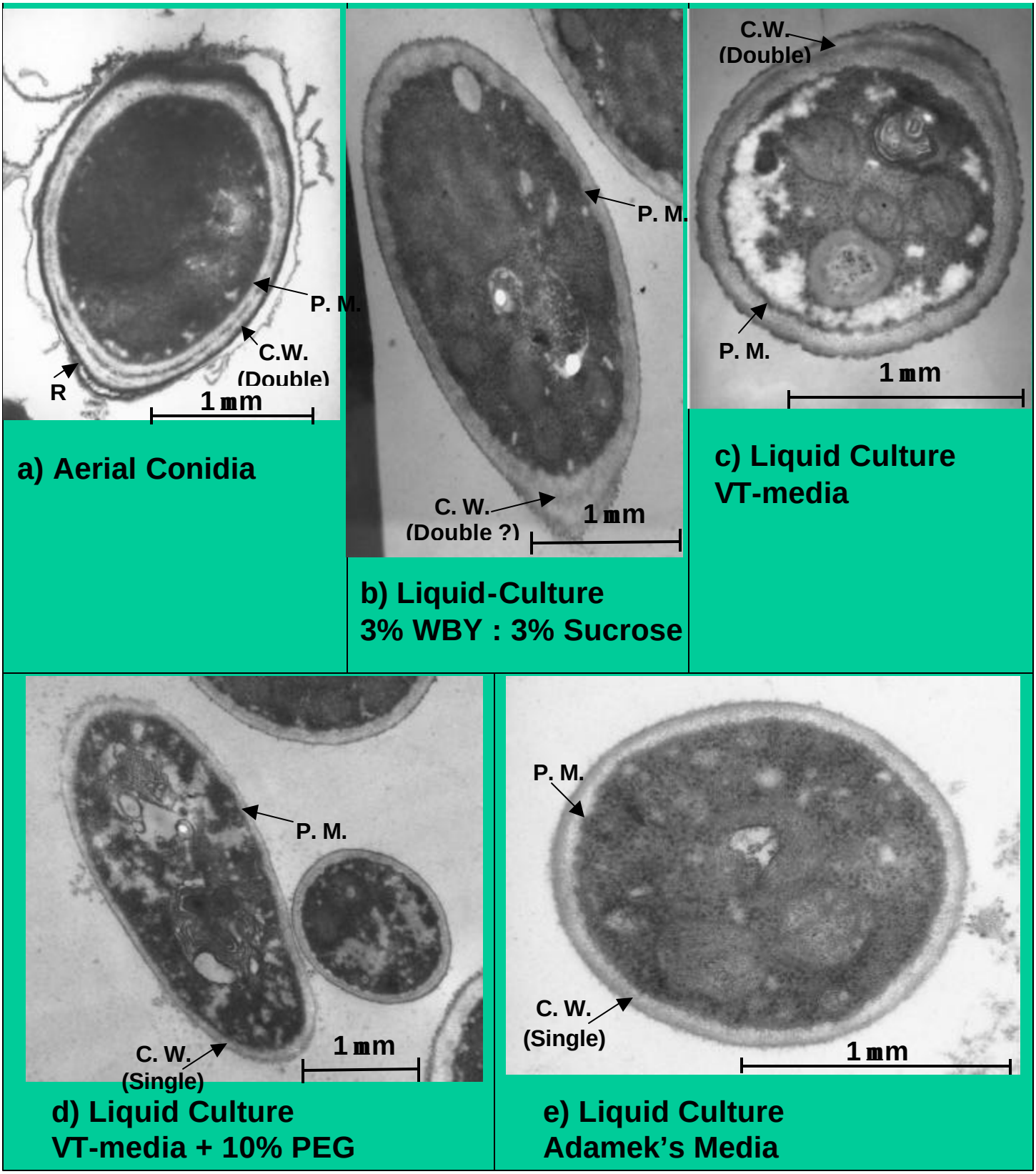

Figure 3-1. Transmission electron micrographs of spores grown under the following conditions: a) aerial conidia from Sabouraud dextrose agar (SDA); b) liquid-culture spores in 3 g/L waste brewers' yeast (WBY) : 3 g/L sucrose (Jenkins and Prior, 1993); c) liquid-culture spores in VT-media (4 g/L WBY : 4 g/L fructose : 5 g/L lecithin); d) liquid-culture spores in VT-media + 10% PEG 200; and e) liquid-culture spores in Adamek's media. CW=cell wall; PM=peritrophic membrane; R=rodlet layer

spores produced in either Adamek's medium or VT medium + 10% PEG ($\alpha = 0.05$). Spores produced in VT medium + 10% PEG had the thinner cell walls than spores from any other medium ($\alpha = 0.05$).

3.4.3. Effects of Liquid Culture Conditions on Cell-Wall-Surface Characteristics

3.4.3.1. Lectin-Binding Characteristics

Lectins play important roles in strengthening binding of spores to host cuticle (Latge et al., 1988), host specificity (Rath et al., 1995), recognition by the host immune system (Pendland et al., 1993), and differentiating spore types (Hegedus et al., 1992, Jeffs et al., 1999). Therefore, the lectin-binding characteristics of *M. anisopliae* spores produced in different liquid media may have practical application related to the ability of spores to bind insect cuticle and also have utility in differentiating spore-types produced in the different liquid media. The results of binding for three FITC-labeled lectins to various spores types of *M. anisopliae* var. *acridum* are summarized in Table 3-4. The α-D-mannose/α-D- glucose specific lectin, concanavalin A (Con-A) from *Conavalia ensiformis* demonstrated greater binding to spores from each of the three liquid media than aerial conidia. There was no difference in the binding of Con-A among the spores from each of the three liquid media. The oligosaccharide specific lectin, *Phaseolus vulgaris* phytohaemagglutinin P (PHA-P) did not bind to any of the spores. The n-acetyl-*B*-D-glucosaminyl and n-acetyl-*B*-D-glucosaminyl oligimer specific oligosaccharide, wheat germ agglutinin from *Triticum vulgaris* bound most strongly to spores produced in either Adamek's media or VT media + 10% PEG. Binding was less pronounced to spores produced in VT media and was least pronounced to aerial conidia.

Table 3-4. Lectin-Binding Characteristics of *M. anisopliae* var. *acridum* Spores Produced in Three Liquid Media and Aerial Culture

Media	Con-A[1]	PHA-P	WGA
SDA (Aerial)	1.5 ± 0.9 a[2, 3]	0.0 ± 0.0 a	0.6 ± 0.7 a
Adamek's media	4.0 ± 0.0 b	0.0 ± 0.0 a	2.9 ± 1.1 b
VT media	4.0 ± 0.2 b	0.0 ± 0.0 a	1.4 ± 1.0 c
VT media + 10% PEG	3.9 ± 0.3 b	0.0 ± 0.0 a	2.6 ± 0.7 b

[1] Con-A = Concanavalin A from *Conavalia ensiformis* , which binds α-D-mannosyl and/or α-D-glucosyl residues; PHA-P = Phytohaemagglutinin from *Phaseolus volgaris* binds oligossaccharides; WGA = Wheat germ agglutinin from *Triticum vulgaris* binds n-acetyl-*B*-D-glucosaminyl and n-acetyl-*B*-D-glucosaminyl oligimers.
[2] Numbers followed by different letters within the same column are significantly different at the alpha=0.05 level (Tukeys HSD) (n = 50 spores). Means +/- standard deviations presented.
[3] Values for fluorescence intensity are based the following ranking (0) = no flourescence; (1) = very weak; (2) = weak; (3) = moderate; (4) = very strong fluorescence over the entire spore surface

Similar lectin binding characteristics have been reported for *M. anisopliae* var *major* hyphae as we found for blastospores from Adamek's media and VT media + 10% PEG (Pendland and Boucias, 1986). However, the binding of ConA, PHA-P, and WGA to *M. anisopliae* var *acridum* isolate IMI330189 aerial conidia were all lower than binding found for three other isolates of *M. anisopliae* (Ma 97, Ma49, and Ma 65) (Jeffs et al., 1999).

Our results indicated that spores from liquid culture had overall higher lectin-binding than aerial conidia, which is contradictory to relative lectin-binding characteristics among *B. bassiana* spore types. Results of Hegedus et al., (1993) and Jeffs et al. (1999) indicated that the lectins, wheat germ agglutinin (WGA), concanavalin A (ConA), phytohaemagglutinin P (PHA-P), soybean agglutinin (SBA), and *Ricinus communis* agglutinin 1 (RCA), had a greater affinity for aerial conidia and submerged conidia than blastospores (Hegedus et al., 1992; Jeffs et al., 1999). Blastospores produced in liquid media are often described as being similar to hyphal bodies produced *in vivo* in insect hemolymph, and are therefore, expected to have reduced lectin binding characteristics to evade host immune systems. However, the cell-wall surfaces of hyphal bodies produced in submerged culture do not necessarily correlate well with those

produced in insect hemolymph. For example, *B. bassiana* has been shown to form protoplast-like cells to evade host immune systems *in vivo* in insect hemolymph. These protoplast-like cells appear to have shed their glycomannan coat, as indicated by lack of Con A binding, and underlying wall layer (chitin and β(1→3) glucans) as indicated by lack of WGA binding (Pendland et al., 1993). Our results, which indicate strong lectin binding characteristics of blastospores, is further evidence that the surface carbohydrate properties of blastospores produced *in vitro* and *in vivo* may be dissimilar.

3.4.3.2. Charge

The potential for spores to bind to charged substrates or hydrophobic substrates was used to determine the presence of charged and hydrophobic regions on the surface of spores produced in four liquid media types and aerial conidia. The charge present on the surface of *M. anisopliae* spores has less of an effect on interaction with insect cuticle than does hydrophobicity (Boucias et al., 1988); however measurement of spore charge may be useful in distinguishing spore types. The electrostatic charges present on the surface of aerial conidia and spores from various liquid media were measured by counting the number of spores adhering to cationic and anionic substrates (Table 3-5). Aerial conidia and spores from the two liquid media, Adamek's and VT+10% PEG, did not demonstrate strong attraction to any of the cationic or anionic substrates. The chitin and chitosan substrates appeared to be very heterogenous substrates leading to high variability in spore binding and thereby make it difficult to distinguish the relative attraction of spores produced in the four liquid media and aerial conidia. Nonetheless, aerial conidia showed a significantly greater attraction to chitin than any of the spores from liquid culture. This is in contrast to the results of Boucias et al. (1988) who found a greater attraction of *M. anisopliae* aerial conidia to chitosan than to chitin. Spores produced in VT media appeared to be much more anionic than any of the other spore-types, as indicated by their attraction to Q-sepharose. Other researchers have found *M. anisopliae* aerial conidia to be more attracted to cationic substrates than anionic. Boucias et al. (1988) also found that *M.*

anisopliae (UF1 5507) aerial conidia were attracted to two cationic substrates chitosan and DEAE-Bio-Gel beads, and that pretreatment with poly-L-lysine inhibited binding to these substrates. Jeffs et al. (1999) found that *M. anisopliae* (SL 297) aerial conidia were moderately attracted to amine-modified latex beads.

Table 3-5. Comparison of Surface Charge and Hydrophobicity of *M. anisopliae* var. *acridum* Spores Produced in Four Liquid Media and Aerial Culture as Determined by Binding to Various Charged and Hydrophobic Substrates

Media	Number of Spores Binding to Cationic (+) and Anionic (-) Substrates[2]				(HMA) Hydrophobic # of Beads / Spore[2]
	Chitin (-)	Chitosan (+)	CM-seph. (-)	Q-seph. (+)	
SDA (Aerial)	5.7 ± 7.3 a	1.2 ± 4.2 a	1.8 ± 2.0a	1.5 ± 1.5 a	1.5 ± 1.5 a
Adamek's	1.0 ± 2.3 b	2.5 ± 8.7 a	1.0 ± 1.1ab	2.3 ± 2.4 a	0.2 ± 0.6 b
VT media	0.2 ± 0.5 b	1.1 ± 1.3 a	1.7 ± 1.6a	12.9 ± 7.3 b	1.3 ± 1.3 a
VT media +10% PEG	0.7 ± 1.2 b	0.8 ± 1.3 a	0.7 ± 1.0b	2.8 ± 2.4 a	1.0 ± 1.5 a

[1]Numbers followed by different letters within the same column are significantly different at the $\alpha = 0.05$ level (Tukeys HSD). For spore dimensions n = 30 to 40 spores; for chitin and chitosan binding n = 20 particles; for Q-sepharose and CM-sepharose n = 30 beads; and for the hydrophobic microsphere assay (HMA) n = 50 spores.
[2] Values presented as means ± standard deviations

3.4.3.3. Hydrophobicity

The adhesion of *M. anisopliae* aerial conidia to insect cuticle is primarily mediated by hydrophobic forces (Boucias et al., 1988). Therefore, the hydrophobicity of spores produced in various liquid media may affect their efficacy as biopesticides by influencing adhesion to insect cuticle as well as having utility in distinguishing spore types. The hydrophobicity of spores produced in the three liquid media and aerial conidia was measured by the number of hydrophobic microspheres binding to spores using a Hydrophobic Microsphere Assay (HMA). Results are presented in Table 3-5 along with data on the binding of spores to various cationic and anionic substrates. Aerial conidia,

VT media spores, and VT media + 10% PEG spores all had similar potential to adhere to hydrophobic microspheres, which was higher than that of spores from Adamek's media ($\alpha = 0.05$).

It has been well established using three different assay methods that *M. anisopliae* aerial conidia are extremely hydrophobic, but it is surprising that spores from liquid culture may have similar hydrophobic properties. Boucias et al. (1988) found that *M. anisopliae* aerial conidia were extremely hydrophobic using both the phase exclusion and the salt aggregation described by Mozes and Rouxhet (1987). Similar results were also found using salt mediated aggregation and sedimentation assays (SAS) (Jeffs and Khachatourians, 1997; Jeffs et al., 1999). These three assays were not practical for this study, however, because VT media contained waste brewer's yeast. These assays require either the observation of spores precipitating from a solution or changes in optical density. None of these methods involve direct examination with a microscope and, therefore, would not differentiate between waste brewer's yeast cells and *M. anisopliae* cells. The hydrophobic microsphere assay (HMA) used microscopic observation of interaction between hydrophobic latex beads and spores. Using this method, spores that were produced in liquid culture (VT media and VT media + 10% PEG), which would be expected to be hydrophilic since they were grown in an aqueous environment, were found to have a similar number of hydrophobic binding sites as aerial conidia. Similar results were found by Jeffs et al. (1999) when using hydrophobic latex beads to measure the hydrophobicity of *B. bassiana* blastospores and aerial conidia. Jeffs et al. (1999) found that both of these spore types were found to have high attraction to hydrophobic latex beads; whereas the hydrophobicity of aerial conidia is much greater than blastospores when using SAS assay.

Jeffs (1999) proposed that hydrophobic sites on the surface of spores may be found adjacent to anionic sites resulting in a net anionic surface charge of the spore. Even though the net effect on the surface of the spore is anionic and hydrophilic, small (1 µm) hydrophobic microspheres are able to bind to the hydrophobic sites. The HMA method may, therefore, provide for the ability to distinguish hydrophobic sites on spores that are masked by adjacent electronegative groups when using the salt aggregation assay (Jeffs et

al., 1999). The high densities of electronegative spore-based charges in the suspension could also increase the attraction of the hydrophobic beads to relatively sparse hydrophobic sites on the spore surface (Jeffs et al., 1999). If the HMA is detecting hydrophobic sites on the surface of spores, which are masked by the adjacent anionic sites when evaluating the net hydrophobicity of the spore surface, it is possible that the HMA may not accurately reflect the way that spores would interact with insect cuticle.

3.4.4. Effects of Liquid Culture Conditions on Drying Stability

The desiccation tolerance of fungal spores may be influenced by the conditions in which they are grown (Harman et al., 1991; Jackson et al., 1997). This has practical application when developing processes for producing a dry formulation of fungal spores and may be used to differentiate spore types. Cliquet and Jackson (1997) compared several drying techniques to establish a standard procedure for evaluating the desiccation tolerance of blastospores of the entomopathogenic fungus *Paecilomyces fumosoreus*. A method that involves rapid drying on silica gel was found to be fast, reproducible and provided a moderate level of spore survival. This method was adapted to evaluate the drying stability of *M. anisopliae* var *acridum* aerial conidia and spores produced in various liquid media. Spores were either not washed and kept in their growth media, washed with distilled water, or washed with polyethylene glycol (PEG) at an osmolality approximately equal to that of the growth media. The non-washed spore treatments were included to evaluate the influence of media on drying stability. The PEG-wash treatments were included to isolate osmotic effects associated with transferring spores from media to distilled water. The non-washed treatment was not conducted for aerial conidia since they were grown on solid media.

The percent of spores germinating was determined after 6 hr and 40 hr of incubation on 2% malt agar. Germination following 6 hr of incubation was used to compare the relative germination rates among spores from various media, from different washing regimes, before and after drying; germination after 40 hr incubation was used to determine percent viability. This was based on the assumption that all live spores would germinate within

40 hr of incubation on 2% malt agar. Percent germination following 6 hr of incubation was corrected for proportion of spores that were viable to remove the effect of spore viability when comparing germination rates. This correction was done by dividing percent germination after 6 hr of incubation by the fraction of spores that were viable (% Germination at 6 hr ÷ Proportion germinating at 40 hr). Spores were considered to be non-viable if they did not germinate after 40 hr of incubation on 2% malt agar. The corrected 6-hr percent germination measurements are presented in Figure 3-2 a, b, and c for distilled water, or washed with isoosmotic PEG solution, respectively. The 6-hr percent germination of aerial conidia was consistently lower than any of the spores from liquid culture in all washing regimes, both before and after drying ($\alpha = 0.05$, Wilcoxon). The 6-hr percent germination of spores from Adamek's media and VT media + 10% PEG were consistently higher than that of spores from VT media before drying ($\alpha = 0.05$, Wilcoxon). After drying, the 6-hr percent germination of spores from Adamek's media remained significantly higher than VT media; whereas spores from VT media +10% PEG no longer had significantly higher 6-hr germination than spores from VT media ($\alpha = 0.05$, Wilcoxon). The effect of drying on germination rate was inconsistent among media types and washing regimes (Greek letters). Drying decreased the germination rates of blastospores produced in VT media + 10% PEG when washed with either water or isoosmotic PEG, whereas it generally increased germination rates of blastospores produced in Adamek's media and aerial conidia, and had no effect on spores from VT media. The change in germination rate in each of these cases, although statistically significant, did not represent large numerical differences. Cliquet and Jackson (1997) also found that silica drying did not affect the germination rate of *P. fumosoreus* blastospores when using this same method; whereas air drying on diatomaceous earth significantly reduced rate of colony formation, indicating that drying method may have an affect on germination rate.

Spores with faster germination rates may have a greater potential for infection by reducing potential for desiccation, effects of other microorganisms, or loss during molting while on the insect cuticle (Charnley, 1984; Dillon and Charnley, 1985; Hassan and Charnely, 1983). The higher germination rates of spores grown in VT media + 10%

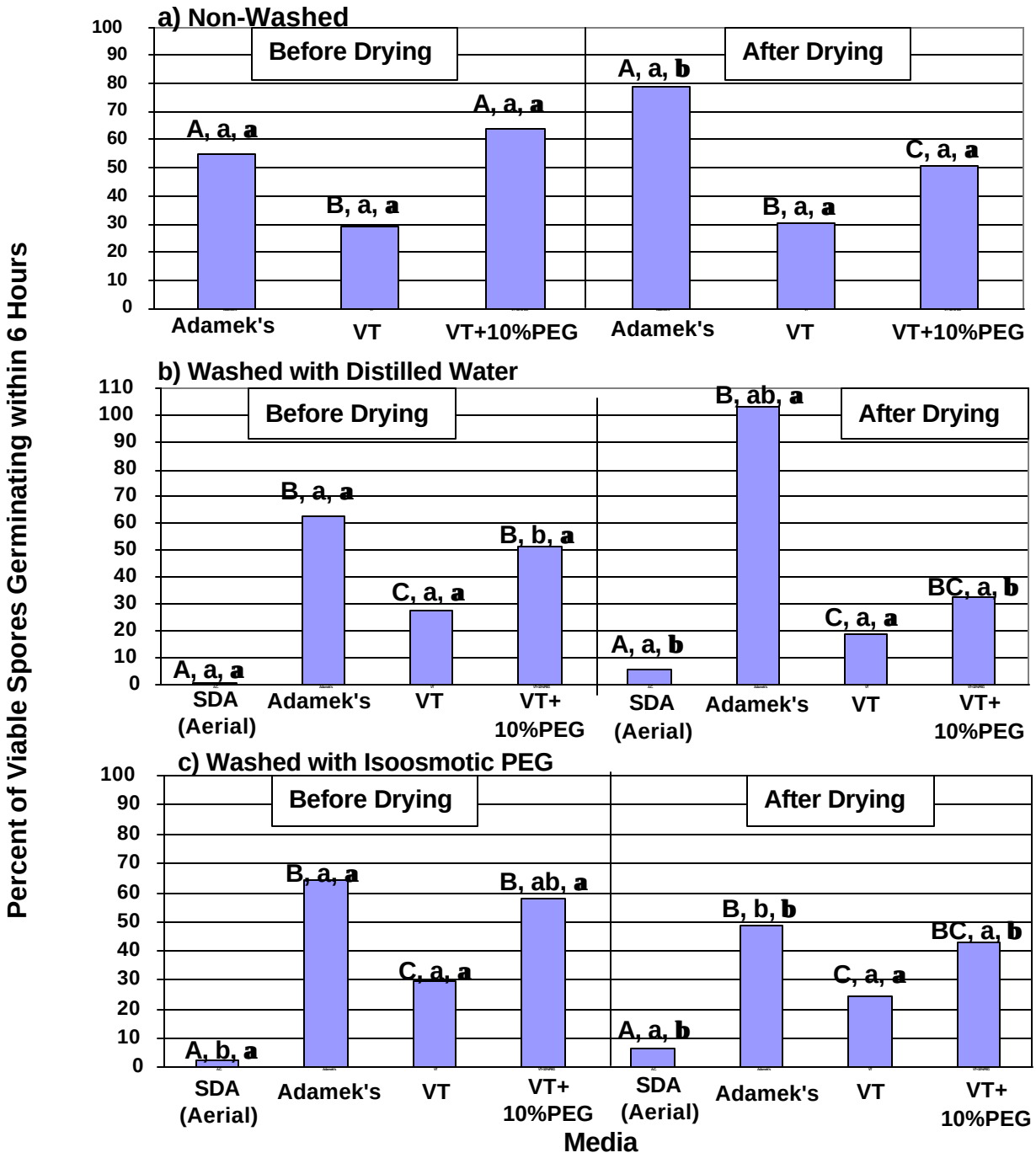

Figure 3-2. Percent of viable *M. anisopliae* var. *acridum* spores produced in three liquid media types and aerial culture germinating within 6 hr of incubation on 2% malt agar before and after drying on silica gel. Viability based on percent germination after 40 hr of incubation. Spores were either **a)** not washed to remove media; **b)** washed with distilled water; or **c)** washed with isoosmostic polyethylene glycol. Capital letters correspond to differences among spores from different media along the x-axis of the same graph (before drying and after drying treatments are compared independently). Lower-case letters correspond to differences between washing regimes within the same medium (**a, b, and c**). Greek letters correspond to differences before and after drying for spores from the same media and washing regime. All comparisons are significant at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

PEG may partially explain higher mortality of *S. americana* exposed to these spores over spores from VT media (See Section 3.4.6. Effects of Osmolality during Liquid Culture on Virulence to *Schistocerca americana*).

In addition to the practical application of enhanced infectivity, germination rate may also be used to help delineate spore types as in the case of comparing germination rates for aerial conidia, submerged conidia and blastospores of *B. bassiana* (Thomas et al., 1987). The similar germination rates of blastospores from Adamek's media and from VT media + 10% PEG is additional evidence that *M. anisopliae* var. *acridum* shifts from producing submerged conidia to producing blastospores at increased osmolality since blastospores are generally thought to have faster germination rates than conidia (Thomas et al., 1987). The lower germination rates of spores from VT media than from Adamek's media and VT media + 10% PEG is additional evidence that spores produced in VT media represent a distinct spore type.

Percent germination of spores after 40 hr of incubation was used to evaluate the viability of the spores from various media, from different washing regimes, before and after drying. These 40-hr percent germination measurements are presented in Figure 3-3 a, b, and c for spores that were not washed to remove media, washed with distilled water, or washed with PEG solution, respectively. Initial viablility was high for aerial conidia, spores from VT media, and from VT media + 10% PEG. Low initial viability of blastospores from Adamek' s media may have resulted from freeze thaw damage or poor storage at -80 C though these propagules were frozen with 20% glycerol. Hegedus et al. (1992) found that the stability of blastospores was lower than that of submerged conidia and aerial conidia for *B. bassiana* stored at -70 C; times for 50% reduction in spore viability (SV50) were 30.7, 15.2, and 5.2 weeks for aerial conidia, submerged conidia, and blastospores, respectively.

There was a dramatic effect of washing regime on the ability of spores to survive drying on silica gel. Spores that were not washed to remove media survived drying much better than those that were washed to remove media. A positive effect of media on drying stability was also observed by Cliquet and Jackson (1997) when testing the drying stability of the entomopathogenic fungus *Paecilomyces fumosoreus* using this same

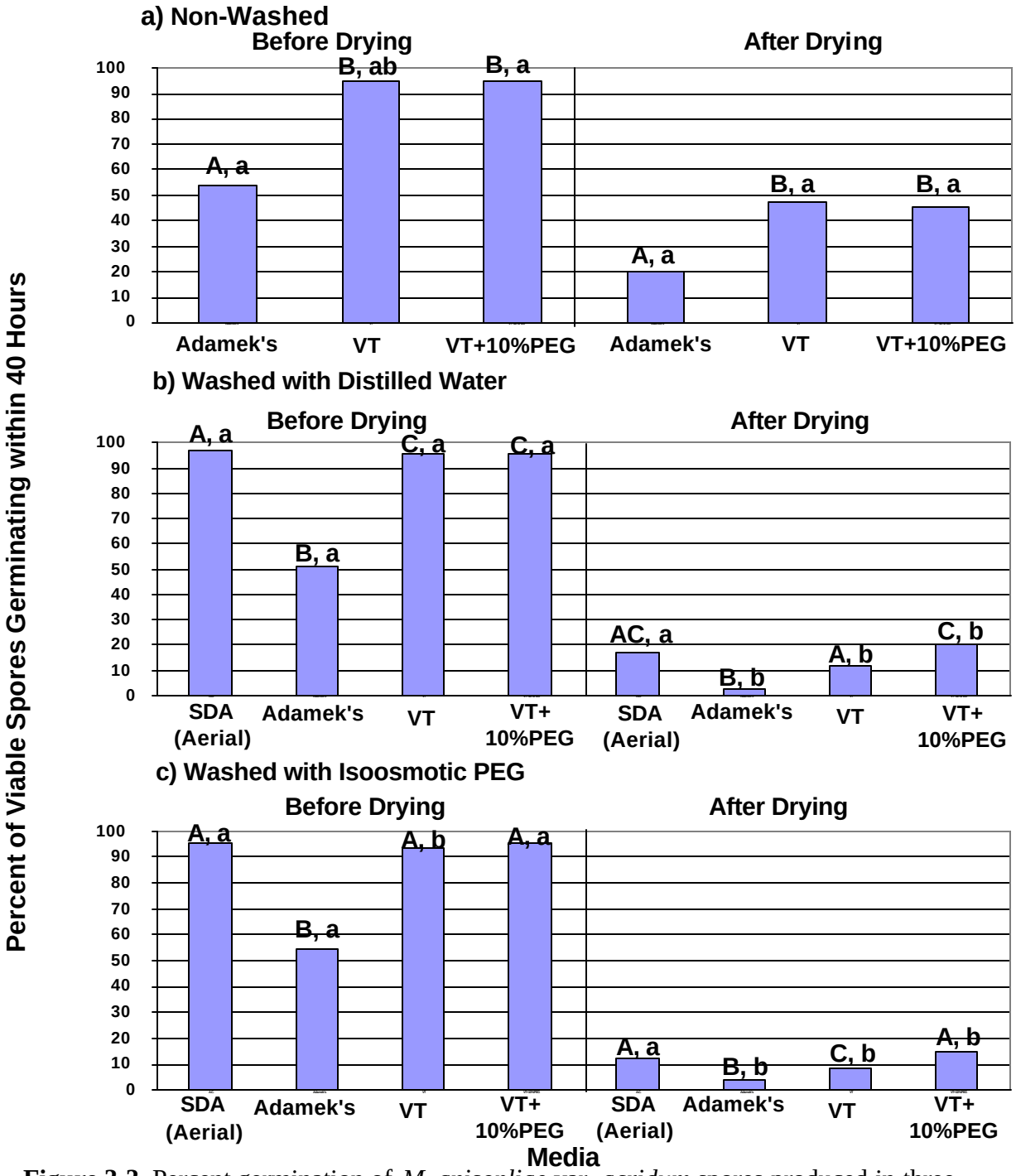

Figure 3-3. Percent germination of *M. anisopliae* var. *acridum* spores produced in three liquid media types and aerial culture within 40 hr of incubation on 2% malt agar before and after drying on silica gel. Spores were either **a)** not washed; **b)** with distilled water; or **c)** washed with isoosmotic polyethylene glycol remove media. Capital letters correspond to differences among spores from different media along the x-axis of the same graph (before drying and after drying treatments are compared independently). Lower-case letters correspond to differences between washing regimes within spores from the same media (a, b, and c). Differences before and after drying for spores from the same media and drying regime were significant in all treatments. All comparisons are significant at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

method. These authors attributed the enhanced drying stability to residual glucose present in the media citing the work of Crowe et al. (1990), which has demonstrated the potential of non-reducing disaccharides to stabilize membranes during desiccation. One difficulty with this explanation is that the stabilizing effect of sugars is typically ascribed to disaccharides, whereas glucose (Cliquet and Jackson, 1997) and fructose (VT media) are both monosaccharides. It is possible that the monosaccharides may have protected cells from leaking of intracellular substances by formation of protein gels within the cell (Beker et al., 1984). This enhanced drying stability was not observed for spores washed in isoosmotic PEG solution indicating that it was not the result of spores losing a stabilizing factor when placed in a hypotonic distilled water environment. Cliquet and Jackson (1997) washed spores with isoosmotic PEG as well as distilled water and also found no enhanced drying stability of PEG-washed blastospores over that of water-washed blastospores.

Spores grown in VT media and VT media + 10% PEG consistently showed greater drying stability than spores from Adamek's media. When media was removed by washing with either distilled water or isoosmotic PEG solution, spores grown in VT media + 10% PEG showed greater drying stability than spores from VT media. Spores grown in VT media + 10% PEG had similar drying stability to aerial conidia in both washing regimes, whereas spores from VT media only had similar drying stability to aerial conidia when washed with distilled water. It is surprising that spores grown in VT media + 10% PEG had greater drying stability than spores from VT media when considering the cell-wall thickness of spores from these media (See Section 3.4.2.2. Cell-Wall Thickness). Spores from VT media + 10% PEG had much thinner walls that were similar to blastospores from Adamek's media; whereas spores from VT media had thick walls similar to aerial conidia. This additional drying stability may relate to the presence of a stabilizing factor that is formed during growth at high osmolality such as increased trehalose concentrations or increased bonding between phospholipids in the cell membrane. Further research is needed to elucidate the nature of this stabilizing factor.

3.4.5. Effects of Osmolality during Liquid Culture on Virulence to *Schistocerca americana*

Mortality of *S. americana* following exposure to four doses aerial conidia, spores from VT media, and spores from VT media + 10% PEG are presented in Figure 3-4. Mortality was corrected for control mortality using Abbott's formula (Finey, 1963). The LT_{50} values and corresponding 95% confidence intervals for aerial conidia, spores from liquid culture in VT media, and VT media + 10% PEG at 1 x 10^6 spores/mLwere 14.6 d (11.9 to 18.0 d), 10.5 d (9.5 to 11.6 d), and 7.8 d (6.7 to 9.0 d). On the basis of these 95% confidence intervals spores grown in VT media + 10% PEG were significantly more virulent than spores from either of the other two media and spores grown in VT media were significantly more virulent than aerial conidia.

Aerial conidia in this experiment, which were formulated in 20% molasses, were less virulent toward *S. americana* than the same isolate formulated in either 70% diesel fuel : 30% peanut oil (See Section 6.4.2. Effects of Coating Spores with a Water-Soluble Lignin Derivative during Air Drying on the Virulence of *Metarhizium anisopliae* var. *acridum* to *Schistocerca americana*) or formulated in pure peanut oil (Sieglaff et al., 1998). Bateman et al. (1993) also found that LD_{50} values for *M. anisopliae* var *acridum* (IMI 330189) to *S. gregaria* were two orders of magnitude lower when applied in cotton seed oil rather than water at low humidity (35% r.h); whereas the virulence was similar for oil and water suspensions when applied at high relative humidity (saturated humidity). The formulation of 20% molasses in water, however, has been shown to be an effective water based formulation for *M. flavoviride* blastospores for use against *S. gregaria* (Stephan et al., 1997). The enhanced infectivity of aerial conidia in oil over water may be the result of increased adhesion of conidia to the insect in oil formulations (Prior et al., 1988).

3.5. Summary

Table 3-6 summarizes the characteristics of spores from various liquid media and from aerial culture that were addressed in this chapter. On the basis of gross spore

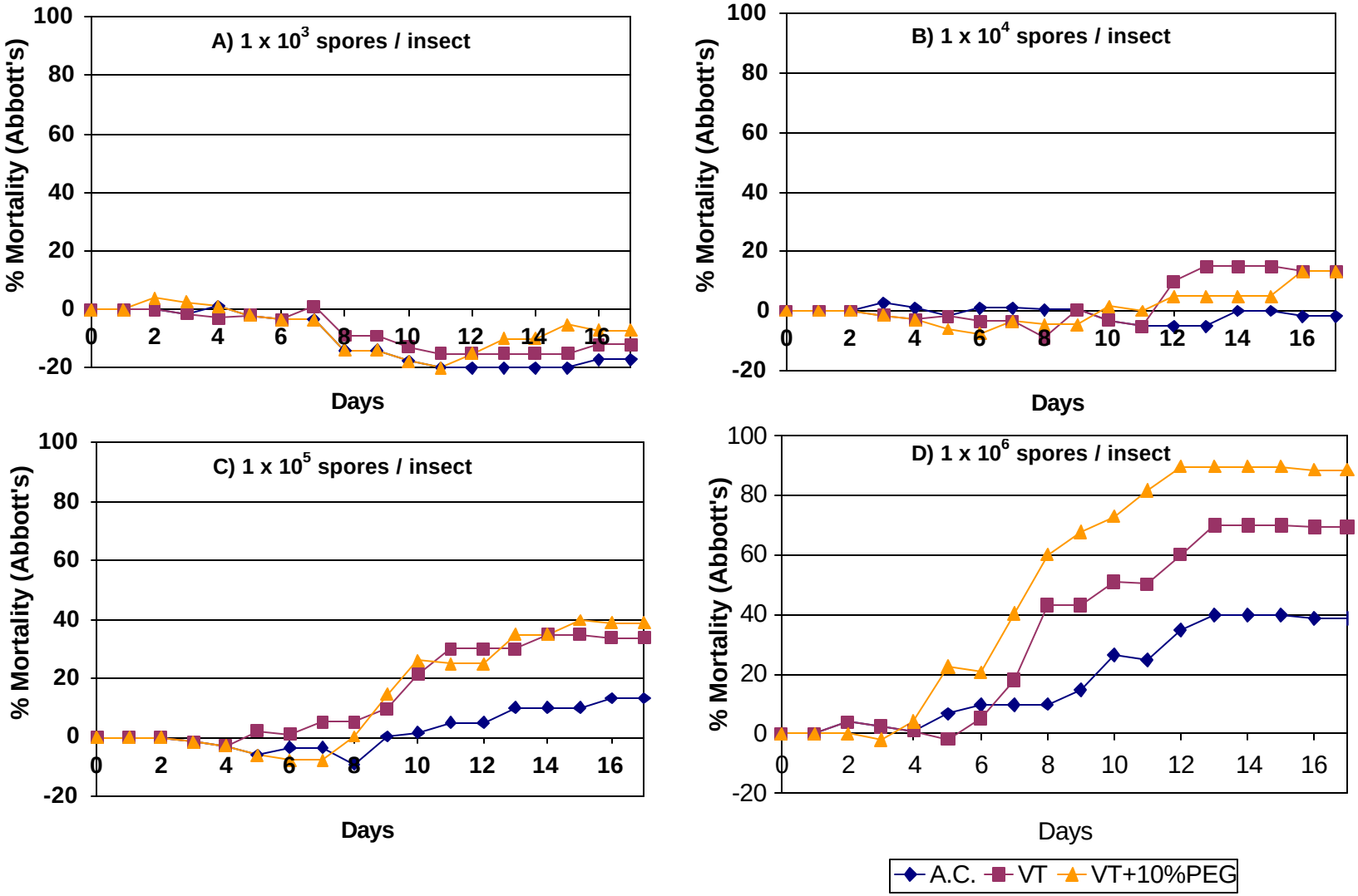

Figure 3-4. Mortality over time for adult (2- to 3- week old) *Schistocerca americana* inoculated by direct dosing (2 µL) with aerial conidia, and spores produced in VT media and VT media + 10% PEG. Individuals were dosed with A) 1 x 10^3, B) 1 x 10^4, C) 1 x 10^5, and D, 1 x 10^6 spores/insect (n = 25 insects / dose). Mortality corrected for controls with Abbott's formula (Finey, 1963).

Table 3-6. Summary of Characteristics[1] of Spores Produced in Various Liquid Media and Aerial Culture.

Media and (Spore Type)	**Dimensions L x W (μm) Tbl. 3-3**	**Wall Thickness (μm) Tbl. 3-3**	**Lectin Binding Tbl. 3-4**	**Anionic Charge Tbl. 3-5**	**Hydrophobic Binding Tbl. 3-5**	**Germination Rate Fig. 3-2**	**Drying Stability Fig. 3-3**	**Virulence Fig. 3-4**
SDA (Aerial) (Aerial Conidia)	4.2 x 5.7	0.17	+	+	+++	+	+++	+
VT media (Submerged Conidia)	3.2 x 5.8	0.15	++	+++	+++	++	++	++
Adamek's media (Blastospores)	4.2 x 6.5	0.10	+++	+	++	+++	+	N/A
VT media + 10% PEG (Blastospores)	3.4 x 6.4	0.07	+++	+	+	+++	+++	+++

[1] Characteristics are presented without standard deviations or statistical comparisons, which are presented in specific tables provided in this chapter. Characteristics that are denoted with +++ represent a strong characteristic; ++ represents and moderate characteristic, and + represents a weak characteristic as compared to spores from the various media. Numerical values for each of these characteristics are provided throughout the body of the text.

morphology, cell-wall thickness, cell-wall surface characteristics, germination rate, and drying stabilityit appears that *M. anisopliae* var *acridum* is able to produce three distinct spore types; aerial conidia, submerged conidia and blastospores. These results support the results of Jenkins and Prior (1993), which indicated that submerged conidia as defined by shape, pigmentation, and presence of phialides could be produced by isolates of *M. anisopliae* in nitrogen limiting media. Submerged conidia were produced in a media consisting of 3% WBY : 3% Sucrose and media consisting of 4% WBY : 4% Fructose and 5% lecithin (VT media), with reduced formation of mycellial pellets (Kleespies and Zimmerman, 1998). Spores from VT media were distinguishable from blastospores produced in Adamek's media on the basis of spore dimensions, cell-wall thickness, lectin binding to wheat germ agglutinin, anionic charge (Q-sepharose binding), and hydrophobicity (HMA). Production of a high concentrations of a stable spore form (submerged conidia) in inexpensive liquid media has certain practical advantages over production in aerial culture (Jenkins and Goettel, 1997). However, without the ability to dry spores from liquid culture they would not be amenable to ultra-low volume application (Moore and Caudwell, 1997), may have decreased thermal stress tolerance (McClatchie et al., 1994) and reduced infectivity at low relative humidity (Bateman et al., 1993; Prior et al., 1988). Practical approaches for drying submerged conidia will be addressed in the following chapter. *Metarhizium anisopliae* var. *acridum* was able to grow at osmolalities up to 1454 mOsm without affecting spore production. The osmolality of VT media was approximately tripled by the addition of 10% polyethylene glycol. Spores produced in VT media had many characteristics of submerged conidia, whereas spores produced at the higher osmolality (VT media + 10% PEG) shared many characteristics with blastospores from Adamek's media. Spores from VT media + 10% PEG and from Adamek's media were similar with regard to dimensions, cell wall thickness, lectin binding to concanavalin A and wheat germ agglutinin, surface charge, and germination rates. However, spores from VT media had a higher potential to bind to hydrophobic microspheres and greater drying stability than blastospores from Adamek's media. The addition of 10% polyethylene glycol to VT media resulted in a spore form that had greater drying stability and greater virulence to *S. americana*.

Enhanced virulence to *S. americana* may relate to more rapid germination rate of spores from higher osmolality,. whereas enhanced drying stability is likely the result of the presence of a stabilizing factor (e.g. trehalose) since these spores possessed thinner cell walls.

Chapter 4. Effect of Non-Reducing Disaccharides and Compatible Solutes on the Desiccation-Tolerance and Shelf Life of *Metarhizium anisopliae* var. *acridum* Spores While Coating Spores for Protection from Ultraviolet Light

4.1 Abstract

A process for air-drying *Metarhizium anisopliae* var. *acridum* submerged conidia and aerial conidia was developed that incorporates coating spores with a water-soluble lignin derivative (Curan 100®) for UV-protection. The effects of three protective agents (skim milk, sucrose, and glycerol) in combination with a lignosulfonic acid (Ultrazine®) and a Kraft lignin (Curan 100®) on spore survival and germination rate after drying, sieving and storage at 28 C was examined. Skim milk enhanced spore survival during air-drying but it produced a dry coated-spore formulation that reduced spore survival during sieving when it was not combined with Curan 100®. The addition of sucrose helped to stabilize formulations when drying with Curan 100®, but was not necessary when skim milk was added to the formulation. In combination with skim milk, higher sucrose concentrations reduced percent germination 12, 24, and 40 hr after drying, and spore survival (60 hr germination) during sieving and storage at 28 C. Submerged conidia did not survive drying in a solution containing only glycerol, but glycerol improved desiccation tolerance at higher concentration in skim-milk based spore-coating formulations. The protective agents we tested are all hygroscopic and increased water retention of formulations after drying at ambient and low relative humidity. A formulation containing Curan 100®, skim milk, and glycerol was selected on the basis of drying stability, survival during sieving, and shelf life at 28 C for further experimentation that compared the UV-tolerance and virulence of coated and non-coated spores.

4.2. Introduction

Suspending *Metarhizium* spp. in an oil carrier offers several advantages over aqueous formulations that are particularly relevant to application in environments of extreme insolation, heat, and dryness such as those targeted for desert locust control. These advantages include: 1) improved survival in simulated sunlight (Alves et al., 1998; Moore et al., 1993); 2) improved infective at low humidity (Bateman et al., 1993; Prior et al., 1988); 3) enhanced thermal-stress tolerance (McClatchie et al., 1994; Hedgecock et al., 1995; Hong et al., 1997, 1998); and 4) compatibility with ultra-low volume application. However, spores produced in liquid culture cannot be directly suspended in oil and must first be dried with a coating material. The method of drying and selection of spore-coating materials may affect spore survival during drying, the shelf-life of spores in the dry material, and spore survival as the particle size of the dry formulation is reduced prior to suspension in oil. Coating materials may directly affect the infection process by influencing adhesion to the cuticle, spore germination on the cuticle, and cuticle recognition leading to formation of infective structures. In addition, the method of drying and selection of coating materials may affect the germination rate of spores in the dry formulation, which can influence infectivity (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al, 1983). By evaluating various combinations of protective materials (skim milk, sucrose, and glycerol) with water-soluble lignins as spore coatings I hope to produce a coated-spore formulation with high spore viability, shelf-life, germination rate, and spore survival as particle size of the formulation is reduced. Further testing of spore survival following exposure to simulated sunlight and infectivity to *Schistocerca americana* will be conducted for a single formulation of submerged conidia and aerial conidia developed in this chapter.

Drying *M. anisopliae* aerial conidia improves their thermal stress tolerance (Hedgecock et al., 1995; McClatchie et al., 1994; Moore et al., 1997; Hong et al., 1997; 1998) and enables spores produced in liquid culture to be suspended in an oil carrier. The additional thermal-stress tolerance provided by preserving *M. anisopliae* var.

acridum aerial conidia in a state of anabiosis may be particularly important in its field application where temperatures may exceed 50 C and storage facilities may not provide adequate temperature regulation (McClatchie et al., 1994). Hong et al., (1997) demonstrated that there was a negative logarithmic relationship between the moisture content of *M. anisopliae* var. *acridum* aerial conidia and their ability to survive thermal stress (50 C). The lower limit of this relationship was 4.6% moisture and the upper limit was between 21.2% and 31.8% moisture (Hong et al., 1998). Below the lower limit, additional drying did not further improve thermal-stress tolerance, and above the upper limit, additional moisture did not further reduce thermal-stress tolerance.

The method of drying spores may influence their ability to survive drying and the thermal-stress tolerance of dried spores (Hong et al., 2000). In the laboratory, spores may be dried by freeze-drying, drying over silica gel, controlled-humidity desiccation in a confined chamber, or air-drying in a transfer hood or in vacuum driers (Humber, 1997; Cliquet and Jackson; 1997; Lievense and van't Riet, 1993). Although several of these methods may be practical for laboratory-scale purposes, industrial-scale processes must be based on requirements of energy input, support material, percent survival, and system-level continuity; and are, therefore, generally limited to spray-drying, fluidized-bed drying, or spray-granulation (Lievense and van't Riet, 1993). In these drying systems, cells are dried relatively rapidly and may be subject to thermal and dehydration inactivation. For industrial purposes, *M. anisopliae* aerial conidia may be dried by a convective-drying process, which involves forcing warm air through the sporulation media, or in low-technology mass production by slow-drying in a dehumidified room (Hong et al., 2000). Although the faster convective air-drying process may remove water more efficiently; aerial conidia of *M. anisopliae* var *acridum* survived better in storage at high temperatures (50 C) when initially dried more slowly at 50 to 60% r.h (Hong et al., 2000). It is likely that *M. anisopliae* undergoes some physiological changes in preparation for anabiosis at the slower drying rates; however, the nature of these changes is not known.

Considering the low moisture contents that aerial conidia are dried to using these methods (Hedgecock et al., 1995; McClatchie et al., 1994; Moore et al., 1997; Hong et

al., 1997; 1998), the layers of "non-freezable" water that surround and stabilizes biomolecules has likely been removed from the spores (Berney., et al., 1991; Crowe et al., 1990), suggesting a role of non-reducing disaccharides in stabilizing membranes (Crowe et al., 1984; 1988; 1990) and enzymes (Carpenter and Crowe, 1988 a, b; Colaco et al., 1992) as described by the "water replacement hypothesis". This property appears to be unique to disaccharides and larger molecular weight sugars and is not a property of other cryoprotectants, such as "compatible solutes", which only provide stability in the presence of free water (Crowe et al., 1990). The thermal stress tolerance of the desiccated microorganism may be related to the glass transition temperature and the hydrogen bonding potential of the stabilizing sugar (Tan et al., 1995). Aerial conidia of *Metarhizium anisopliae* have been shown to produce trehalose in low concentrations (Hallsworth and Magan, 1994), and this disaccharide is particularly suited for stabilizing membranes in a glass state and has a high glass transition temperature. Several researchers have demonstrated the beneficial effects of various non-reducing disaccharides, particularly trehalose, on survival of yeast during freeze-drying (Berny et al., 1991; Diniz-Mendez et al., 1999; Lodato et al., 1999; Tan et al., 1995). In addition, maltose has been used to protect mycelia of *M. anisopliae* during air-drying (Krueger et al., 1992).

The work described in this chapter involves combining *M. anisopliae* var *acridum* submerged conidia and aerial conidia with various ratios of three protective agents (sucrose, glycerol and skim milk) in combination with two water-soluble lignin derivatives (Ultrazine® and Curan 100®) in aqueous suspensions that are referred to as "spore-coating formulations" throughout this chapter. The objectives of this research are to examine the effects of these spore-coating formulations on percent germination over time and spore survival under the following conditions: 1) when spores are suspended in the spore-coating formulations prior to drying; 2) after air-drying spores suspended in spore-coating formulations at ambient relative humidity followed by low relative humidity using silica gel; 3) after reducing the particle size of the coated spore formulations for suspension in oil; and 4) following storage at 28 C for 6 d. Experiments in Chapter 5 involved testing the UV-tolerance and virulence of the

formulated spore; which placed certain practical restrictions on formulation characteristics including: 1) the formulation must be dry so that it can be suspended and applied in an oil carrier; 2) the coated-spore formulation must be presented as small particles (<100 µm) to be used in ultra-low volume application and; 3) the coated material must have a shelf life that is at least long enough to conduct bioassays at Virginia Tech survive shipment to collaborators at Institut National de la Recherche Agronomique (INRA), Montferrier-sur-Lez, France for testing of tolerance to simulated sunlight. A schematic flow chart that describes the strategy for developing this formulation is presented in Figure 3-1.

4.3. Materials and Methods

4.3.1. Production of Submerged Conidia and Aerial Conidia for Air-Drying Experiments

Metarhizium anisopliae var. *acridum* isolate IMI 330189, which was isolated from the grasshopper species *Ornithacris cavroisi* (Finot) *(Orthopera : Acrididae)* in Niger, was obtained from LUBULOSA. These cultures were transferred to Sabouraud dextrose agar (SDA), grown at 24 C and then conidia from these plates were harvested and stored with in 1 mL aliquots in 20% glycerol at -80 C to be used as a starting culture for all experiments. Aerial conidia were produced on SDA from this frozen stock culture to be used as inoculum for producing the spores described in this chapter. Aerial conidia of *M. anisopliae* var. *acridum,* were harvested from 2- to 3-week old cultures on SDA and suspended in 0.05% Tween 80 in sterile distilled water at an approximate concentration of 6 x 10^6 conidia/mL for use as an inoculum. Liquid media in each of the following experiments was inoculated to produce an initial concentration of 1.2 x 10^5spores/mL of media. Viability of the inoculum was determined as percent germination after 24 hr at 24 °C on 2% malt agar.

Three air-drying experiments are described in this chapter; the first two experiments used spores produced in a 1-L fermenter and the third used spores produced

in shake-flask culture. Spore production conditions varied between Air-Drying Experiments 1 and 2 because the 1-L fermenter was also being used to determine the effects of dissolved oxygen on spore production and, therefore, the availability of spores from the 1L-fermenter was limited to the protocols of these experiments. Shake-flask culture was used for Air-Drying Experiment 3 to provide replication at the level of spore production.

Submerged conidia for Air-drying Experiments 1 and 2 were produced in a 1-L fermenter (BioFlo III System; New Brunswick Scientific). The liquid media consisted of 40 g/L waste brewers' yeast (WBY) (Stroh's Batch, NPC Inc., Eden, N.C.) and 40 g/L sucrose (Sigma Chemical Co.) (Jenkins and Prior, 1993). The pH of the media was adjusted to 7.0 with sterile 1 N NaOH before autoclaving. Temperature in the 1-L fermenter was maintained at 24 °C and the air-flow rate was 0.5 L/min (0.5 VVM). In Air-Drying Experiment 1, spores were produced using the incubation conditions of 50% dissolved oxygen and constant pH 7 resulting in a final spore concentration of 1×10^9 spores/mL. In Air-Drying Experiment 2, spores were produced using the incubation conditions of 5% dissolved oxygen for Air-Drying Experiment 2, and pH was not regulated producing a final spore concentration of 9.8×10^8 spores/mL. The dissolved oxygen levels were maintained by allowing the agitation rate to automatically adjust between 130 RPM and 500 RPM. Antifoam 289 was delivered by an automated delivery system that monitored and controlled foaming. The content of the bioreactor was sampled approximately every 5 hr, at which time the dissolved oxygen, temperature, and agitation rate were recorded.

Spores for Air-Drying Experiment 3 were produced in shake flask culture as described in Chapter 3, Section 3.3.1. The final spore concentration was $5 \pm 0.7 \times 10^8$ spores/mL (mean ± st. dev.). Aerial conidia for this experiment were harvested from 2- to 3-week old cultures on Sabouraud dextrose agar, grown at 24 C.

4.3.2. Methods for Determining Percent Germination

Dry formulations were placed in a 100% relative humidity (r.h.) environment for 1 hr to reduce the stress of rapid rehydration before dilution to approximately 1 x 10^6 spores/mL with sterile distilled water (Moore et al., 1997). The spore suspensions (30 µL) were spread on 30 mm Petri plates containing 2% malt agar plus 0.001% benomyl and 0.02% chloramphenicol. Details on the development of this germination technique are describde in Appendix II. Germination plates were incubated at 24 C and germination was stopped with 20% formalin at specified time invervals. Percent germination was determined for at least 200 spores at 400 x magnification by counting all of the spores in a microscopic field and examining transects across the Petri plate. Spores were considered to have germinated if the germ tube was more than half the diameter of the spore. Percent germination was used to determine both percent germination at various time intervals and total spore viability. The longest incubation time in each germination test was used to as a measure of viability, based on the assumption that all viable spores had germinated within this incubation time. A similar approach has been used in experiments that test the UV-tolerance of *Metarhizium* spp. These papers used germination at 24-hr to examine the effects of UV on germination rate and germination at 48-hr as total viability (Alves et al., 1998, Hunt et al., 1994, Moore et al., 1993). Shorter incubation times were corrected for total viability to isolate the effect of percent germination at various time intervals from total viability (corrected % germination = % germination at the shorter incubation time ÷ proportion germination at the final germination time).

4.3.3. Preparation of Spores for Air-drying

Three air-drying experiments were used to investigat the effects of a non-reducing disaccharide (sucrose) and a compatible solute (glycerol) in combination with skim milk and two lignin derivatives on the spore survival after air-drying, sieving, and storage at

28 . Selection of coating materials in Air-Drying Experiments 1 and 2 was, in part, based on producing a formulation of skim milk-coated submerged conidia that could serve as an appropriate control for lignin-coated submerged conidia coated in UV-tolerance tests and bioassays (Chapter 5). The skim milk control was needed because submerged conidia could not be suspended in oil for UV-tests and bioassays without first being dried and they do not survive drying without a coating material. This approach was changed in Air-Drying Experiment 3 because it became clear that skim milk-coated spores were not appropriate controls for lignin-coated spores due to problems encountered in sieving and storing the skim milk-coated spores produced in Air-Drying Experiments 1 and 2. Air-Drying Experiment 1 was designed to evaluate the survival of submerged conidia during air-drying with a skim-milk coating (1 : 0.5 spores : skim milk; dry mass ratios) and increasing sucrose concentrations (1 : 0.5, 0.25, 0.125 spores : sucrose; dry mass ratios), and the presence of two lignin derivatives (Ultrazine® and Curan 100®; 1 : 0.5 spores to lignin; dry mass ratios) added to the skim milk formulation with the highest sucrose concentration. Curan 100® is a Kraft alkali lignin and Ultrazine® is a sodium salt of a lignosulfonic acid (Aldrich # 37-095-9 and # 37-097-5, respectively). Air-Drying Experiment 2 was designed to evaluate the survival of submerged conidia during air-drying with varying proportions of skim milk, Curan 100®, and a single sucrose concentration. Air-Drying Experiment 3 was designed to evaluate the survival of both aerial conidia and submerged conidia in varying proportions of skim milk, Curan 100®, sucrose, and glycerol. Aerial conidia were tested because non-coated aerial conidia could be suspended in oil and serve as an appropriate control for lignin-coated aerial conidia for UV-tolerance tests and bioassays (Chapter 5).

Spores that were to be used for all three Air-Drying Experiments were washed three times by centrifuging for 5 min (2800 x g; Fisher Scientific Marathon 6K centrifuge) in 50-mL sterile centrifuge tubes and re-suspending in sterile distilled water. For Air-Drying Experiment 3, spores from four different 250-mL shake flasks were kept separate and each flask represents a replicate. The spore-coating formulations used for Air-Drying Experiments 1, 2, and 3 are provided in Tables 4-1, 4-2, and 4-3, respectively. Individual components of the suspension media were autoclaved at 114 C for 20 min to

Table 4-1. Air Drying Experiment 1: Submerged Conidia Spore-Coating Formulations

	Dry Mass Ratios[1] of Spores and Coating Material					
Test Group	Spores	Skim Milk	Glycerol	Curan	Ultrazine	Sucrose
1	1	0.5	0.1	0	0	0.5
2	1	0.5	0.1	0	0	0.25
3	1	0.5	0.1	0	0	0.125
4	1	0.5	0.1	0	0	0
5	1	0.5	0.1	0	0.5	0.5
6	1	0.5	0.1	0.5	0	0.5
7	1	0	0.1	0	0	0

[1] Dry mass ratios are based on 100 g/L of spores (i.e. the dry mass ratios of spores, skim milk, sucrose and glycerol for Test Group 1 were 1: 0.5 : 0.5 : 0.1 x 100 = suspension media containing 100 g/L spores : 50 g/L skim milk : 50 g/L sucrose : 10 g/L glycerol).

Table 4-2. Air Drying Experiment 2: Submerged Conidia Spore-Coating Formulations

	Dry Mass Ratios[1] of Spores and Coating Material				
Test Group	Spores	Skim Milk	Glycerol	Curan	Sucrose
1	1	0	0.025	1	0
2	1	1	0.025	0	0
3	1	0	0.1	1	0
4	1	1	0.1	0	0
5	1	0	0.1	1	0.125
6	1	1	0.1	0	0.125
7	1	0.5	0.1	0.5	0.125
8	1	0.5	0.1	0	0.125

[1] Dry mass ratios are based on 100 g/L of spores (i.e. the dry mass ratios of spores, Curan 100® and glycerol for Test Group 1 were 1: 1 : 0.025 x 100 = suspension media containing 100 g/L spores : 100 g/L Curan 100® and 2.5 g/L glycerol).

Table 4-3. Air Drying Experiment 3: Submerged Conidia Spore-Coating Formulations

	Dry Mass Ratios[1] of Spores and Coating Material				
Test Group	Spores	Skim Milk	Glycerol	Curan	Sucrose
1	1	0	0.025	1	0
2	1	0	0.1	1	0
3	1	0	0.1	1	0.125
4	1	0.5	0.1	0.5	0.125
5	1	0.5	0.1	0.5	0

[1] Dry mass ratios are based on 100 g/L of spores (i.e. the dry mass ratios of spores, Curan 100® and glycerol for Test Group 1 were 1: 1 : 0.025 x 100 = suspension media containing 100 g/L spores : 100 g/L Curan 100® and 2.5 g/L glycerol).

avoid denaturing of the skim milk (Humber, 1997) and chemical reaction among coating products at higher temperatures. The pH's of the various suspension media were adjusted to 7.0 ± 0.5 with 1 N NaOH and 2 N HCl. The germination rate and viability of spores in each of the suspension media were determined as described in Section 4.3.2. Each spore suspension was replicated 3 times in Air-Drying Experiment 1 and 4 times in Air-Drying Experiments 2 and 3. Comparison of percent germination among spores in the various suspension media was made using a non-parametric test (Wilcoxon two-sample test for non-paired ranked observations).

4.3.4. Air-drying of Spore Formulations at Ambient and Low Relative Humidities

Spore suspensions described in Tables 4-1, 4-2, and 4-3 were placed into sterile disposable Petri plates. For Air-Drying Experiment 1, 7 mL of suspended spores were dried in a 100 mm Petri plates. For Air-Drying Experiment 2, 3.5 mL of suspended spores were dried in 60 mm Petri plates. For Air-Drying Experiment 3, 4.5 mL of suspended submerged conidia were dried in 60 mm Petri plates and 1.3 mL of suspended aerial conidia were dried in 30 mm Petri plates. Each Petri plate was replicated 3 times in Air-Drying Experiment 1 and 4 times in Air-Drying Experiments 2 and 3.

Samples were dried at ambient relative humidity (r.h.) under a sterile transfer hood for 60 hr, 52 hr and 60 hr for Air-Drying Experiments 1, 2 and 3, respectively. Relative humidity was monitored using a Fisher Scientific traceable printing hygrometer/thermometer (Model # 11-661-17A) and varied ranging from 23 to 43%, 25 to 36%, and 29 to 40% for Air-drying Experiments 1, 2, and 3, respectively. After drying at ambient r.h., the coated-spore formulations were scraped from the surface of the Petri plate with a sterile metal spatula, then Petri plates containing the coated-spore formulations were transferred to a desiccation chamber containing silica gel, which maintained a constant r. h. of 8 to 10%. Samples were dried in this desiccation chamber until they reached stable moisture contents as determined by change in mass (161, 117, and 82 hr for Air-Drying Experiments 1, 2, and 3, respectively). Differences in the drying time required for coated-spore formulations to reach a stable moisture content

among the three experiments during low-humidity drying likely reflects different concentrations of hydroscopic coating materials and variability in ambient r.h. during the ambient-r.h. drying step among the experiments. The moisture content of samples was determined throughout the drying process to describe the rate of drying. For Air-Drying Experiment 1, moisture content was determined for subsamples (approximately 0.1 g) by change in mass after heating at 100 C for 24 hr. For Air-Drying Experiments 2 and 3, moisture content was determined for the initial spore suspension by measuring the change in mass after heating at 100 C for 24 hr. Water loss during drying was measured by re-weighing the entire sample to measure the decrease in total mass. The germination rate and viability of spores after the complete drying process was determined as described in Section 4.3.2. Comparison of percent germination among spores in the various suspension media was made using a non-parametric test (Wilcoxon two-sample test for non-paired ranked observations). Dry formulations were stored in air-tight containers with dried packets of silica gel at 4 C.

4.3.5. Survival of Coated Spores after Storage at 28 Cand after Sieving

Subsamples of the dry formulations were incubated at 28 C for 6 d in microcentrifuge tubes sealed in two zip-lock bags containing silica gel. Each formulation was replicated 3 times in Air-Drying Experiment 1 and 4 times in Air-Drying Experiments 2 and 3. After 6 d of incubation, the germination rate and viability of spores were determined as described in Section 4.3.2. Comparison of percent germination among spores in the various suspension media was made using a non-parametric test (Wilcoxon two-sample test for non-paired ranked observations).

Subsamples of the dry samples were ground against the surface of a microsieve (170 mesh) using a glass rod until the entire subsample passed through the sieve producing particles less than 80 µm. Each formulation was replicated 3 times in Air-Drying Experiment 1 and 4 times in Air-Drying Experiments 2 and 3. The viability of the sieved spores was determined as described in Section 4.3.2. Comparison of percent

germination among spores in the various suspension media was made using a non-parametric test (Wilcoxon two-sample test for non-paired ranked observations).

4.4. Results and Discussion

4.4.1. Air-Drying Experiment 1

Percent germination over time (12- and 24-hr) in seven spore-coating formulations before drying is compared in Figure 4-1. Percent germination was used to establish both percent germination at increasing incubation times and viability. The final germination time (24-hr) was considered to reflect the percentage of spores that were viable based on the assumption that all live spores would have germinated within this time interval. The shorter time interval (12-hr) was used to compare the rate of germination of the spores. These values were corrected for total viability to isolate the affect of germination rate from spore viability using the equation (corrected % germination = % germination at 12 hr ÷ proportion germination at 24 hr). The corrected percent germination values are presented in parentheses in each of the figures. Spores that were formulated in either a 10% glycerol solution only (formulation #7), or in the formulation containing Ultrazine® (formulation #5) had a lower 12-hr percent germination (lower-case letters) and viability (24-hr germination; capital letters) compared to all other formulations before drying ($\alpha = 0.05$). There was no difference in viability among any of the other formulations. The skim-milk based formulation with the with the highest sucrose concentration had the highest 12-hr percent germination of all formulations (formulation #1; lower-case letters, $\alpha = 0.05$). Germination prior to drying was used to determine if the formulations had direct inhibitory effects on germination or toxic effects. Ultrazine® appears to be slightly toxic to *M. anisopliae* var *acridum* submerged conidia.

The moisture contents of the seven spore-coating formulations from Air-Drying Experiment 1 after drying at ambient r.h. (23-43%) and low r.h. (7-10%) are presented in Table 4-4. Higher sucrose concentrations increased water retention at ambient and low

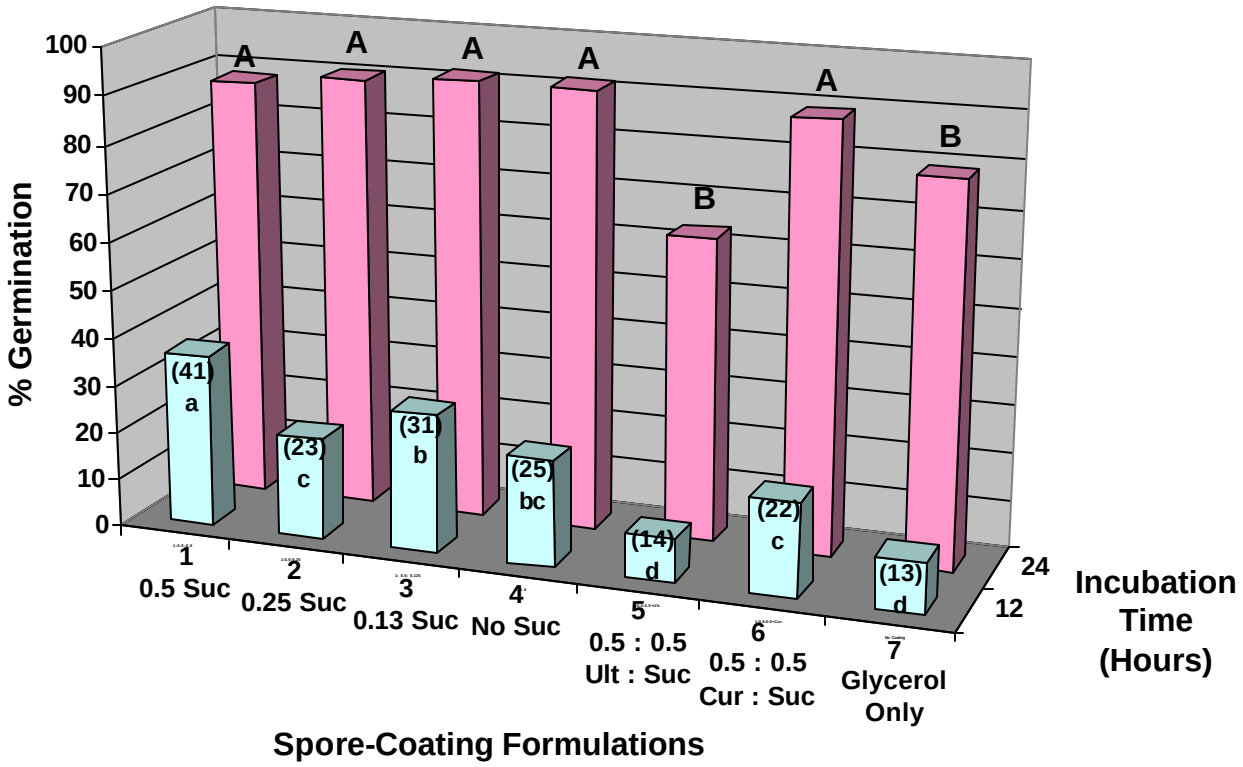

Figure 4-1. Air-Drying Experiment 1: Comparison of percent spore germination in seven spore-coating formulations before drying. Values on columns presented in parentheses represent 12-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 12 hr ÷ proportion germination at 24 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (12-hr corrected) and capital letters signify difference in viability (24-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are described further in Table 4-1 (Suc = sucrose; Ult = Ultrazine®; Cur = Curan 100®). Each formulation contained 50% skim milk (dry wt) and 10% glycerol (dry wt) relative to the dry mass of spores, with the exception of Test Group 7, which contained only 10% glycerol (dry wt.).

Table 4-4. Air-drying Experiment 1: Moisture Contents of Seven Spore-Coating Formulations after Drying at Ambient and Low Relative Humidity (R.H.)[1]

Spore-Coating Formulation[2] (Dry-Mass Ratio; Components)	% Moisture After Ambient R.H. Drying	% Moisture After Low R. H. Drying
1) 0.5 : 0.5; SM : Suc	22 ± 0.6 a[3], α[4]	15 ± 0.1 a, β
2) 0.5 : 0.25; SM : Suc	16 ± 2 bc, α	15 ± 1.2 a, α
3) 0.5 : 0.125; SM : Suc	14 ± 0.5 bc, α	12 ± 0.1 bc, β
4) 0.5 ; SM	12 ± 0.4 d, α	11 ± 1.3 c, α
5) 0.5 : 0.5 : 0.5; SM : Ult : Suc	18 ± 0.4 b, α	13 ± 0.5 b, β
6) 0.5 : 0.5 : 0.5; SM : Cur : Suc	17 ± 0.2 b, α	13 ± 0.2 b, β
7) Glycerol Only	13 ± 1 cd, α	N/A

[1] Ambient r.h. was 23-43% and low r.h. was maintained at 7-10% with silica gel.
[2] Formulations are presented as dry mass ratios relative to 1 part dry spores. These formulations are presented in greater detail in Table 4-1 (SM = skim milk; Suc = sucrose; Ult = Ultrazine®; Cur = Curan 100®).
[3] Values are presented as means ± standard deviations. Numbers followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).
[4] Numbers followed by different Greek letters are significantly different than values within the same row at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

r.h. (formulations #1 through #4; $\alpha = 0.05$; lower-case letters). The addition of lignin derivatives to the formulation reduced water retention at ambient and at low r.h (formulations #5 and #6 vs. #1; $\alpha = 0.05$; lower-case letters). The ability of drying at low r.h. to remove additional water was inconsistent among the different sugar levels (formulations #1 through #4; $\alpha = 0.05$; Greek letters). Additional water was removed from formulations from containing lignin derivatives by drying at low r.h. (formulations #5 and #6; $\alpha = 0.05$; Greek letters). The final moisture content of all formulations were higher than the 4.6% moisture content limit that was found to be optimal for improving thermal-stress tolerance of aerial conidia (Hong et al., 1998). However, if submerged conidia in these formulations behave similarly to aerial conidia, the formulations with lower moisture contents would be expected have greater thermal- stress tolerance than those with higher moisture contents (Hong et al., 1998). The moisture content of spores

dried in suspension media containing only glycerol (formulation #7) was not determined after silica gel drying because these spores were not viable after drying at ambient r.h.

Comparisons of percent spore germination over four time intervals for the seven formulations following air-drying at ambient and low r.h. is presented in Figure 4-2. There was a negative relationship between sugar concentration and 12- and 24-hr percent germination after drying, with the highest 12- and 24-hr percent germination corresponding to the lowest sugar concentrations (formulation #3; $\alpha = 0.05$; lower-case letters). The sugar concentration did not affect the viability of spores (60-hr germination) after drying when comparing among the skim milk formulations with lower sucrose levels (formulations #1 through #4; $\alpha = 0.05$; capital letters). However, the only significant loss of viability among the first four formulations from the drying process was found in the highest sugar formulation (#1) and in formulation containing no sugar (formulation # 4) ($\alpha = 0.05$, Greek letters X / Δ). The addition of Ultrazine® to the highest sugar formulation significantly reduced the viability of spores after drying relative to all other formulations (formulation #5) ($\alpha = 0.05$, capital letters). However, this may be the result of spore inactivation that occurred in the suspension media before drying since there was not significant loss in viability for formulation #5 during the drying process ($\alpha = 0.05$, Greek letters X / Δ). The addition of Curan 100® to the highest sugar formulation significantly increased 12-, 24, and 40 hr percent germination (lower-case letters) and viability (capital letters) after drying (formulation #6 vs. #1; $\alpha = 0.05$). Spores did not survive drying in a solution containing only 10% glycerol, with almost complete loss of viability after drying at ambient r.h. only (#7).

Comparison of spore viability in the dry spore-coating formulations prior to and after reducing the particle size (less than 80 μm) is presented in Figure 4-3. There was a negative relationship between the spore survival during sieving and the concentation of sugar (formulation #1 through #4), with the highest spore survival corresponding to the lowest sugar concentrations (formulations #3 and #4) ($\alpha = 0.05$). The higher sugar concentrations were also qualitatively more difficult to fracture on the surface of the sieve. One possible explanation is that the higher sugar concentrations may have increased hydrogen bonding in the formulation making the cell walls the weakest

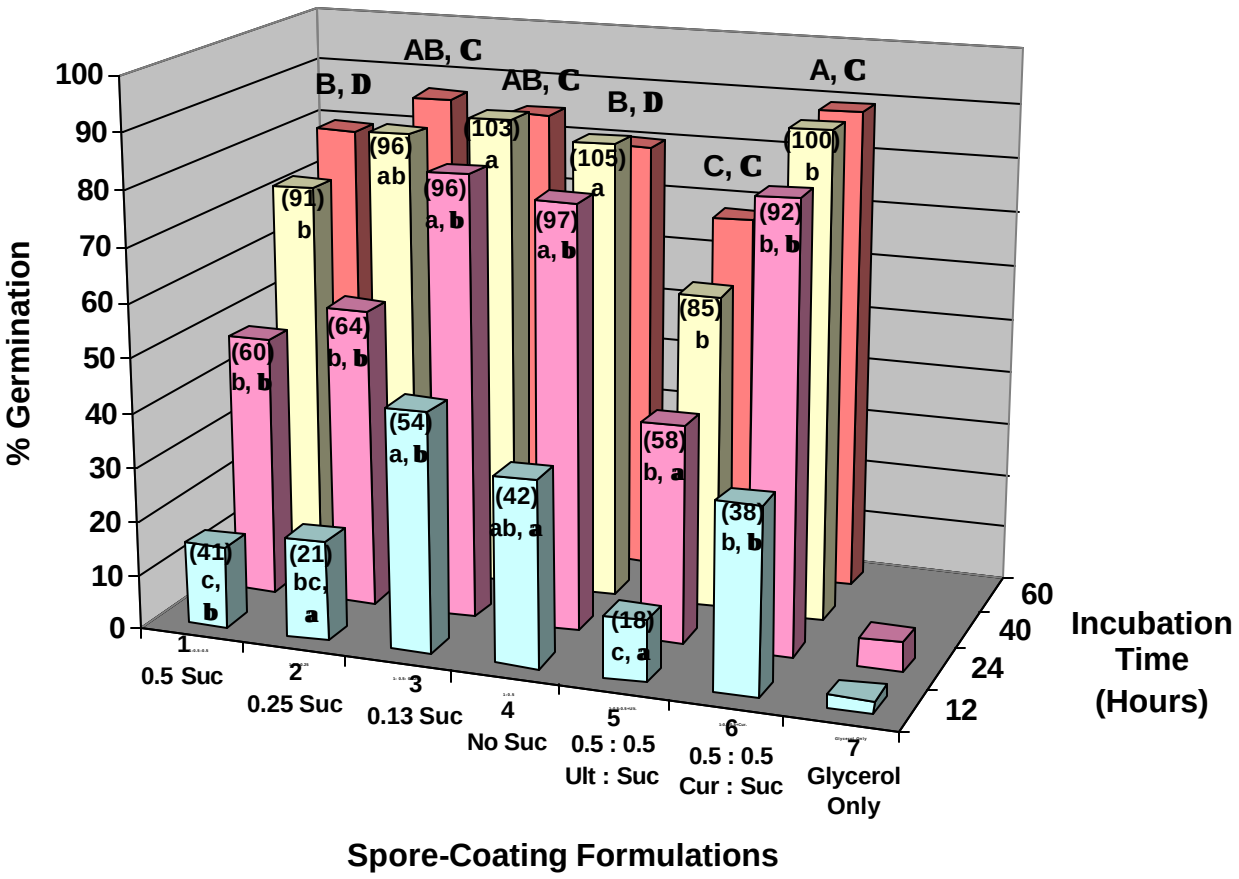

Figure 4-2. Air-drying Experiment 1: Comparison of percent spore germination in seven spore-coating formulations after drying. Values on columns presented in parentheses represent 12-, 24-, and 40-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 12, 24, and 40 hr ÷ proportion germination at 60 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (12-, 24-, and 40-hr corrected) and capital letters signify differences in viability (60-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are described further in Table 4-1 (Suc = sucrose; Ult = Ultrazine®; Cur = Curan 100®). Each formulation contained 50% skim milk (dry wt.) and 10% glycerol (dry wt.) relative to the dry mass of spores, with the exception of Test Group 5, which contained only 10% glycerol (dry wt.). *Germination in Test Group 7 was determined after drying at ambient r. h. only.

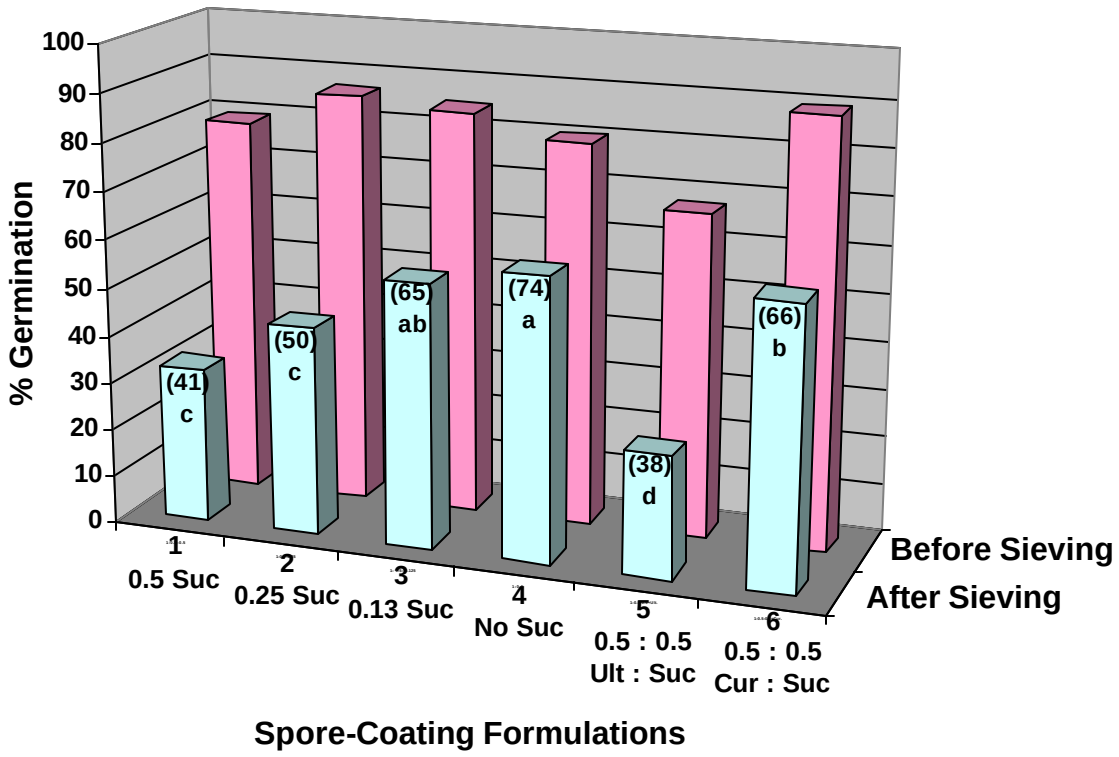

Figure 4-3. Air-drying Experiment 1: Comparison of spore viability (60-hr germination) in six spore-coating formulations prior to and after sieving. Lower-case letters signify differences among formulations for values corresponding to the percent of spores that survived sieving ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Values on columns presented in parentheses represent viability after sieving that was corrected for viability before sieving (corrected % germination = % germination after sieving ÷ proportion germination before sieving). Comparisons among values for viability after sieving were made using these corrected values. The effect of sieving on viability was significant for all of the formulations ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group is presented in Table 4-1 (Suc = sucrose; Ult = Ultrazine®; Cur = Curan 100®). Each formulation contained 50% skim milk (dry wt) and 10% glycerol (dry wt) relative to the dry mass of spores.

($\alpha = 0.05$); whereas, the addition of Curan 100® to the highest sugar concentration increased spore survival during sieving (formulation # 6 vs. #1) ($\alpha = 0.05$). The improvement in sieving survival observed for the spore-coating formulation containing Curan 100® may result from providing fracture points in the dry formulation matrix that are weaker than the spore cell walls.

Comparisons of percent spore germination over three time intervals for the six dry spore-coating formulations following storage at 28 C for 6 d is presented in Figure 4-4. The 24-hr percent germination for the two lowest sugar concentration (formulations #2 and #3), and the skim milk formulation containing no sugar (formulation #4) were lower than the 24-hr percent germination in the dry formulation before 28 C storage ($\alpha = 0.05$,Greek letters α / β). The only formulation that lost a significant amount of viability after 28 C storage was the formulation with the highest sugar concentration (formulation #1; $\alpha = 0.05$; Greek letters X / Δ). The 24-hr percent germination of the Curan 100® formulation (formulation #6) was significantly higher than all other formulations ($\alpha = 0.05$; lower-case letters) and showed a significant increase after 28 C storage as compared to the dry formulation before 28 C storage ($\alpha = 0.05$; Greek letters α / β). The results of Air-Drying Experiment 1 indicate that the lowest sugar concentration is the best concentration for desiccation tolerance and spore survival during sieving for formulated submerged conidia with skim milk coatings. However, percent germination over time and spore survival following drying, sieving, and 28 C storage were not significantly improved by the addition of sucrose to the skim milk based formulation. Ultrazine® reduced the viability of submerged conidia when it was added to suspension media before drying and also reduced survival during sieving. The negative effect of Ultrazine® on spores was likely a direct toxic effect since these spores were inactivated in the suspension media. However, the addition of Curan 100® to the formulation did not reduce the viability spores in suspension media, increased spore survival during drying and sieving, and increased the germination rate of formulated spores after 28 C storage. The beneficial effects of Curan 100®, however, may not necessarily be attributed to direct physical stabilization of the spores. Since Curan 100® enhanced the water removal from the spore-coating formulation, the beneficial effects may have been due to an indirect

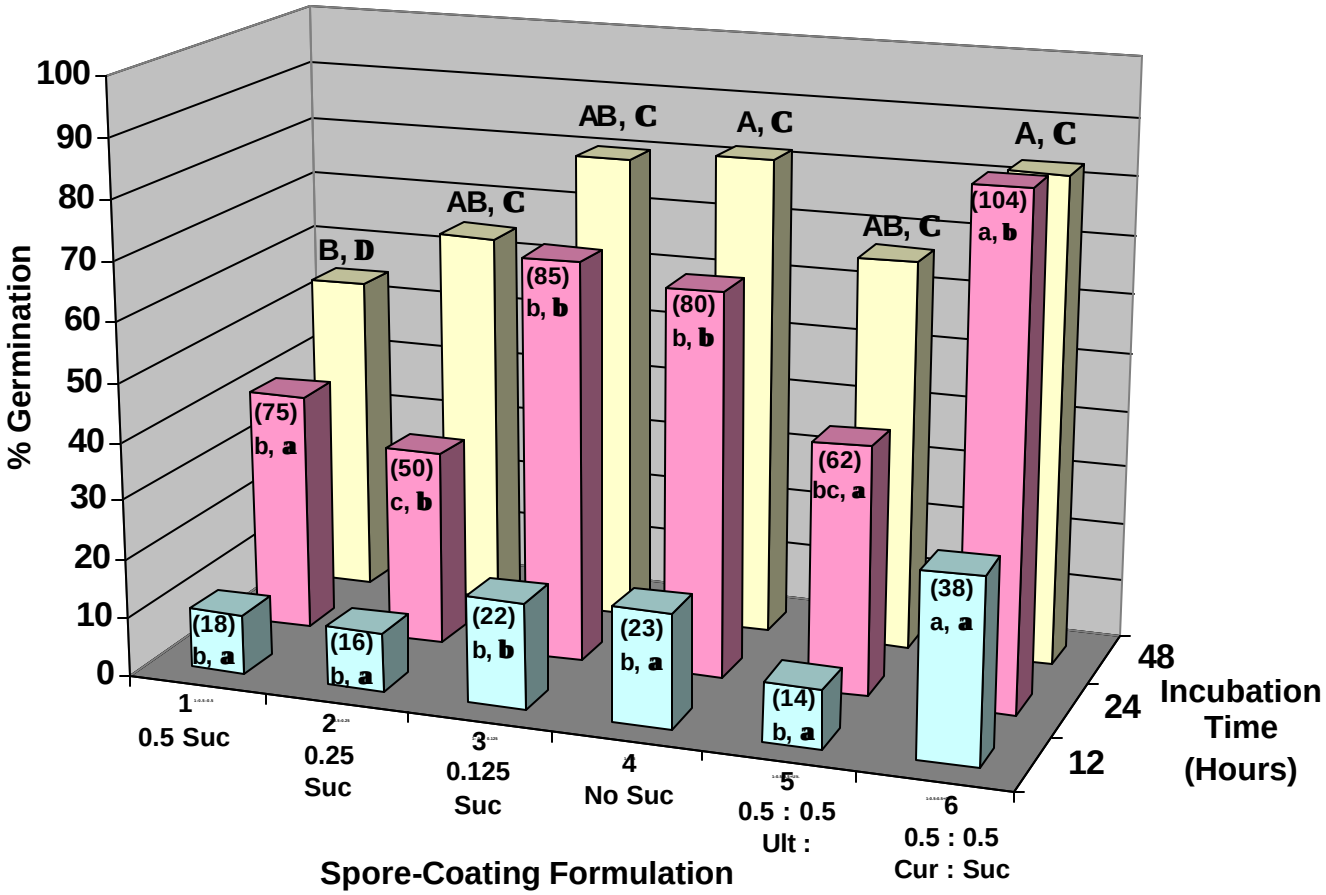

Figure 4-4. Air-drying Experiment 1: Comparison of percent spore germination in six dry spore-coating formulations after storage at 28 C for 6 days. Values on columns presented in parentheses represent 12-, 24-, and 40-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 12, 24, and 40 hr ÷ proportion germination at 60 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (12-, 24-, and 40-hr corrected) and capital letters signify differences in viability (60-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are further described in Table 4-1 (Suc = Sucrose; Ult = Ultrazine®; Cur = Curan 100®). Each formulation contained 50% skim milk (dry wt) and 10% glycerol (dry wt) relative to the dry mass of spores.

4.4.2. Air-Drying Experiment 2

Initial viability of spores in this experiment was low (approximately 65%). The viability of spores from the 1-L fermentation system was typically greater than 95%. The source of this loss in viability has not been determined. These data were used to focus the selection of spore-coating formulations for Air-drying Experiment 3. Formulated spores were not used for further experimentation on UV-tolerance and virulence due to this low viability. Comparison of percent germination over four time intervals in eight spore-coating formulations before drying is presented in Figure 4-5. Spores that were not suspended in any spore-coating formulation had a significantly higher 12-hr percent germination than spores suspended in all of the spore-coating formulations, and there was no difference in 12-hr percent germination among all of the spore coating formulations (α = 0.05; lower-case letters). Viability (36-hr germination) was highest in the skim milk : high glycerol formulation (#4) and in the skim milk : sucrose : glycerol formulations (#6 and #8) but the differences in viability were generally low (less than 10%) among all of spore-coating formulations.

The moisture contents of the eight spore-coating formulations from Air-Drying Experiment 2 after drying at ambient r.h. (23-43%) and low r.h. (7-10%) are presented in Table 4-5. The presence of skim milk, glycerol, and sucrose as components in the spore-coating formulations reduced water removal after drying both at ambient and low r. h. This is not surprising given the hygroscopic nature of each of these formulation components. As noted in the results of Air-Drying Experiment 1, increasing moisture contents between 4.6 and 21% has been shown to decrease the thermal stress tolerance (50 C) of *M. anisopliae* var. *acridum* aerial conidia (Hong et al., 1998).

Comparison of percent germination over four time intervals in eight spore-coating formulations after drying at ambient followed by low r.h. is presented in Figure 4-6. The concentration of glycerol did not have a significant effect on 7-, 12- or 27-hr percent germination (lower-case letters) or spore viability (capital letters) after drying in combination with Curan 100® (formulation #1 and #3; α = 0.05). However, the higher

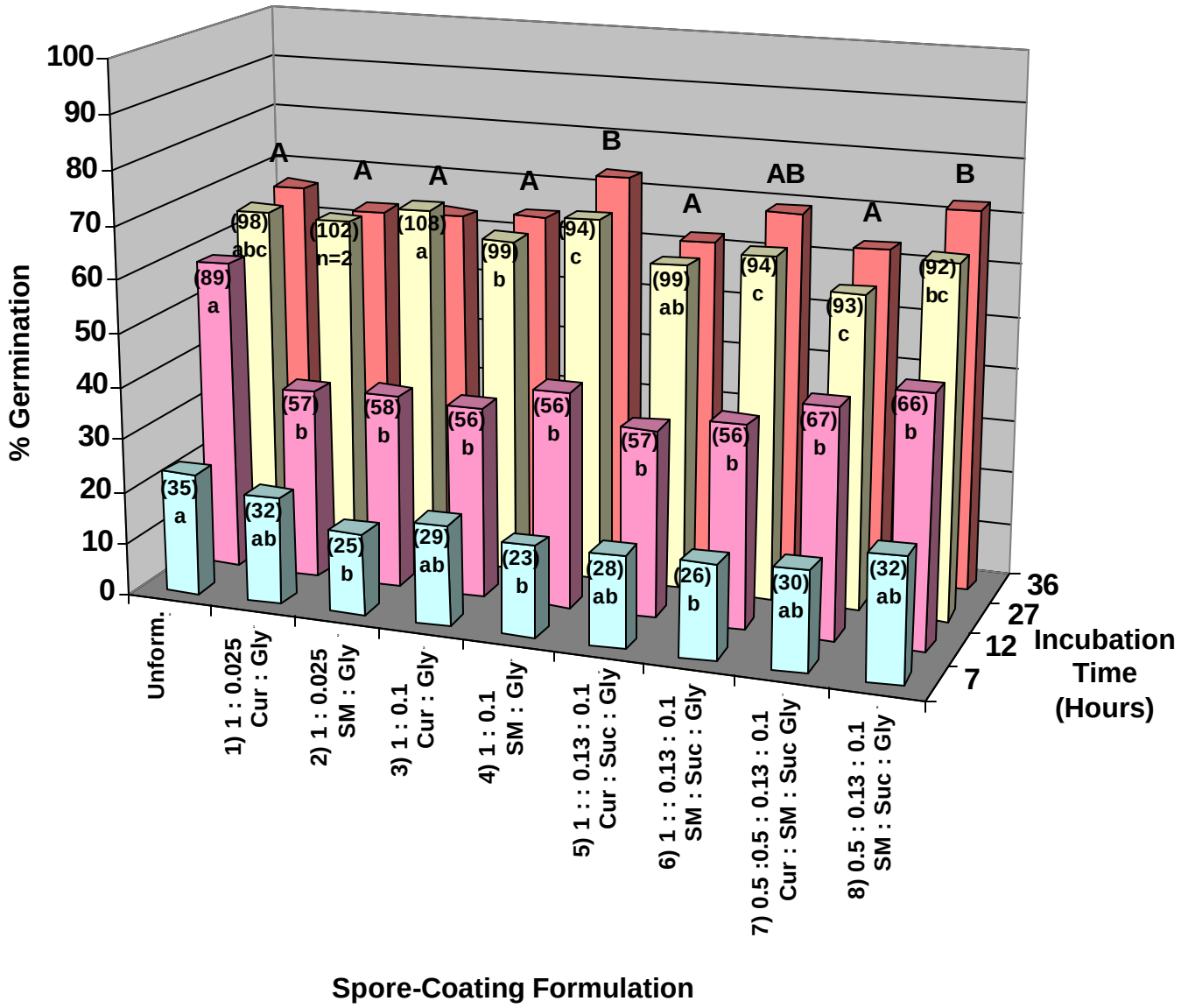

Figure 4-5. Air-Drying Experiment 2: Comparison of percent spore germination in eight spore-coating formulations before drying. Values on columns presented in parentheses represent 7-, 12-, or 27-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 7-,12-, or 27-hr ÷ proportion germination at 36 hr). Lower-case letters signify differences in germination rate (7-,12, and 27-hr corrected) and capital letters signify difference in viability (36-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are further described in Table 4-2 (SM = Skim Milk; Cur = Curan 100®; Suc = Sucrose; Gly = glycerol).

Table 4-5. Air-drying Experiment 2: Moisture Contents of Eight Spore-Coating Formulations after Drying at Ambient and Low Relative Humidity (R.H.)[1]

Spore-Coating Formulation[2] (Dry-Mass Ratio; Components)	% Moisture After Ambient R.H. Drying	% Moisture After Low R. H. Drying
1) 1 : 0.025; Cur : Gly	6 ± 0.9 a[3], α[4]	4 ± 0.9 a, α
2) 1 : 0.025; SM : Gly	17 ± 0.5 b, α	15 ± 0.5 b, β
3) 1 : 0.1; Cur : Gly	7 ± 0.4 c, α	6 ± 0.4 c, β
4) 1 : 0.1; SM : Gly	20 ± 0.5 d, α	18 ± 0.5 d, β
5) 1 : 0.13 : 0.1; Cur : Suc :Gly	11 ± 0.6 e, α	9 ± 0.5 e, β
6) 1 : 0.13 : 0.1; SM : Suc :Gly	21 ± 0.4 f, α	20 ± 0.3 d, β
7) 0.5 : 0.5: 0.13 : 0.1; Cur : SM : Suc : Gly	15 ± 0.9 g, α	13 ± 1 f, α
8) 0.5 : 0.13 : 0.1; SM : Suc :Gly	20 ± 0.7 df, α	18 ± 0.9 d, β

[1] Ambient r.h. was 25-36% and low r.h. was maintained at 7-10% with silica gel.
[2] Formulations presented as dry mass ratios relative to 1 part dry spores. These formulations are presented in greater detail in Table 4-2 (SM = skim milk; Suc = sucrose; Cur = Curan 100®: Gly = glycerol).
[3] Values are presented as means ± standard deviations. Numbers followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).
[4] Numbers followed by different Greek letters are significantly different than values within the same row at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

level of glycerol increased the viability after drying (capital letters), but not the 7-, 12- or 27-hr percent germinations (lower-case letters) in combination with skim milk (formulation #2 and #4; $\alpha = 0.05$). The addition sucrose did not have a significant effect on 7-, 12- or 27-hr percent germinations (lower case letters) or spore viability (capital letters) after drying in combination with skim milk (formulation #4 and #6; $\alpha = 0.05$). However, the addition of sucrose increased the viability after drying (capital letters), but not the 7-, 12- or 27-hr percent germinations (lower-case letters) in combination with Curan 100® (formulation #3 and #5; $\alpha = 0.05$). Replacement of half of the skim milk in formulation #6 with Curan 100® (formulation #7) resulted in an increase in the 12-hr percent germination, but not the 7- or 27-hr ($\alpha = 0.05$, lower-case letters). Spores formulated with Curan 100® generally had higher percent germination at 12-hr than spores from formulated with skim milk after (significant for formulations #1 vs. #2 and

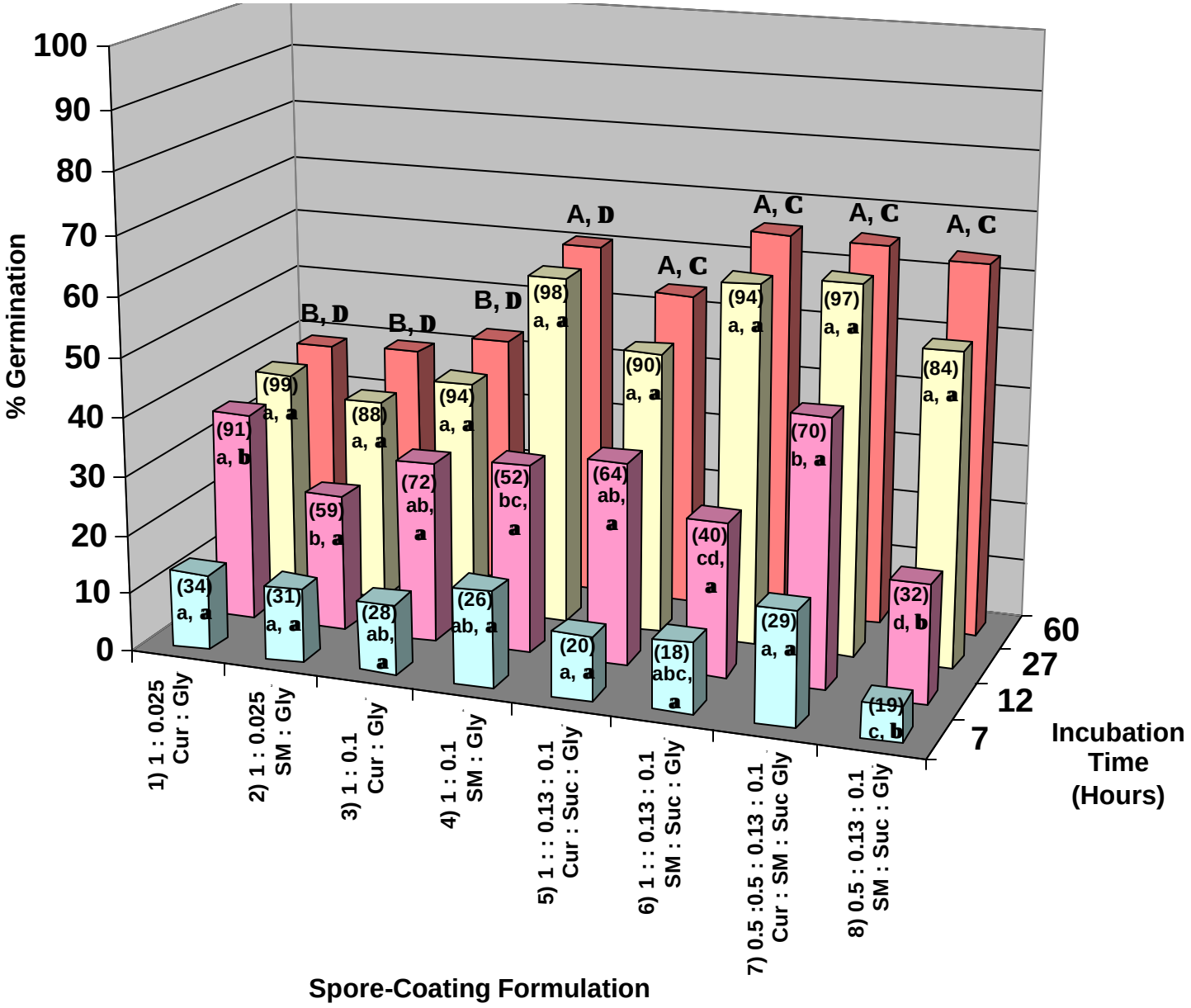

Figure 4-6. Air-drying Experiment 2: Comparison of percent spore germination in eight spore-coating formulations after drying. Values on columns presented in parentheses represent 7-, 12-, and 27-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 7, 12, and 27 hr ÷ proportion germination at 60 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (7-, 12-, and 27-hr corrected) and capital letters signify differences in viability (60-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are further described in Table 4-2 (SM = skim milk; Cur = Curan 100®; Suc = sucrose; Gly = glycerol).

#5 vs. #6 but not #3 vs. #4) ($\alpha = 0.05$, lower-case letters). The only formulations to show no significant loss in viability during the drying process contained sucrose (formulation #5 through #8) ($\alpha = 0.05$, Greek letters X / Δ). This effect cannot be attributed soley to the final moisture content of the formulation since two of the formulations that had significant viability loss also had high final moisture contents (15% and 18%).

Comparison of spore viability in the eight dry spore-coating formulations from Air-drying Experiment 2 prior to, and after reducing the particle size (less than 80 µm) is presented in Figure 4-7. The concentration of glycerol in the formulation did not have an effect on spore survival during sieving (formulation #1 vs. #3 and #2 vs. #4; $\alpha = 0.05$). The addition of sugar reduced spore survival during sieving in combination with skim milk (formulation #4 vs. #6) but not in combination with Curan 100® (formulation #s 3 vs. #5) ($\alpha = 0.05$). The lower concentration of skim milk did not affect spore survival during sieving (formulation #6 vs. #8; $\alpha = 0.05$). Spores formulated in a combination of Curan 100® and skim milk demonstrated the greatest potential to survive sieving (77%) relative to all other formulations (formulation #7; $\alpha = 0.05$).

Comparisons of percent spore germination over three time intervals for the eight dry spore-coating formulations following storage at 28 C for 6 d is presented in Figure 4-8. Spores formulated with skim milk and no sucrose (formulations #2 and #4) were the only formulations that lost significant spore viability during storage at 28 C ,based on comparing to viability after drying (Figure 4-6) ($\alpha = 0.05$, Greek letters X / Δ). Storage for 6 d at 28 C also had little effect on germination over time with the only significant reduction in percent germination being for 12-hr percent germination spores formulated with a mixture of Curan 100® and skim milk (formulation #7; $\alpha = 0.05$, Greek letters α / β).

Results from Air-Drying Experiment 2 indicated that the highest spore survival during air-drying was in formulations that contained sucrose (formulation #5 through #8) and in the skim milk formulation with the higher glycerol concentration (formulation #4). There was no significant difference among these formulations in spore viability after drying (Figure 4-6; capital letters; $\alpha = 0.05$). The 12-hr percent germination values were generally higher in Curan 100®-based formulations than skim milk-based after drying,

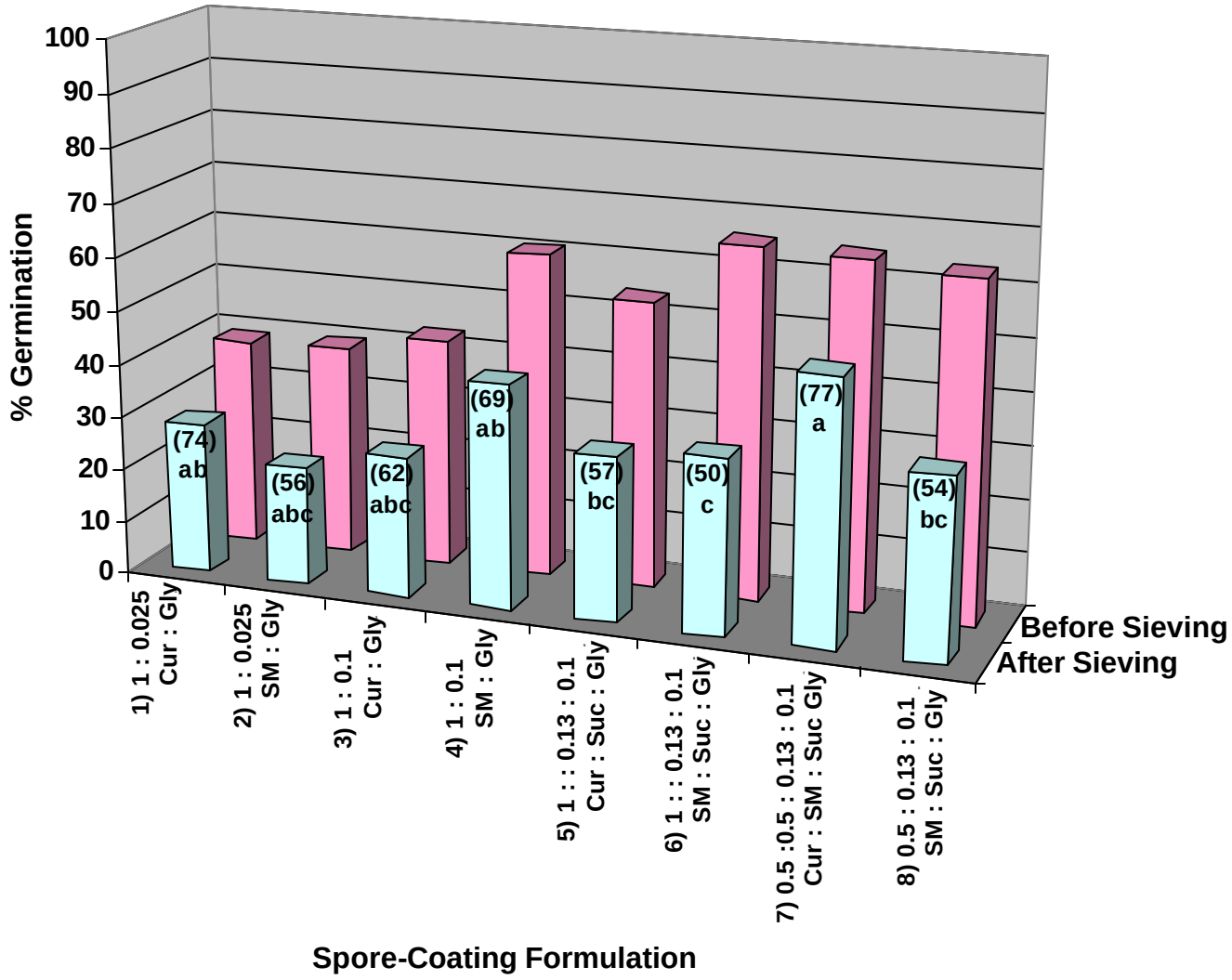

Figure 4-7. Air-drying Experiment 2: Comparison of spore viability (60-hr germination) in eight spore-coating formulations prior to and after sieving. Lower-case letters signify differences among formulations for values corresponding to the percent of spores that survived sieving ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Values on columns presented in parentheses represent viability after sieving that was corrected for viability before sieving (corrected % germination = % germination after sieving ÷ proportion germination before sieving). Comparisons among values for viability after sieving were made using these corrected values. The effect of sieving on viability was significant for all of the formulations ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are further described in Table 4-2 (SM = skim milk; Suc = sucrose; Cur = Curan 100®; Gly = glycerol).

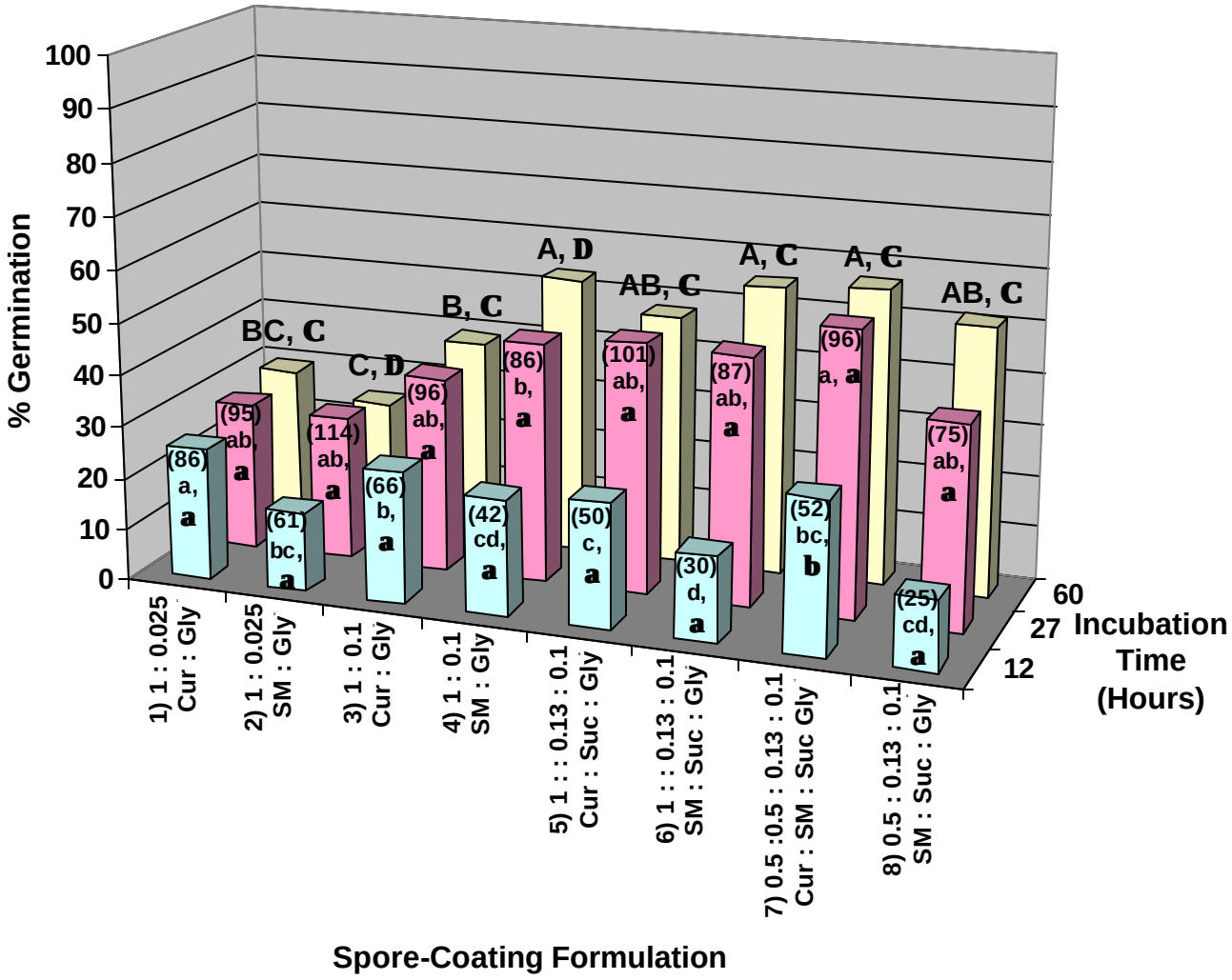

Figure 4-8. Air-drying Experiment 2: Comparison of percent spore germination in seven dry spore-coating formulations after storage at 28 C for 6 days. Values on columns presented in parentheses represent 12- and 27-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 12 and 27 hr ÷ proportion germination at 60 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (12- and 27-hr corrected) and capital letters signify differences in viability (60-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). The formulations corresponding to each test group are further described in Table 4-2 (SM = Skim Milk; Cur = Curan 100®; Suc = Sucrose; Gly = glycerol).

but the 6- and 27-hr percent germination were generally not different. Among the formulations that contained sucrose, spores in formulation #7, containing a mixture of Curan 100® and skim milk, had the greatest survival during sieving. As mentioned in Air-drying Experiment 1, it may be that the combination of Curan 100® with skim milk provides fracture points in the formulation that are weaker than spore cell walls. The only formulations that lost significant viability during storage for 6 d at 28 C were those containing skim milk and no sucrose. Storage at 28 C generally did not significantly reduce percent germination over time. The results from Air-drying Experiment 2 indicated that skim milk-coated spores could not serve as appropriate controls for lignin-coated spores. Thus experiment s using skim milk-coated spores could not serve as appropriate controls for lignin-coated spores for UV-tolerance experiments and bioassays described in Chapter 5. Spores survival during sieving is low in skim milk formulations, and they have slower germination rates. Therefore, in Air-Drying Experiment 3, aerial conidia were included in the drying experiment so that non-coated aerial conidia could be compared to lignin-coated aerial conidia in UV-tolerance experiments and bioassays described in Chapter 5.

4.4.3. Air-Drying Experiment 3

Percent spore germination over time (6, 13, 24 and 42 hr) for five spore-coating formulations of submerged conidia and aerial conidia before drying are presented in Figures 4-9 and 4-10, respectively. Although there were some significant differences in 6-hr percent germination values among the spore-coating formulations (formulation #1 through #5) of submerged conidia, spore coating formulation did not have a great effect on 6-, 13-, or 24-hr percent germination resulting in differences less than 10% (Figure 4-9; lower-case letters; $\alpha = 0.05$). Aerial conidia suspended in a mixture of Curan 100®, skim milk, sucrose, and glycerol (formulation #5) had the highest 24-hr percent germination (88%) and unformulated aerial conidia had the slowest 24-hr percent germination rates (67%) (Figure 4-10; lower-case letters; $\alpha = 0.05$). The 6- and 13-hr

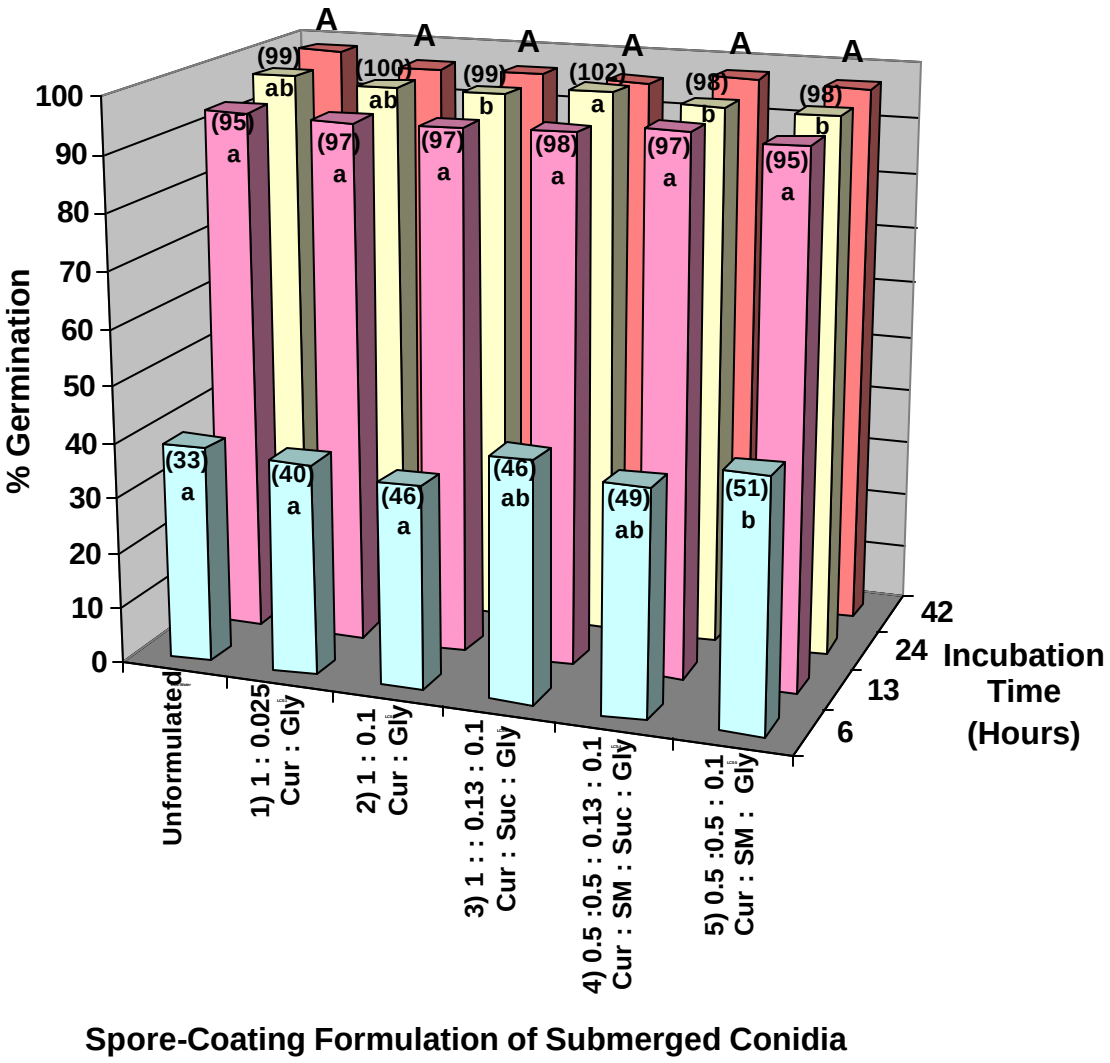

Figure 4-9. Air-Drying Experiment 3: Percent of submerged conidia germinating in five spore-coating formulations before drying. Values in parentheses on columns represent 6-, 13-, or 24-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 6-,13-, or 24-hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-,13-, and 24-hr corrected) and capitol letters signify difference in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is further described in Table 4-3 (SM = skim milk; Suc = sucrose; Cur = Curan 100®; Gly = glycerol).

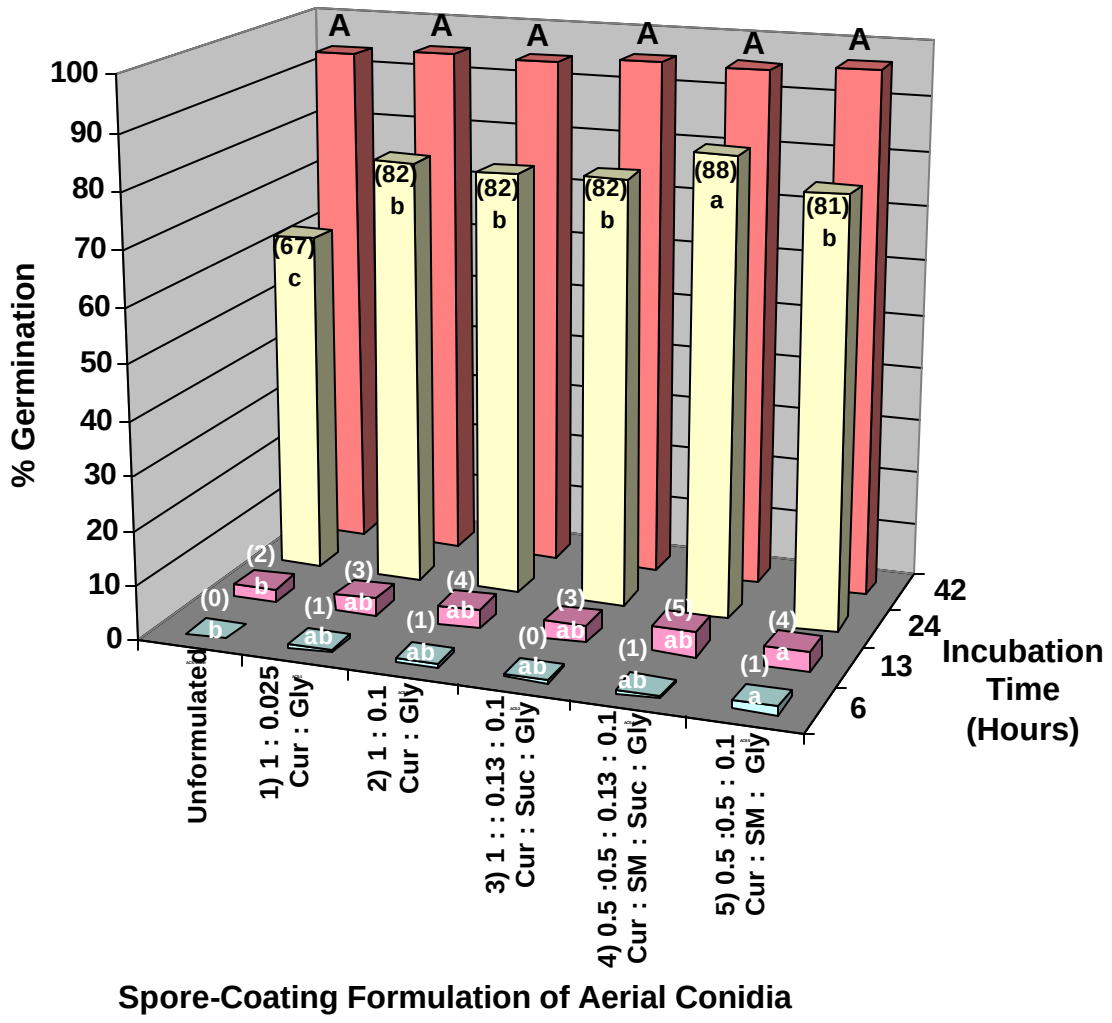

Figure 4-10. Air-Drying Experiment 3: Percent of aerial conidia germinating in five spore-coating formulations before drying. Values in parentheses on columns represent 6-, 13-, or 24-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 6-,13-, or 24-hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-,13-, and 24-hr corrected) and capitol letters signify difference in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is further described in Table 4-3 (SM = skim milk; Suc = sucrose; Cur = Curan 100®; Gly = glycerol).

percent germination was very low for unformulated and all spore-coating formulations of aerial conidia. There was no effect of formulation on viability (42-hr germination) of either submerged conidia (Figure 4-9) or aerial conidia (Figure 4-10) (capital letters; α = 0.05).

The moisture content of five spore-coating formulations of submerged conidia and aerial conidia after drying at ambient and low (7-10%) r.h. are presented in Table 4-6. A higher concentration of glycerol in the formulation increased water retention in the

Table 4-6. Air-Drying Experiment 3: Moisture Content of Five Spore-Coating Formulations for Submerged Conidia and Aerial Conidia after Drying at Ambient and Low Relative Humidity (R.H.) [1]

Formulation[2]		% Moisture After Ambient Drying	% Moisture After Silica Drying
Submerged Conidia Formulations			
1) 1 : 0.025;	Cur : Gly	7.0 ± 3.0 a[3], α[4]	2.0 ± 3.2 a, α
2) 1 : 0.1;	Cur : Gly	11.4 ± 2.1 bc, α	7.9 ± 4.0 ab, α
3) 1 : 0.13 : 0.1;	Cur : Suc : Gly	11.5 ± 2.0 b, α	7.3 ± 2.1 a, α
4) 0.5 : 0.5 : 0.13 : 0.1;	Cur : SM : Suc : Gly	16.3 ± 2.1 c, α	12.9 ± 2.2 b, α
5) 0.5 : 0.5: 0.1;	Cur : SM : Gly	15.8 ± 1.9 c, α	12.4 ± 1.9 b, α
Aerial Conidia Formulations			
1) 1 : 0.025;	Cur : Gly	13.2 ± 0.3 a, α	7.9 ± 0.2 a, β
2) 1 : 0.1;	Cur : Gly	12.8 ± 0.4 a, α	7.5 ± 0.3 a, β
3) 1 : 0.13 : 0.1;	Cur : Suc :Gly	14.7 ± 0.6 b, α	9.6 ± 0.5 a, β
4) 0.5 : 0.5 : 0.13 : 0.1;	Cur : SM : Suc : Gly	17.9 ± 0.2 c, α	13.0 ± 0.3 c, β
5) 0.5 : 0.5: 0.1;	Cur : SM : Gly	18.6 ± 0.2 d, α	13.9 ± 0.2 d, β

[1] Ambient r.h. was 29-40% and low r.h. was maintained at 7-10% with silica gel.
[2] Formulations presented as dry mass ratios relative to 1 part dry spores. These formulations are presented in greater detail in Table 4-3 (SM = skim milk; Suc = sucrose; Cur = Curan 100®: Gly = glycerol).
[3] Values presented as means ± standard deviations. Numbers followed by different lower-case letters are significantly different than values for the same spore type within the same column at the α = 0.05 level (Wilcoxon two-sample test for non-paired ranked observations).
[4] Values followed by different Greek letters are significantly different than values within the same row at the α = 0.05 level (Wilcoxon two-sample test for non-paired ranked observations).

submerged conidia formulation during ambient r.h. drying (formulation #1 vs. #2), but did not effect ambient- or low-r.h. drying of aerial conidia formulations ($\alpha = 0.05$, lower-case letters). The replacement of one-half of the Curan 100® with skim milk in the formulation increased water retention after both ambient- and low-r.h. drying for both submerged- and aerial-conidia formulations (formulation #3 vs. #4) ($\alpha = 0.05$, lower-case letters). Although, the moisture contents of aerial conidia formulations with sucrose was statistically higher than corresponding formulations without sucrose after drying at ambient r.h. (formulation #2 vs. #3 and #4 vs. #5; $\alpha = 0.05$, lower-case letters), the difference in percent moisture content was only approximately 1 to 2%. Additional drying at low r.h. did not have a significant effect on the final moisture contents of any of the submerged conidia formulations; whereas it significantly reduced moisture contents of all aerial conidia formulations ($\alpha = 0.05$; Greek letters). This may be partially explained by the high variability of submerged conidia formulation moisture.

Percent spore germination over time (6, 13, 24, 42 hr) for five spore-coating formulations of submerged conidia and aerial conidia after drying are presented in Figures 4-11 and 4-12, respectively. Differences in percent germination over time and viability were generally greater among submerged conidia (Figure 4-11) than aerial conidia (Figure 4-12) in the various spore-coating formulations. The concentration of glycerol in formulation of submerged conidia (Figure 4-11; formulation #1 vs. #2) did not significantly effect 6-, 13-, or 24-hr percent germination (lower-case letters) or viability (42-hr germination, capital letters) after drying ($\alpha = 0.05$). The addition of sucrose to Curan 100®-based formulations of submerged conidia (Figure 4-11; formulation #3 vs. #2) improved spore viability (42-hr germination; capital letters; $\alpha = 0.05$) but did not effect the 6-, 13-, or 24-hr percent germination (lower-case letters; $\alpha = 0.05$) after drying. Replacement of one-half of the Curan 100 with skim milk in submerged conidia formulations (Figure 4-11; formulation #3 vs #4) reduced spore viability after drying (42-hr germination; capital letters; $\alpha = 0.05$), but did not effect the 6-, 13-, or 24-hr percent germination (lower- case letters; $\alpha = 0.05$). The 6-hr percent germination values were significantly reduced by the drying process for all submerged conidia in all formulations other than formulation #5, which contained Curan 100®, skim milk, and glycerol (Figure

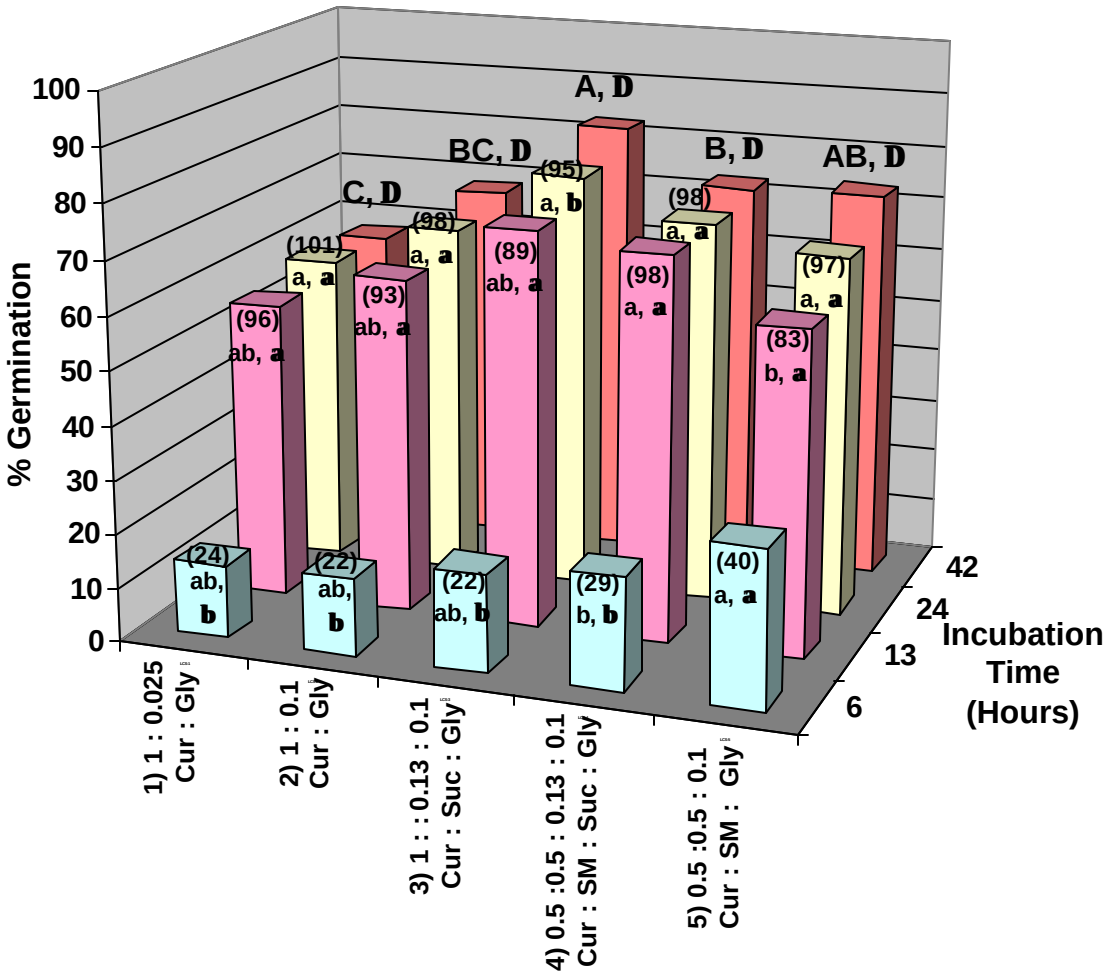

Figure 4-11. Air-drying Experiment 3: Percent of submerged conidia germinating in five spore-coating formulations after drying. Values in parentheses on columns represent 6-, 13-, and 24-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 6, 13, and 24 hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-, 13- and 24-hr corrected) and capital letters signify differences in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups are presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is presented in Table 4-3 (SM = skim milk; Cur = Curan 100®; Suc = sucrose; Gly = glycerol).

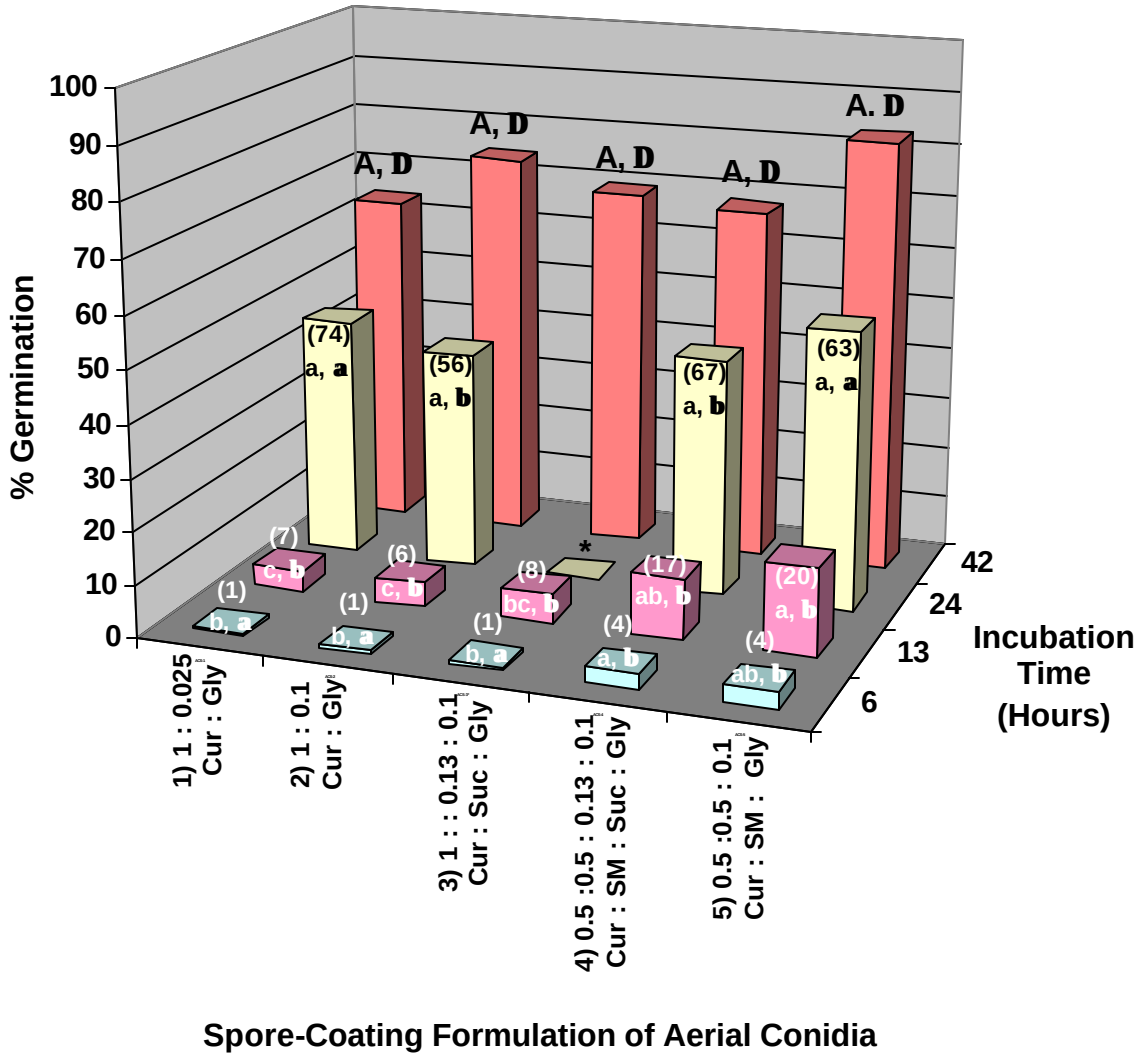

Figure 4-12. Air-drying Experiment 3: Percent of aerial conidia germinating in five spore-coating formulations after drying. Values in parentheses on columns represent 6-, 13-, and 24-hr percent germinations that were corrected for total viability (corrected % germination = % germination at 6, 13, and 24 hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-, 13- and 24-hr corrected) and capital letters signify differences in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups are presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is presented in Table 4-3 (SM = skim milk; Cur = Curan 100®; Suc = sucrose; Gly = glycerol). *missing data for formulation # 3.

4-11; Greek letters α / β; $\alpha = 0.05$). The drying process resulted in a significant decline in viability (42-hr germination) for submerged conidia in all formulations (Figure 4-11, Greek letters X / Δ; $\alpha = 0.05$).

There were not as many differences in germination over time for aerial conidia as there were for submerged conidia in the various spore-coating formulations. The two aerial-conidia formulations containing a combination of Curan 100® and skim milk (Figure 4-12; formulations #4 and #5) had the highest 6- and 13-hr percent germination (lower-case letters; significant for #5 and 13 hr and #4 at 6 hr; $\alpha = 0.05$). Other than this comparison, there was no difference in germination over time for aerial conidia in any of the spore-coating formulations after drying (Figure 4-12; lower-case letters; $\alpha = 0.05$). The drying process resulted in a significant decline in viability for aerial conidia in all formulations (Figure 4-12, Greek letters X / Δ; $\alpha = 0.05$).

Submerged conidia from formulations #3 (Curan 100®, sucrose and glycerol) and #5 (Curan 100®, skim milk, and glycerol) had the best characteristics after drying on the basis of spore viability and germination over time. Aerial conidia in formulation #5 had higher 13-hr germination than aerial conidia in formulation #3. Therefore, on the basis of spore characteristics (gemination over time and viability) after drying, formulation #5 appears to be the most promising formulation. However, differences in spore survival after sieving and effects on storage at 28 C on germination over time and spore viability will also need to be considered when selecting a final formulation for further development.

The viability of submerged conidia in the dry formulations from Air-Drying Experiment 3 before and after sieving (less than 80 μm) is presented in Figure 4-13. Evaluation of aerial conidia sieving survival among the various spore-coating formulations was not determined because there was a limited supply of material available for formulated aerial conidia. In addition, it was assumed that submerged conidia would be the more sensitive of the two spore types to physical damage during sieving. Glycerol concentration did not affect the submerged conidia survival during sieving (formulation #1 vs #2; $\alpha = 0.05$). The addition of sugar in combination with Curan 100® improved the submerged conidia

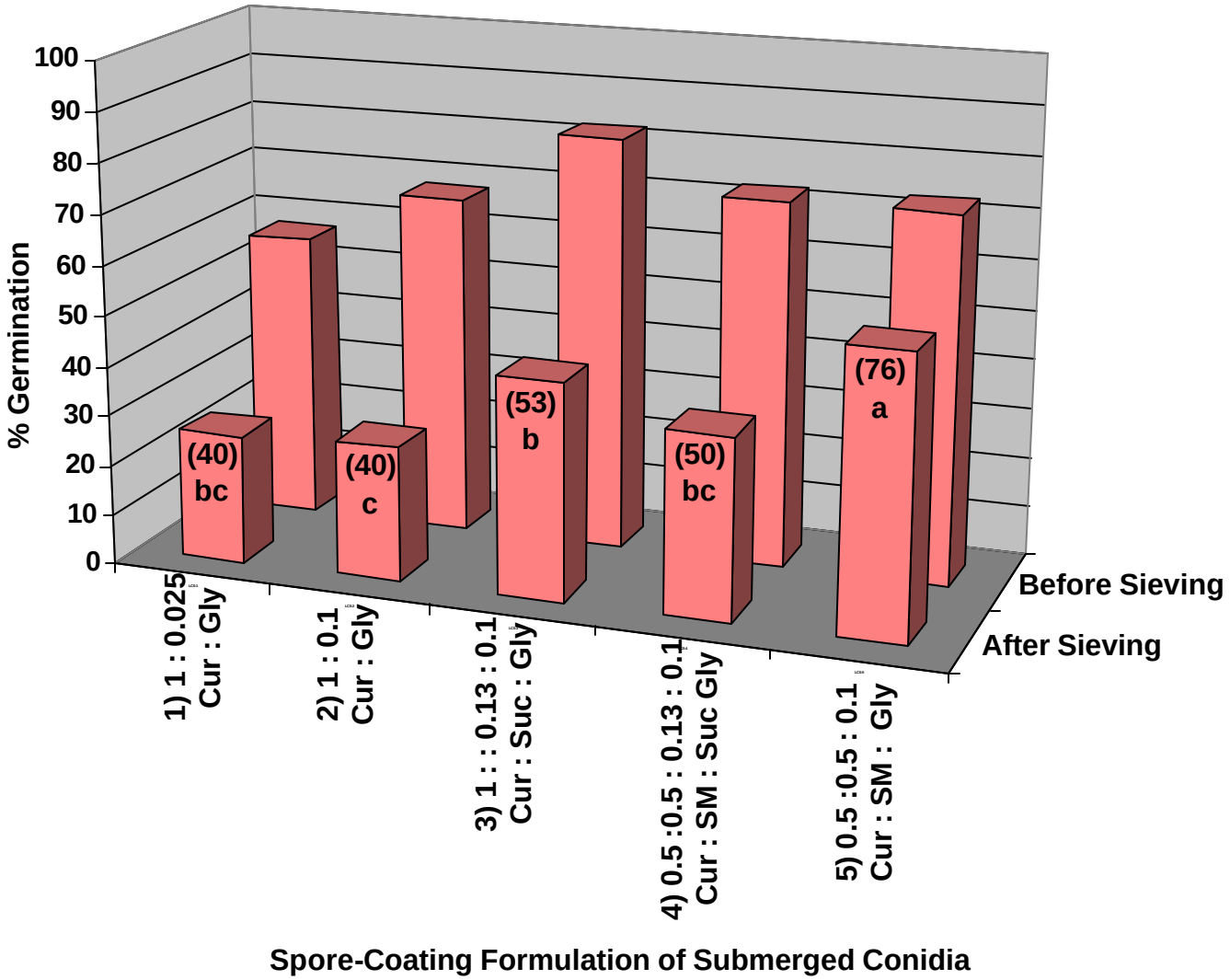

Figure 4-13. Air-drying Experiment 3: Comparison of submerged conidia viability (42-hr germination) in five spore-coating formulations prior to and after sieving. Lower-case letters signify differences among formulations for values corresponding to the percent of spores that survived sieving ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Values on columns presented in parentheses represent viability after sieving that was corrected for viability before sieving (corrected % germination = % germination after sieving ÷ proportion germination before sieving). Comparisons among values for viability after sieving were made using these corrected values. The effect of sieving on viability was significant for all of the formulations ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups are presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group further described in Table 4-3 (SM = Skim Milk; Cur = Curan 100®; Suc = Sucrose; Gly = glycerol).

survival during sieving (formulation #2 vs #3; $\alpha = 0.05$). Replacement of half of the Curan 100® with skim milk in sucrose-containing formulations did not significantly affect the ability of submerged conidia to survive sieving (formulation #3 vs #4; $\alpha = 0.05$). Spores in the formulation containing a combination of Curan 100® and skim milk without sucrose (formulation #5), had the best survival during sieving of all formulations ($\alpha = 0.05$). On the basis of submerged conidia survival during sieving, formulation # 5 (Curan 100®, sucrose, and glycerol) had the best characteristics, which was also one of the two formulations (#5 and #3) with the best germination over time and viability after drying.

The percent germination over time (13, 24, and 42 hr) of submerged conidia and aerial conidia in dry formulations following storage for 6 d at 28 C are presented in Figures 4-14 and 4-15, respectively. The only formulation of submerged conidia that lost significant viability during storage at 28 C as compared to viability after drying (Figure 4-12) was the formulation containing sucrose in combination with Curan 100® (Figure 4-14; formulation #3; Greek letters Δ / X, $\alpha = 0.05$), and none of the aerial conidia formulations lost significant viability during storage at 28 C (Figure 4-15; Greek letters X / Δ; $\alpha = 0.05$). Storage at 28 C generally decreased the germination rate of submerged conidia at 13- and 24-hr incubation (Figure 4-14; Greek letters α / β; $\alpha = 0.05$); this effect was not significant for formulations #1 and #5 at 24-hr). Storage at 28 C decreased the 13-hr percent germination for aerial conidia in all spore coating formulations (Figure 4-15; Greek letters α / β; $\alpha = 0.05$). However, storage at 28 C generally increased the 24-hr percent germination aerial conidia in spore-coating formulations (Figure 4-15; formulations #1, #2, and #5; Greek letters α / β), but the effect was not significant for formulation #4 and couldn't be determined for formulation #3 because of missing data in Figure 4-12 on the dry formulation before 28 C storage ($\alpha = 0.05$; Greek letters α / β). Because of the decrease in germination rate that occurs in submerged conidia and the increase in germination rate of aerial conidia after being stored at 28 C, the germination rates of these two spore-types becomes increasingly similar. The similarities in germination rates of these two spore types after being stored as a dry formulation is in stark contrast to their relative germination rates before drying, where submerged conidia

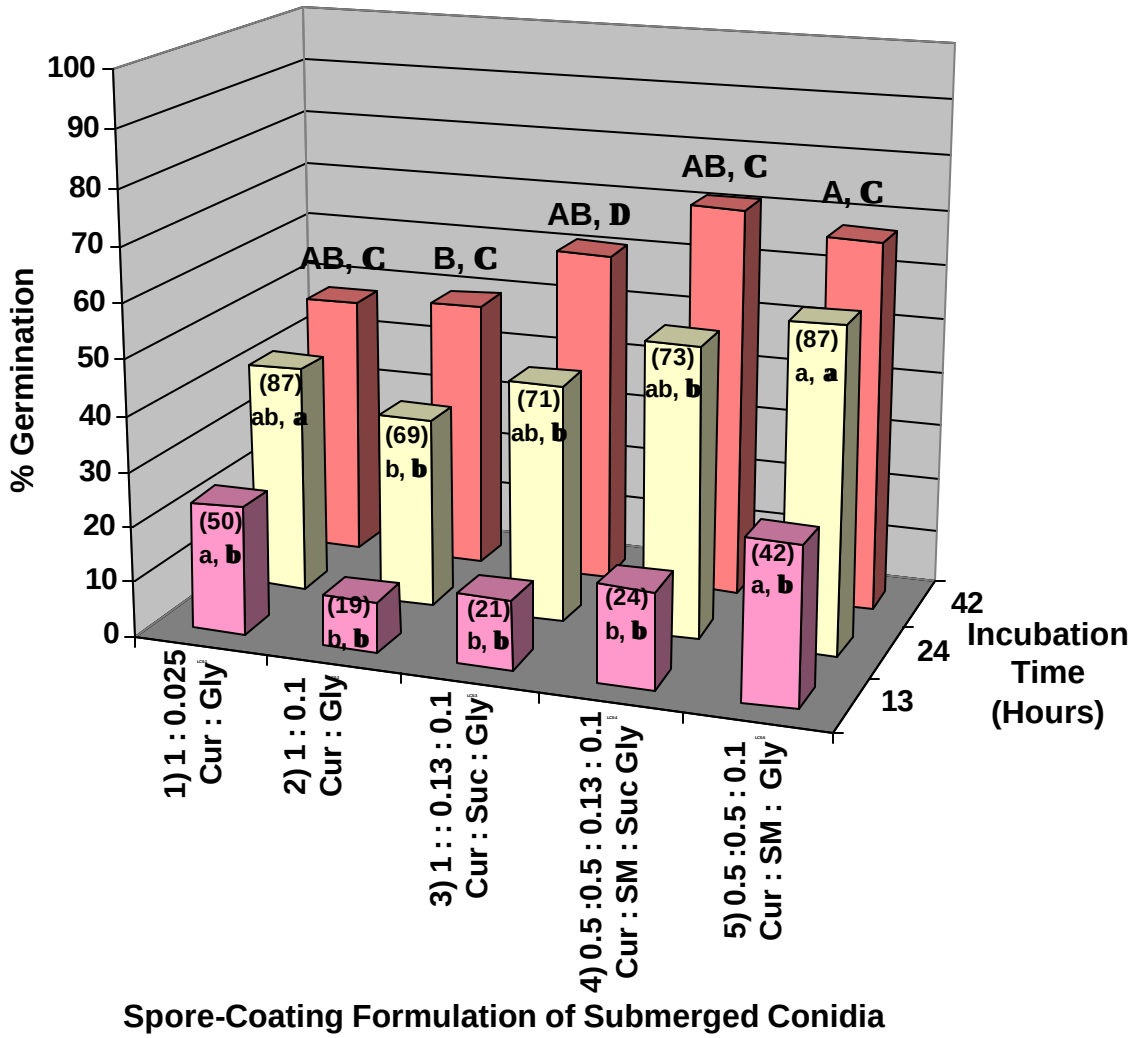

Figure 4-14. Air Drying Experiment 3: Percent of submerged conidia germinating in five dry spore-coating formulations after storage at 28 ºC for 6 d. Values in parentheses on columns represent 13- and 24-hr germination rates that were corrected for total viability (corrected % germination = % germination at 13 and 24 hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-, 13- and 24-hr corrected) and capital letters signify differences in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups are presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is presented in Table 4-3 (SM = skim milk; Cur = Curan 100®; Suc = Sucrose; Gly = glycerol).

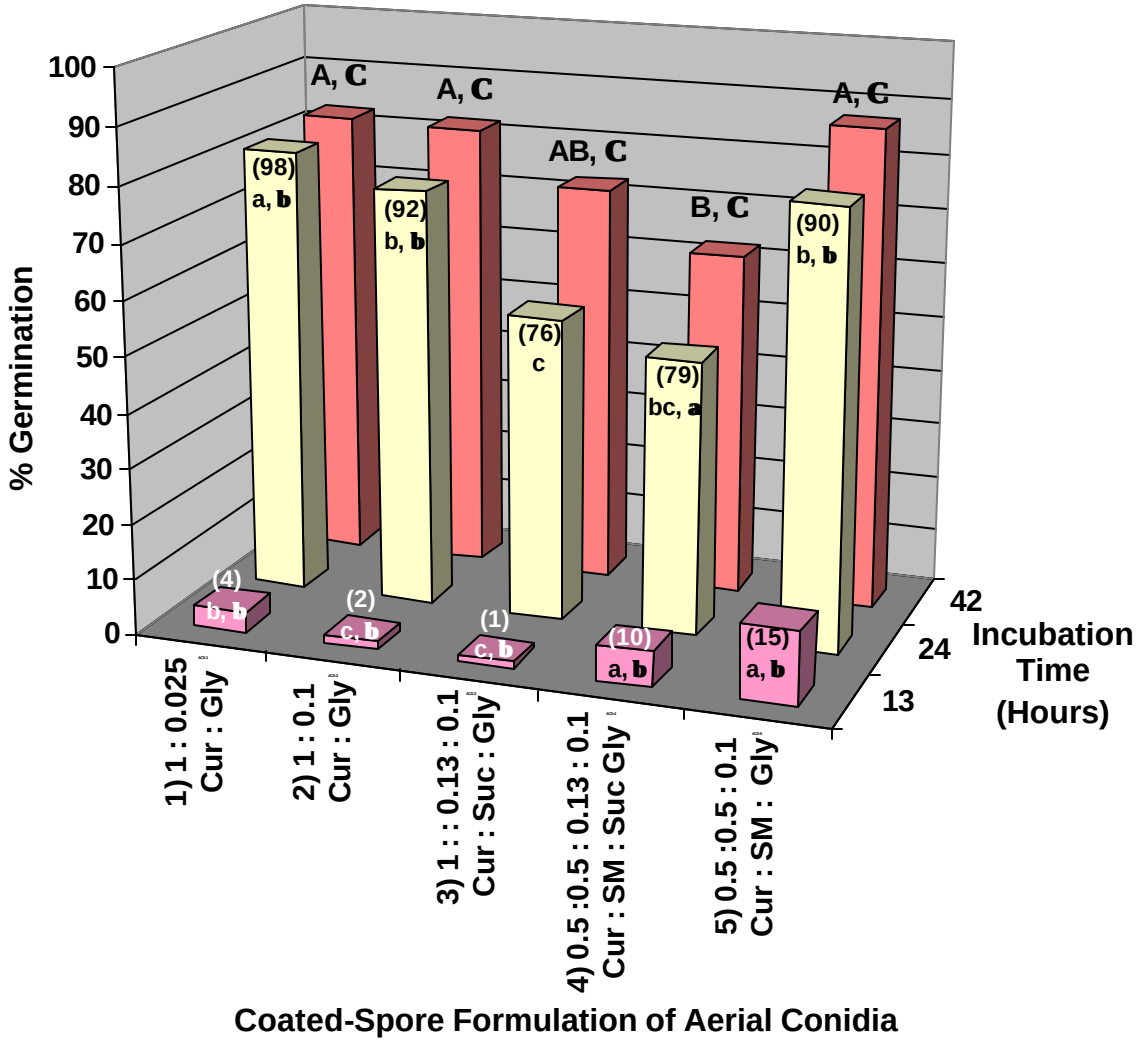

Figure 4-15. Air Drying Experiment 3: Percent of aerial conidia germinating in five dry spore-coating formulations after storage at 28 ºC for 6 d. Values in parentheses on columns represent 13- and 24-hr germination rates that were corrected for total viability (corrected % germination = % germination at 13 and 24 hr ÷ proportion germination at 42 hr). Comparisons among germination rates were made using these corrected values. Lower-case letters signify differences in germination rate (6-, 13- and 24-hr corrected) and capital letters signify differences in viability (42-hr) among formulations at the same incubation time ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Greek letters indicate a significant change in germination for the same incubation time or in viability for the longest incubation time from before drying to after drying for the same formulation (for corrected germination rates α = not significant and β = significant; for viability X = not significant and Δ = significant) ($\alpha = 0.05$; Wilcoxon two-sample test for non-paired ranked observations). Test groups are presented as dry mass ratios relative to 1 part dry spores. The formulations corresponding to each test group is presented in Table 4-3 (SM = skim milk; Cur = Curan 100®; Suc = Sucrose; Gly = glycerol).

germinated much faster than aerial conidia. This point will be discussed further in Chapter 5 when comparing the relative virulence of the two formulated spore types. Submerged conidia viability was highest in formulations #3, #4 and #5 after 28 C storage for 6 d, although these were not significantly greater than formulation #1 due to high variability for formulation #1 (Figure 4-14; capital letters, $\alpha = 0.05$). When comparing germination over time among submerged conidia in these three formulations (#3, #4, and #5), submerged conidia in formulation #5 had the highest 13-hr percent germination and there were no significant differences in 24-hr percent germination (Figure 4-14; lower-case letters, $\alpha = 0.05$). Aerial conidia in formulation #5 also demonstrated high 13- and 24-hr percent germination after storage at 28 C (Figure 4-15). When comparing germination over time and viability for aerial conidia among these three formulations (#3, #4 and #5); the 13- and 24-hr percent germination was significantly higher for aerial conidia in formulation #5 than #3 (Figure 4-15; lower-case letters; $\alpha = 0.05$) and the viability was significantly higher for aerial conidia in formulation #5 than #4 3 (Figure 4-15; capital letters; $\alpha = 0.05$). Therefore, on the basis of germination over time and viability after storage at 28 C, formulation #5 appears to be the best formulation for further evaluation.

Formulation #5, containing Curan 100®, skim milk and glycerol (0.5 : 0.5 : 0.1; dry mass ratios relative to spore dry weight) was also selected as the best formulation following air-drying on the basis of gemination rate and viability of submerged conidia and aerial conidia. Survival of submerged conidia during sieving was also significantly higher in this formulation than all other formulations (76%). Therefore, this formulation was selected for further testing of UV-tolerance and infectivity to *Schistocerca americana* in Chapter 5.

1.4. General Discussion

Two water-soluble lignins were initially tested in air-drying experiments, lignosulfonic acid (Ultrazine®) and Kraft lignin (Curan 100®), however Ultrazine® was

eliminated from further experimentation after Air Drying Experiment 1 because it appeared to be toxic to spores. Further research will be needed to determine if this entire class of lignin derivatives (lignosulfonic acids) is inappropriate for use with formulation of entomopathogenic fungi. Air-drying was an effective means for producing viable dry formulations of coated submerged and aerial conidia. Submerged conidia and aerial conidia survived this air drying process with either low viability loss (less than 30%) and in some cases no significant viability loss. The presence of sucrose in low concentrations (0.13% relative to spore dry mass) or skim milk (50% relative to spore dry mass) in the spore-coating formulation generally improved spore survival after drying. Spore survival while reducing the particle size of the spore-coating formulations to less than 80 µm was highly variable among the formulations, and was increased by combining Curan 100® and skim milk in the spore-coating formulation. Storage at 28 C for 6 d did not reduced the viability of either submerged conidia or aerial conidia in the majority of the formulations. Percent germination was lower for submerged conidia after 28 C storage than after drying in all formulations at 13 hr of incubation and in formulations 2, 3, and 4 at 24 hr of incubation. However, the percent germination was higher for aerial conidia after 28 C storage at 24 hr incubation than after drying. As a result, the 24-hr percent germination for submerged conidia and aerial conidia was more similar after drying and storage at 28 C than in suspension media before drying. On the basis of spore germination over time and viability after drying, sieving and 28 C storage a single formulation containing Curan 100®, skim milk and glycerol (0.5 : 0.5 : 0.1; dry mass ratios relative to spore dry weight) was selected for further experiments on UV-tolerance and infectivity to *S. americana*.

Drying at ambient r.h. followed by low (7 to 10%) r.h. produces dry spore-coating formulations ranging from 2 to 20% moisture, depending on the concentration of hydroscopic formulation components (skim milk, glycerol and sucrose). If spores in these spore-coating formulations behave in a similar manner to non-formulated aerial conidia, formulations with lower moisture content should have greater thermal stress tolerance (50 C) (Hong et al., 1998). At these moisture contents (2 to 20%) it is likely that non-freezable water has been removed from the spores, suggesting a potential role for

disaccharides in stabilizing membranes and proteins as described by the "water replacement hypothesis" (Crowe et al., 1990; Beker et al., 1984). Adding exogenous sucrose to the formulations containing only Curan 100® and glycerol consistently improved the desiccation tolerance submerged conidia, however, these experiments do not provide definitive evidence that the addition of exogenous sucrose protects *M. anisopliae* var *acridum* submerged conidia from desiccation by mechanisms described in the "water replacement hypothesis". Interpreting the mechanisms by which skim milk and sucrose improved desiccation tolerance of submerged conidia during air-drying is compounded by two factors: 1) the potential for uptake of exogenous sucrose and lactose and 2) the potential for changes in spore physiology during air-drying. The potential for uptake of exogenous sucrose and lactose by *M. anisopliae* var. *acridum* during air-drying is not known. This information could have important consequences because disaccharides provide increased desiccation protection when present on both sides of the phospholipid bilayer (Crowe et al., 1986). However, exogenous disaccharides have also been shown to enhance the ability of microorganisms to survive desiccation (Leslie et al., 1995). Interpreting the mechanisms by which sucrose and skim milk improved desiccation tolerance is further complicated by the fact that spores may have been physiologically active during the drying process. *Metarhizium anisopliae* aerial conidia are known to produce small concentrations (less than 5 mg/g) of endogenous trehalose (Hallsworth and Magan, 1994), and it is possible that hydroscopic formulation components served to decrease the rate of drying thereby giving spores time to prepare physiologically (e.g. production of endogenous trehalose) for anabiosis. However, non-reducing disaccharides (sucrose and trehalose) improved the desiccation tolerance of submerged conidia during freeze-drying when spores would not be physiologically active (See Appendix I). Developing a better understanding of the physiological changes that occur in *M. anisopliae* in preparation for anabiosis may have practical applications toward selecting drying processes to optimize these changes (Hong et al., 2000).

When considering how this drying process may be practically applied to a large-scale production system for formulating entomopathogenic fungi, it is worth considering the results of the silica gel drying experiments described in Chapter 3. This study

demonstrated that spores left in their growth media had greater drying stability than spores that were washed to remove the growth media prior to drying. It would be interesting to try an approach that involved adding coating materials directly to the growth media at the end of the fermentation process. The final product of the fermentation process, with added water-soluble lignin derivatives, might then be dried in thin layers in a dehumidified room, and then transferred to bags containing silica gel for further drying; followed by sieving. Also, considering the enhanced drying stability of spores produced at higher osmolality (VT-media + 10% PEG), the addition of coating material could potentially also be used to increase media osmolality for a short time at the end of the fermentation. A process that uses a similar approach, called the Stabileze process, was developed by USDA researchers at the European Biological Control Laboratory (EBCL), Montipillier, France. This process involves the addition of sucrose at the end of the fermentation process "sugar shock" followed by air-drying of the spore in the media. The practical steps involved in the scale-up and development of such a system is beyond the scope of this study, but information from Chapter 3 and 4 combined with research being conducted at EBCL may provide a foundation for the development of such a system.

4.6. Conclusions

Spore-coating formulations using varying ratios of skim milk, sucrose, glycerol, and two lignin derivatives, a lignosulfonic acid (Ultrazine®) and a Kraft lignin (Curan 100®) were tested for their ability to enhance spore survival during air drying, sieving, and storage at 28 C. The lignosulfonic acid (Ultrazine®) was toxic to submerged conidia in aqueous medium as determined by spore germination. Submerged conidia and aerial conidia of *M. anisopliae* var. *acridum* survived air-drying to moisture contents ranging from 2 to 20%. Spore survival after drying was enhanced by the addition of sucrose to spore-coating formulations containing only a Kraft lignin (Curan 100®) and glycerol. Sucrose did not enhance spore survival after drying in skim milk based spore-coating

formulations. Spore survival, while reducing the particle size of coated-spore formulations to less than 80 μm, was negatively affected by increasing concentrations of sucrose and was highest in formulations containing a combination of skim milk and Curan 100®. Short shelf-life experiments (6 d) at moderate temperature (28 C) were conducted for the practical purpose of ensuring that formulations could survive overseas shipment at ambient temperature for further experimentation. The spores in these dry spore-coating formulations were generally stable under these conditions without significant loss in viability. The germination rate of submerged conidia, however, generally decreased during storage; whereas it did not for aerial conidia. The result of this trend was increasing similarity between the germination rates of aerial conidia and submerged conidia after drying and storage of the coated-spore formulations as compared to relative germination rates before drying and storage. This may have practical consequences for the relative virulence of the coated-spore formulations of the two spore types, since rapid germination rates generally correlate with enhanced virulence. When combining information about the desiccation tolerance, ability of spores to survive sieving, and shelf-life at 28 C, formulation # 5 (containing Curan 100®, skim milk, and glycerol) was selected for further experimentation on UV-tolerance and virulence in Chapter 5.

4.7. Acknowledgments

Bob Wright and Dr. Wolgang Glasser, Brooks Forest Products Center, Department of Wood Science and Forest Products provided advice on selection of lignin derivatives and water-soluble lignin samples for experimentation. Spores from the 1-L fermentation system used this chapter were produced in the laboratory of Dr. Foster Agblevor in Biological Systems Engineering, with the technical assistance of Heather Hardcastle.

Chapter 5. Effect of Coating *Metarhizium anisopliae* var. *acridum* Aerial Conidia and Submerged Conidia with Water-Soluble Lignin Derivatives on Tolerance to Simulated Sunlight and Infectivity to *Schistocerca americana*

5.1 Abstract

Fungal biopesticides are highly susceptible to the effects of ultraviolet radiation, and attempts to protecting spores from sunlight using oil-soluble sunscreens and oil-emulsions have been only marginally successful. The influence of coating *Metarhizium anisopliae* var. *acridum* aerial and submerged conidia with a combination of Kraft lignin (Curan 100®), skim milk, and glycerol on spore tolerance to simulated sunlight and the infectivity to *Schistocerca americana* in oil was investigated. Coated-aerial and coated-submerged conidia had higher survival following exposure to simulated sunlight than non-coated aerial conidia. The LT_{50} values (95% confidence intervals) for viability based on germination after 48 hr incubation following exposure to simulated sunlight for non-coated aerial and coated-aerial conidia were 4.0 hr (3.1 - 5.1 hr) and 17.0 hr (12.5 - 23.0 hr), respectively. Therefore, coating aerial conidia increased survival time following exposure to simulated sunlight by approximately three times. Coated submerged conidia did not lose more than 50% viability following exposure to the longest exposure time (16 hr) to simulated sunlight. Coating spores reduced their infectivity to *Schistocerca americana* adults. The LT_{50} values (95% confidence intervals) for non-coated aerial, coated-aerial and coated-submerged conidia at 1×10^5 viable spores / insect were 5.8 d (4.9 - 6.9 d), 8.2 d (7.3 - 9.3d), and 10.4 d (8.2 - 13.3 d), respectively. When considering the large improvements on tolerance to simulated sunlight and the small decrease in infectivity, the use of water-soluble coated spores in oil may be an effective new strategy for protecting entomopathogenic fungi from solar degradation.

5.2. Introduction

One of the major challenges to the use of microbial biopesticides as inundative biocontrol agents is their lack of persistence in the field due to environmental factors such as sunlight, high temperature, and water stress. Sunlight is likely the most destructive of these environmental factors resulting in direct structural effects on DNA or indirect damage caused by the formation of reactive oxygen molecules, with the indirect action by reactive oxygen molecules likely being the most significant damaging factor (Ignoffo and Garcia, 1978, 1994; Ignoffo, 1992). The half-life of most entomopathogenic fungal conidia ranges from 1 to 4 hr in simulated sunlight and 4 to 400 hours in natural sunlight on foliage; the difference likely being the effects of shading and the diurnal cycle in field studies (Ignoffo, 1992). Strategies for protecting microbial biopesticides from solar degradation have used the following approaches: 1) the use of oil-soluble sunscreens with oil-carriers (Hunt et al., 1994; Moore et al., 1993; Shah et al., 1998); 2) the use of oil-water emulsions (Alves et al., 1998); 3) the use of water-soluble or suspendable absorbers or blockers with water carriers (Cohen et al., 1991; Ignoffo et al., 1997; Shapiro 1989, 1992, Shapiro and Robertson, 1990; Shasha et al., 1998) or 4) encapsulation (e.g. non-soluble starch) with a water carrier (Behle, et al., 1997; Ignoffo et al., 1991; McGuire and Shasha, 1995; McGuire et al., 1990, 1994, 1996; Shasha and Dunkle, 1989; Shasha and McGuire, 1989; Tamez-Guerra, et al., 1996).

The use of oil-based formulations appear to provide four important advantages over water-based formulations: 1) *Metarhizium anisopliae* generally has higher tolerance to simulated sunlight in oil-based formulations or oil emulsions than in aqueous formulations (Alves et al., 1998; Moore et al., 1993); 2) *Metarhizium anisopliae* var. *acridum* is more infective at low humidity in oil-based formulations (Bateman et al., 1993; Prior et al., 1988); 3) oil-based formulations allow the fungal biopesticide to be applied in a desiccated state, which reduces effects of thermal stress (McClatchie et al., 1994; Hedgecock et al., 1995; Hong et al., 1997, 1998); and 4) oil-based formulations are compatible with ultra-low volume application, which is important for reducing spray volume when controlling desert locusts in remote areas. These advantages may be of

particular important when using entomopathogenic fungi to control insect pests in environments of high heat, low humidity, and high insolation, such as Desert Locust control in the Sahel.

Oil-soluble sunscreens and sunblockers have failed to enhance efficacy *Metarhizium anisopliae* var. *acridum* in field trials, although some of these materials enhance UV-protection in laboratory experiments (Hunt et al., 1994, Moore et al., 1993; Shah et al., 1998). The discrepancy between sunscreen efficacy in laboratory studies and field trials may be due to two factors: 1) oil formulations in the laboratory were applied in 100 µm films in Petri plates (Hunt et al., 1994; Moore et al., 1993); whereas in the field, oil formulations spread out over hydrophobic (plant) surfaces leaving the spores exposed to direct sunlight (Shah et al., 1998; Burgess, 1998) and; 2) the sunscreens may be absorbed by the plant surfaces further decreasing the degree of protection (Burgess, 1998).

In an effort to improve the protection of microbial biopesticides in oil-based formulation on hydrophobic surfaces, a new strategy was tested that involves coating the microbial biopesticide with a water-soluble UV-protective material. After an extensive review of the literature and U.S. patents, I found no documented work describing the use of water-soluble coatings in an oil-based formulation for protecting viruses, bacteria, protozoa, or nematodes from solar radiation. This strategy was used to protect *M. anisopliae* var. *acridum* spores from solar radiation. Aerial conidia and submerged conidia were coated with a water-soluble lignin derivative in combination with skim milk and glycerol followed by suspension of the coated spores in an oil carrier.

The potential for the coating material to interfere with the infection process was also considered. To infect the host, entomopathogenic fungi must attach to the insect cuticle, obtain adequate moisture and potentially nutrients from their microenvironment to germinate, obtain cues from the cuticle to induce formation of penetrating structures, and penetrate the host cuticle. The second step in this infection process can be particularly problematic in areas of low relative humidity, such as the Sahel, where the ambient relative humidity is too low to allow for germination. It is hypothesized that

spores must obtain moisture from the relatively humid microenvironments of intersegmental membranes (Bateman et al., 1993; Burgess, 1998; Prior et al., 1988).

Experiments in this chapter examine the effects of a water-soluble lignin coating formulation on the UV-tolerance and infectivity of *M. anisopliae* var *acridum* aerial conidia and submerged condia. The specific objectives were: 1) to determine if a water-soluble coatings can enhance the tolerance of aerial conidia to simulated sunlight in oil, and 2) to determine if a water-soluble coating reduces the infectivity of aerial conidia to *Schistocerca americana* using direct dose bioassays in oil. Comparisons were also made to submerged conidia coated in the same formulation, but the effect of the coating on UV-tolerance and infectivity could not be determined directly for submerged conidia because it was not possible to suspend non-coated submerged conidia in oil.

5.3. Materials and Methods

5.3.1. Coating Spores with a Water-Soluble Lignin Derivative during Air-drying

A detailed description of coatings, development of the coating formulation, and the coating process is provided in Chapter 4. The spore-coating formulation used for submerged and aerial conidia used for these experiments was formulation #5 produced from Air-Drying Experiment 3. Submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) were produced using liquid shake flask culture in 4% waste brewers' yeast (Stroh's Batch, NPC Inc., Eden, NC) : 4% Sucrose (Sigma Chemical Co.) (Jenkins and Prior, 1993) in 250 mL baffled flasks at 24 C and 150 RPM continuous agitation. Final spore concentration was $5 \pm 1 \times 10^8$ spores/mL (mean ± st. dev.). Aerial conidia were harvested from 2- to 3-week old Petri plate cultures grown on Sabouraud dextrose agar at 24 C. A suspension of aerial conidia was adjusted to 7.4×10^8 spores/mL in 0.05% Tween 80. Both spore types were washed three times by centrifuging (2,800 x g, Fisher Scientific Marathon 6K) and re-suspending in sterile distilled water. The washed pellet of submerged conidia was suspended in medium containing 50 g/L Curan100®; 50 g/L

skim milk (Difco Co.); 10 g/L glycerol. Curan 100® is a Kraft alkali lignin (Aldrich # 37-095-9). The amount of suspension medium added to the pellet was calculated to produce a dry mass ratio of 1 : 0.5 : 0.5 : 0.1 for spores : Curan 100®: skim milk : glycerol based on a pellet moisture content of 70%. Aerial conidia were suspended in the same suspension medium and the volume of suspension medium was calculated to produce the same number of spore/g (dry wt.) of formulation as present in the coated-submerged conidia formulation. The spore suspensions were dried at ambient relative humidity for 60 hr; followed by low (7 to 10%) relative humidity in a desiccation chamber containing silica gel until they reached equilibrium (82 hr). Dry coated-spore formulations were ground against the surface of a metal sieve (170 mesh) to produce particles less than 80 µm.

5.3.2. Effects of Spore-Coating on Tolerance to Simulated Sunlight

Experiments on the effects of simulated sunlight were conducted in collaboration with the Centre de Biologie et de Gestation des Populations (CBGP), Institut Nationale de Recherche Agronomique (INRA), Monferrier-sur-Lez, France. I traveled to INRA to conduct preliminary simulated sunlight experiments described in Appendix III. For the experiments described in this chapter, the coated-spore formulations were produced at Virginia Tech as described in Chapter 4. Material was then shipped on cold packs to INRA. Upon arrival at INRA, the formulation was stored at 4 C until it could be used for simulated sunlight experiments. Viability of the material was determined before shipping to INRA and immediately before the simulated sunlight experiments were conducted at INRA. Petri plates containing 2% malt, 0.001% benomyl, and 0.02% chloramphenicol, which were used to determine percent germination, were inoculated with spore suspensions and incubated at INRA, then killed with 20% formalin as described in Section 4.3.2. These plates were shipped to Virginia Tech packed with cold packs where percent germination was determined.

Non-coated aerial conidia (NAC) from 2- to 3- week old cultures on SDA, coated-aerial conidia (CAC), and coated-submerged conidia (CSC) were suspended in 70% diesel fuel:30% peanut oil at a concentration of 1 x 10^7 spores/mL. The concentration of NAC was determined using a hemacytometer. The spore concentration for the CAC and CSC was based on mass of dry formulation and a predetermined spore concentration (spores/g of freeze-dried material). For each treatment, 10 mL of 1 x 10^7 spores/mL were deposited onto a 0.45 µm membrane filter (ME25 Schleicher and Schuell, Dassel, Germany). Deposition onto the filter was done using a Millipore vacuum filtration system (Millipore 10-047-04, 10-047-02). The total area of the inoculum deposit using this system is 10 cm^2. Six membrane filters were prepared for each treatment.

Laboratory irradiation experiments consisted of exposing the NAC, CAC and CSC in oil-based formulations on membrane filters to broad wavelengths greater than 295 nm, at increasing amounts of time. Irradiation tests were conducted in a controlled environment chamber using an artificial sunlight device. The artificial sunlight device consisted of two, 400 W high pressure metallic halogen lamps (HQI-TS, OSRAM, F67120, Molsheim, France), which emit a continuous spectrum from 270 to 1100 nm. A long pass glass filter (WG295-Schott Glaswerke, Mainz, Germany) was used to block shorter wavelengths (under 295 nm) to simulate natural sunlight. The relative UV-tolerance of the three formulation types (NAC, CAC, CSC) was compared in two simulated sunlight experiments. An initial experiment was conducted to determine an exposure time that would be appropriate for comparing each of the three treatments. For this experiment, an equal mass of coated-spore formulation from the four replicates of each formulation type was combined to produce the spore suspension. Spores were exposed to UV-irradiance energy of 0.585 W m^{-2} for 0, 4, 8, 12 or 16 hr, which correspond to UV-irradiance energies of 0, 4.32, 8.46, 12.78, and 17.10 kJ m^{-2}, respectively. Membrane filters covered with a metal plate to block radiation were used as controls for each of the different formulations and were placed in the controlled environment chamber for 16 hr. For the first experiment (UV-Experiment 1), a single filter membrane was used for each exposure time. For the second experiment (UV-Experiment 2), each of the 4 replicate formulations were kept separate and only the 0-

and 16-hr exposure times were used, for a total of 4 replicate membranes per formulation per exposure time. During the exposure period, the surface temperature of the membrane filters was regulated at 25 ± 1 °C and the air humidity ranged from 40 to 50% r.h.

After exposure to simulated sunlight, spores were removed from the filter by placing the membrane in 10 mL of sterile distilled water in 25-mL flasks and shaking for 5 minutes at 700 oscillations min^{-1} (10 cm vertical travel) on a mechanical shaker. Suspensions (30 µL) of approximately 1 x 10^6 spores/mL were plated onto 2% malt agar + benomyl in 30 mm Petri plates and percent germination after 24- and 48-hr exposure was determined as described in Chapter 4, Section 4.3.2. For UV-Experiment 1, three replicate Petri plates were counted from each membrane filter; for UV-Experiment 2, a single Petri plate was counted from each membrane filter. In addition, colony forming units (CFUs) were determined for suspensions of irradiated spores on four replicate Petri plates containing Paris medium using a spiral plating method (Gilchrist et al., 1973). The spore suspension used for CFU counts was approximately 3.3 x 10^5 spores/mL before the spiral plating. After 4 days of incubation in the dark at 25° ± 1°C, colonies were enumerated on three consecutive days to ensure that CFU's accounted for delayed growth.

Two criteria were used to examine the viability of *M. anispliae* var. *acridum* propagules after exposure to simulated sunlight, percent germination and colony forming units (CFU's). Viability estimates based on CFU's are concentration dependant and, therefore, may be affected by percent recovery of spores from the membrane filter and clumping of spores in the suspension. Viability estimates based on percent germination use direct observation and, therefore, are independent of these factors. A discussion of the rationale for this approach is provided in Appendix IV.

5.3.3. Effects of Spore-Coating on Infectivity to *Schistocerca americana*

Non-coated aerial conidia (NAC) from 2- to 3- week old cultures on SDA, coated-aerial conidia (CAC), and coated-submerged conidia (CSC) were suspended in 70% diesel fuel:30% peanut oil at concentrations of 5 x 10^8, 5 x 10^7, 5 x 10^6, 5 x 10^5 viable

spores/mL. The concentration of NAC was determined using a hemacytometer. The spore concentration for CAC and CSC was based on the dry mass of freeze-dried material and a predetermined spore concentration (spores/g of freeze-dried material). Spore viability was based on percent germination after 48 hr on 2% malt agar plus benomyl as described in Section 4.3.2.

Bioassays were performed on 2- to 3-week old *Schistocerca americana* adults. An initial bioassay (Lignin Bioassay 1), was used to determine an appropriate dose for comparing the infectivity of the three spore types. Coated-spore formulation material from each of the four replicates of each formulation type was combined for this bioassay. For Lignin Bioassay 1, 10 adults with a 50:50 sex ratio were inoculated per dose level. Adults were dosed with 2 µL of formulated inoculum using topical application directly under the pronotal shield. These doses corresponded to 1×10^6, 1×10^5, 1×10^4, and 1×10^3 viable spores/insect. For Lignin Bioassay 2, the four replicates of each formulation type were kept separate. Each replicate was used to dose five insects at 1×10^5 viable spores/insect as described above for a total of 20 insects per treatment.

Insects were caged individually in wire-screened cages (27 cm x 19 cm x 13 cm) and maintained at 32 C, 50± 5% r.h., with a photoperiod of 14 hr : 10 hr (L:D). The r.h. in the chamber was maintained using with a humidity regulating system consisting of forced air exchange over a cheesecloth wicking system saturated with K_2CO_3. The r.h was monitored with a Fisher Scientific traceable printing hygrometer/thermometer (Model # 11-661-17A). Mortality was checked and a fresh 6-cm diameter Romaine lettuce disk was provided daily. Mortality was compared among spores from the various media by determining the 95% confidence intervals LT_{50}'s at a dose of 1×10^5 viable spores/insect using probit analysis (Finey, 1971). Dead grasshoppers were removed and surface sterilized using a method described by Lacey (1997). Dead grasshoppers were soaked in 70% ethanol for a few seconds and then rinsed in sterile distilled water. The cadavers were then placed into 0.5% sodium hypochlorite for two minutes, followed by two rinses with sterile distilled water. They were then incubated in a Petri dish with moist filter paper under sterile conditions until sporulation occurred.

5.4. Results and Discussion

5.4.1. Effects of Spore-Coating on Tolerance to Simulated Sunlight

Germination was tested for each of the treatments (non-coated aerial conidia (NAC), coated-aerial conidia (CAC), and coated-submerged conidia (CSC) before they were added to the oil carrier (70% diesel fuel : 30% peanut oil) to ensure that the carrier did not affect germination. These values were used as a baseline germination percentage to which all other germination values were compared and are presented in Table 5-1 and Table 5-2 for UV-Experiment 1 and 2, respectively. (i.e. the percent germination values for spores after being suspended in oil and exposure to simulated sunlight that are presented in Figure 5-1 and Table 5-3 were corrected for the 48-hr Percent Germination Values in Table 5-1 and 5-2 using the following equation corrected percent germination for oil formulations following 0, 4, 8, 12 or 16 hr of UV-exposure = percent germination following 0, 4, 8, 12 or 16 hr of UV-exposure ÷ proportion of spores germination prior to formulation in oil after 48-hr of incubation).

The viability lost in the CAC and CSC treatments occured during the formulation process, primarily during drying and sieving (Chapter 4). Both the CAC and the CSC treatments had lower 24-hr percent germination as compared to the NAC treatment (α = 0.05; Wilcoxon two sample test for non-paired observations). Germination over time in these treatments (CAC and CSC) were affected by the drying process during formulation (See Chapter 4).

Table 5-1. UV-Experiment 1. Initial Germination of Three Formulations Suspended in Water Prior to Formulation with Oil and Exposure to Simulated Sunlight

Formulation	24-hr Germination (Means ± Standard Dev.)	48-hr Germination (Means ± Standard Dev.)
Non-coated Aerial Conidia	81 ± 3	86 ± 1
Coated-Aerial Conidia	46 ± 6	65 ± 4
Coated-Submerged Conidia	39 ± 3	51 ± 3

Table 5-2. UV-Experiment 2. Initial Germination of Three Formulations Suspended in Water Prior to Formulation with Oil and Exposure to Simulated Sunlight

Formulation	24-hr Germination (Means ± Standard Dev.	48-hr Germination (Means ± Standard Dev.
Non-coated Aerial Conidia	87 ± 2 a	89 ± 2 a
Coated-Aerial Conidia	48 ± 2 b	66 ± 3 b
Coated-Submerged Conidia	47 ± 7 b	51 ± 5 b

The percent germination after a) 24-hr incubation and b) 48-hr incubation on 2% malt agar following exposure to five time intervals of simulated sunlight are presented in Figure 5-1 a and b, respectively. The LT_{50} values (95% confidence intervals) for percent germination at 48-hr incubation following exposure to simulated sunlight for NAC and CAC were 4.0 (3.1 - 5.1 hr) and 17.0 hr (12.5 - 23.0 hr), respectively. The LT_{50} could not accurately be determined for CSC since they did not lose greater than 50% of initial viability after the longest simulated sunlight-exposure time (16 hr). Germination following exposure to simulated sunlight was determined after 24- and 48-hr of incubation because exposure to UV light is known to reduce the germination rate of *Metarhizium* spp. (Alves et al., 1998; Hunt et al., 1994; Moore et al., 1993). The delay in germination following exposure to simulated sunlight may be the result of damage to proteins and nucleic acids leading to slower growth rates, a defense response of the conidia, or the result of time and energy being devoted to repair mechanisms (Moore et al., 1993). It is possible that some of the apparent loss in viability for the CAC and CSC treatments (as determined by 48-hr incubation) was due to delayed germination from UV-exposure since the germination rates of these spore treatments were slow relative to NAC treatments prior to UV-exposure. For these treatments, a longer incubation time (e.g. 60 hr) may have provided a higher estimate of viability but may not have been biologically significant, since it is likely slow germinating spores would have reduced infectivity (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al., 1983).

The second simulated sunlight experiment (UV-Experiment 2) was conducted using more replicates at 0 hr and 16 hr exposure intervals. The four replications of each formulation

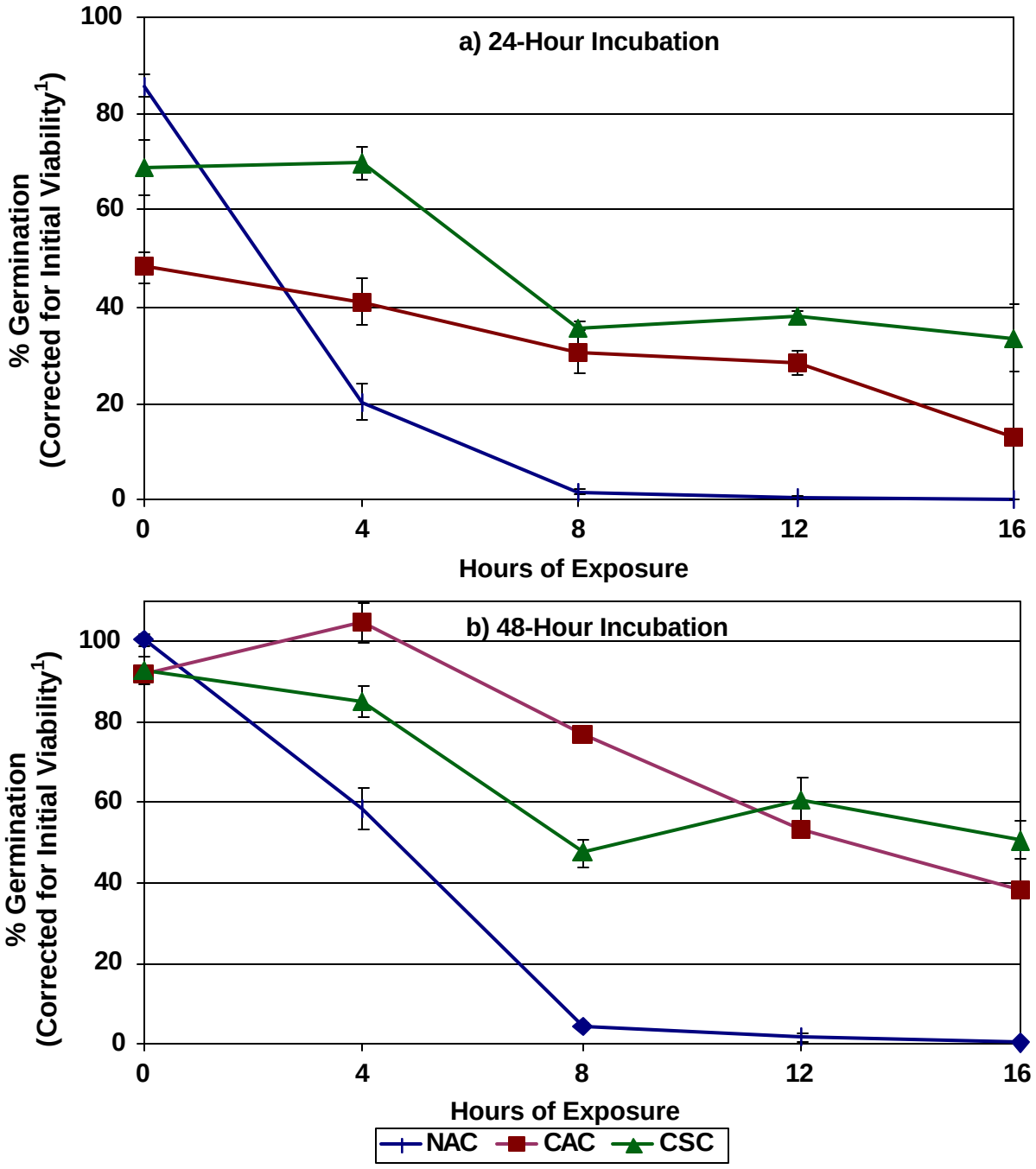

Figure 5-1. Comparison of percent germination for three formulations of *Metarhzium anisopliae* var. *acridum* after incubation for a) 24 hr and b) 48 hr on 2% malt agar following exposure to simulated sunlight (NAC = non-coated aerial conidia; CAC = coated-aerial conidia; CSC = coated-submerged conidia). All formulations were suspended in 70% diesel fuel : 30% peanut oil. Percent germination was corrected for initial viability, defined as the percent of spores that germinated after 48 hr before suspension in oil to isolate the effect of simulated sunlight from viability that was lost during the formulation process (corrected % germination = % germination in oil at 24 or 48 hr ÷ % germination in water at 48 hr). Each data point represents the mean and standard deviation of three replicate germination plates.

type were kept separate during this experiment. The percent germination after 24- and 48-hr incubation on 2% malt following exposure to simulated sunlight is presented in Table 5-3. Both of the coated formulations (CAC and CSC) had significantly higher 24-hr percent germination compared to NAC following 16 hr of exposure to simulated sunlight and the 24-hr percent germination of CSC spores were higher than that of CAC spores ($\alpha = 0.05$). The higher germination of CSC spores verses CAC spores at 24-hr incubation was due to the faster germination rate of CSC than that of CAC since the viability (48-hr percent germination) of these two formulations after 16 hr of exposure was not significantly different ($\alpha = 0.05$). The viability of both of the coated formulations (CAC and CSC) following 16-hr of UV-exposure were higher than that of the non-coated aerial conidia (NAC) by approximately an order of magnitude ($\alpha = 0.05$).

The results of Experiment 2 were generally in agreement with UV-Experiment 1 with the exception that the survival of NAC appeared to be greater in the second

Table 5-3. Percent Germination of Three Spore Formulations Corrected for Initial Viability[1] After 24 and 48 Hours of Incubation on 2% Malt Agar + 0.001% Benomyl Following Exposure to 0 and 16 Hours of Simulated Sunlight

Formulation	0 Hours of UV-Exposure	16 Hours of UV-Exposure
	24-Hour Percent Germination	
Non-Coated Aerial Conidia	96 ± 4.1 a[2]	0 ± 0.3 a
Coated-Aerial Conidia	75 ± 9.9 b	21 ± 6.0 b
Coated-Submerged Conidia	68 ± 6.6 b	34 ± 9.1 c
	48-Hour Percent Germination	
Non-Coated Aerial Conidia	N/A[3]	6 ± 0.9 a
Coated-Aerial Conidia	106 ± 9.3 a	62 ± 18.0 b
Coated-Submerged Conidia	95 ± 13.4 a	67 ± 9.6 b

[1] Percent germination was corrected for initial viability, defined as the percent of spores that germinated after 48 hr before suspension in oil to isolate the effect of simulated sunlight from viability that was lost during the formulation process (corrected % germination = % germination in oil at 24 or 48 hr ÷ % germination in water at 48 hr).
[2] Values are presented as means ± standard deviations. Numbers followed by different letters are significantly different than values within the same column at the same incubation time (24- or 48 hour) ($\alpha = 0.05$) (Wilcoxon two sample test for non-paired samples).
[3] Although benomyl was used with these % germination plates, the growth of germinated spores was too fast to accurately estimate % germination at 48 hr for the non-coated aerial conidia before UV-Exposure.

experiment. In UV-Experiment 1 the viability of NAC was reduced to 1.6% after only 8 hr of UV-exposure and was 0 after 16 hr of exposure. This disparity may in part be due to clusters of aerial conidia in the initial oil suspension of NAC of UV-Experiment 2, that were not present in UV-Experiment 1. These clusters may have resulted in shading of a proportion of the population. This is addressed in greater detail in the Appendix IV, where the clustering of aerial conidia in each of the experiments was quantified.

The tolerance of non-coated aerial conidia to simulated sunlight were generally in agreement with that which has been reported in the literature. Combining data from several publications, Ignoffo (1992) estimated the half-life of *M. anisopliae* aerial conidia on glass to be 1.3 - 4.0 hours. Because of shading and the diurnal cycle, entomopathogenic fungi are generally found to have much longer half-lives in field tests using natural sunlight than laboratory tests using direct exposure to simulated sunlight (Ignoffo, 1992). Fargues et al. (1996) found the percent survival (based on CFU's) of *M. anisopliae* var *acridum* (IMI 330189) aerial conidia suspended in water to be 46, 23, 8, and 5% following 1, 2, 4, and 8 hours of exposure to simulated sunlight, respectively. Percent survival of 14 *M. flavoviride* isolates and 23 *M. anisopliae* isolates ranged from 0.1 to 11%, and from 0 to 1%, respectively, after 8 hr of exposure (Fargues et al., 1996). Alves (1998) found that the viability (48 hr germination) for *M. anisopliae* ranged from 5 to 73% following the longest exposure time to solar radiation (6 hr) depending on the type of water-based or oil emulsifiable formulation that was used. Protection of *Metarhizium* spp. in oil-based formulations has focused exclusively on the use of oil-soluble sunscreens. Hunt et al. (1994) tested the effects 16 sunscreens in a kerosene carrier on tolerance of two isolate of *M. anisopliae*, one of which was *M. anisopliae* var. *acridum* (IMI 330189). Several of the sunscreens reduced the negative effects of solar radiation on germination rate (24-hr) but only one (Eusolex 8021) enhanced the viability of spores (48-hr germination) following exposure to 2 hr of simulated sunlight; and Eusolex 8021 failed to protect conidia from 5 hr of exposure. Moore et al. (1993) tested the UV-tolerance of *M. anisopliae* var *acridum* (IMI 330189) suspended in either water, oil, or oil plus an oil-soluble sunscreen (oxybenzone). Survival (48-hr germination) was

higher an oil than in water, 4% and 37%, respectively after 1 hr of exposure. Adding oxybenzone to the oil formulation increased survival (48-hr germination) from 28% to 82% following 3 hr of exposure. Field tests with oil formulations of *M. anisoplaie* var *acridum* (IMI 330189) containing oxybenzone were not as promising since no significant difference mortality of *Kraussella amabile* (Orthoptera : Acrididae) was observed between formulations with and without oxybenzone in both sweep net and field cage studies (Shah et al., 1998). It may be that the oxybenzone does not provide as much protection on hydrophobic plant surfaces were it spreads with the oil carrier to form thin films as in does in laboratory studies in Petri plates where the thickness of oil film is greater (approximately 100 µm) (Burgess, 1998). Direct comparison of LT_{50} values with the results of sunscreen formulations in the literature are difficult because the exposure times (2 to 6 hr) reported in the literature were not long enough to cause greater than 50% spore mortality in their most promising formulations (Alves et al., 1998; Hunt et al., 1994; Moore et al., 1993). Nonetheless, survival of aerial conidia in the Curan 100® -based coatings used in these experiments (62% after 16-hr exposure) was higher than any of the sunscreen trials reported. Further, this water-soluble coating would be expected to continue to provide protection of the spores even as oil spreads over hydrophobic surfaces because the coating is intimately associated with spores rather than dissolved in the oil carrier.

5.4.2. Effects of Spore-Coating on Infectivity to *Schistocerca americana*

The relative infectivity of non-coated aerial conidia (NAC), coated-aerial conidia (CAC), and coated-submerged conidia (CSC) in an oil carrier (70% diesel fuel : 30% peanut oil) to *Schistocerca americana* was compared in two bioassays. The first bioassay included four dosage levels of each spore treatment with 10 insects per dose. This bioassay was used to select an appropriate dose for comparing the LT_{50} values of the spore treatments for a subsequent experiment with a larger number of insects (20 insects/dose). The results of the first bioassay are presented in Figure 5-2. The LT_{50}

values and 95% confidence intervals for the three highest doses of each spore treatment are presented in Table 5-4. The infectivity of the CAC and NAC treatments were not significantly different at 1 x 10^4 submerged conidia as determined by 95% confidence intervals for LT_{50} values. At the two higher doses (1 x 10^5 and 1 x 10^6) the infectivity of CAC and NAC treatments were not significantly different as determined by 95% confidence intervals for LT_{50} values. The LD_{50} values for the three formulation types could not be accurately determined using this data.

Comparison of mortality over time resulting from a single dose of 1 x 10^5 viable spores/insect, is presented in Figure 5-3. Coating spores reduced their infectivity to

Table 5-4. Comparison of LT_{50} Values for *Schistocerca americana* Mortality After Direct Dosing of Non-coated Aerial Conidia (NAC), Coated-Aerial Conidia (CAC); and Coated-submerged Conidia (CSC) at Three Spore Concentrations

Spore Treatment	1 x 10^4 spores/insect LT_{50} (95% C.I.)	1 x 10^5 spores/insect LT_{50} (95% C.I.)	1 x 10^6 spores/insect LT_{50} (95% C.I.)
NAC	7.0 (5.9 - 8.2)	***	***
CAC	7.22 (4.1 - 12.7)	7.8 (6.8 - 9.0)	6.5 (5.6 -7.6)
CSC	< 50% mortality	7.2 (6.3 - 8.5)	6.3 (5.4 - 7.5)

1 Numbers are presented as LT_{50} values followed by 95% confidence intervals in parentheses.
2 Treatments designated with *** indicate that the LT_{50} could not accurately be determined because there was an insufficient number of values that were between 0 and 100% mortality to accurately estimate an LT_{50} for these concentrations.

Schistocerca americana adults. The LT_{50} values (95% confidence intervals) for non-coated aerial conidia, coated-aerial conidia and coated-submerged conidia at 1 x 10^5 viable spores / insect were 5.8 d (4.9 - 6.9 d), 8.2 d (7.3 - 9.3d), and 10.4 d (8.2 - 13.3 d), respectively. Coating aerial conidia significantly reduced their infectivity at 1 x 10^5 viable spores/ insect. There was not a significant difference in infectivity between CAC and CSC at 1 x 10^5 spores/insect.

The infectivity of aerial conidia in 70% diesel fuel : 30% peanut oil was considerably higher than in 20% molasses in water as compared to bioassays described in Chapter 3. For example, the LT_{50} values (95% C.I.) for *S. americana* exposed to a dose

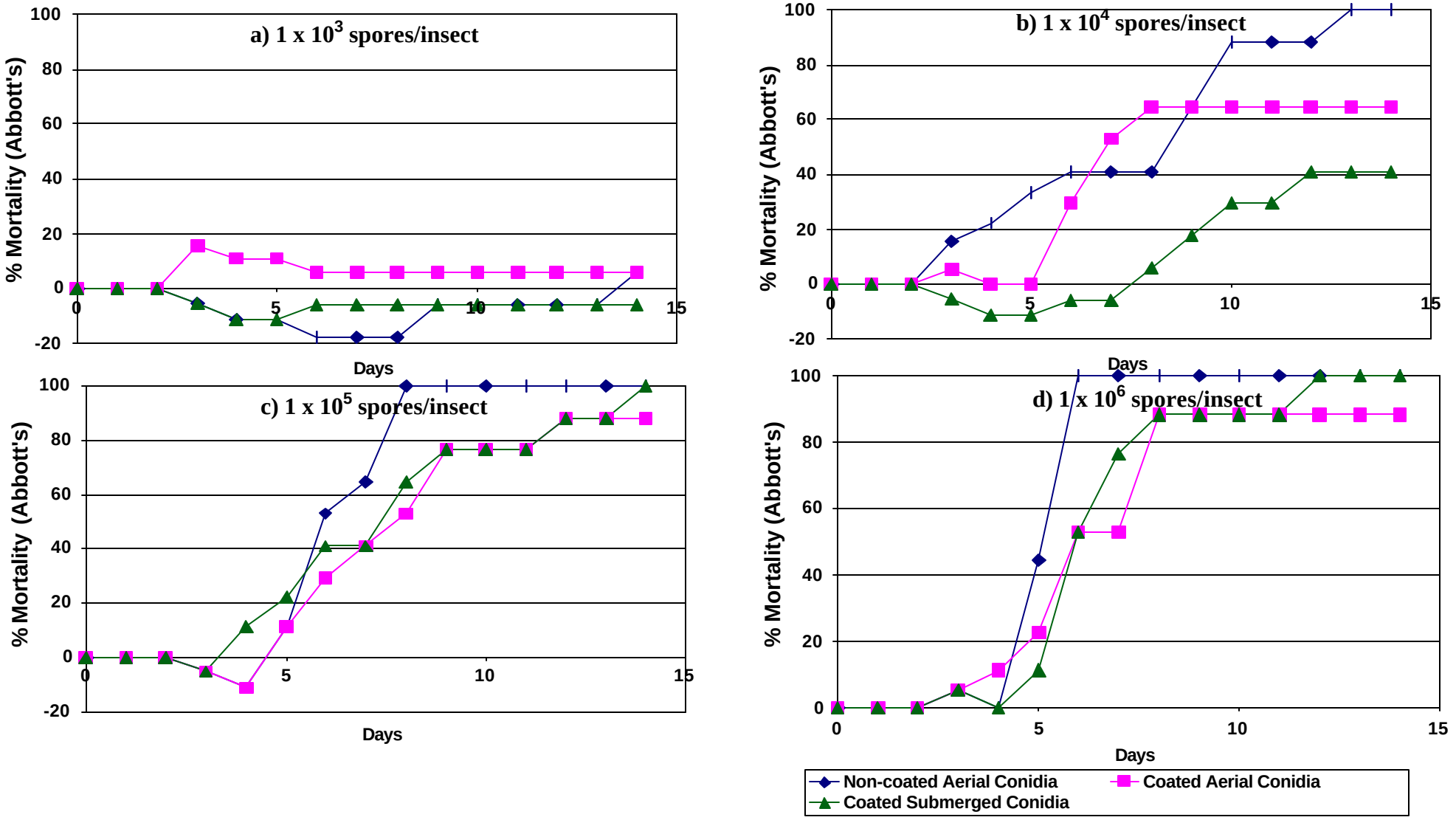

Figure 5-2. Mortality over time for adult (2- to 3- week old) *Schistocerca americana* inoculated by direct dosing (2 µL) with non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia. Individuals were dosed with a) 1 x 10^3, b) 1 x 10^4, c) 1 x 10^5, and d), 1 x 10^6 spores/insect (n = 10 insects / dose). Mortality corrected for controls with Abbott's formula (Finney, 1971).

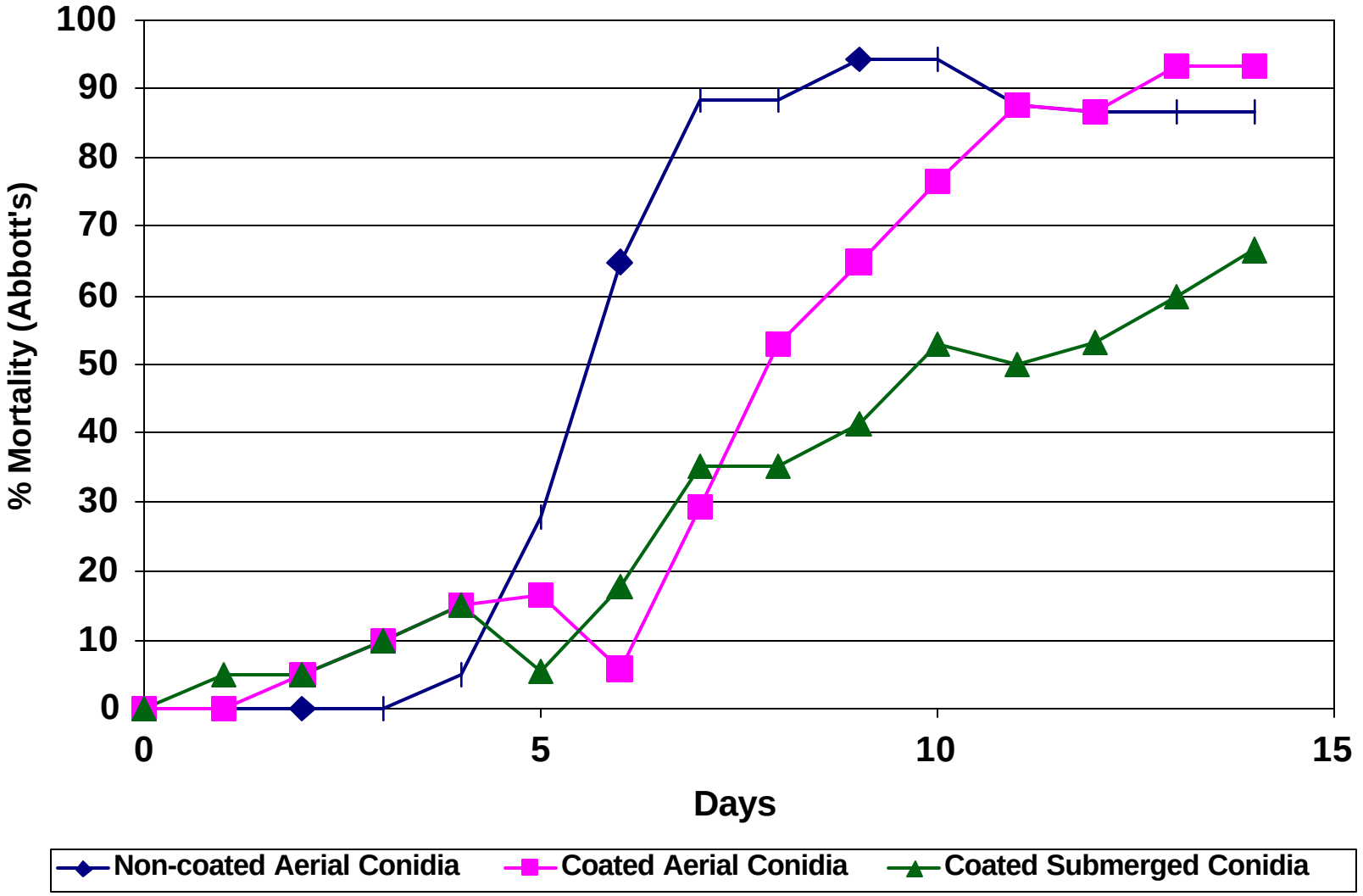

Figure 5-3. Mortality over time for adult (2- to 3- week old) *Schistocerca americana* inoculated by direct dosing (2 µL) with non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia. Individuals were dosed with 1 x 10^5 spores/insect (n = 20 insects / dose). Mortality corrected for controls with Abbott's formula (Finney, 1971).

of 1 x 10^6 aerial conidia/mL of 20 % molasses solution was 14.6 d (11.9 to 18.0 d); whereas, at an order of magnitude lower concentration (1 x 10^5 aerial conidia/mL of oil) the LT_{50} (95% C.I.) was only 5.8 (4.9-6.9). These results are in agreement with the results of Bateman et al (1993) who found that *M. anisopliae* var *acridum* (IMI 330189) was more infective to *S. gregaria* when suspended in oil than in water when conducting bioassays at low humidity (35%). Prior et al (1988) speculated that this enhanced infectivity may be the result of increased adherence to the insect cuticle. Results from Chapter 3 indicated that spores from liquid culture (VT-medium and VT-medium + 10% PEG) were more virulent than aerial conidia when applied in an aqueous 20% molasses solution (See Chapter 3, Figure 3-4). LT_{50} values and corresponding 95% confidence intervals for aerial conidia, spores from liquid culture in VT medium, and VT medium + 10% PEG at 1 x 10^6 spores/mL were 14.6 d (11.9 to 18.0 d), 10.5 d (9.5 to 11.6 d), and 7.8 d (6.7 to 9.0 d). The enhanced infectivity of spores from liquid culture in the 20% molasses formulation may in part be explained by their more rapid germination rates (See Chapter 3, Figure 3-2) (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al., 1983). The 6-hr percent germination for aerial conidia and submerged spores produced in VT-medium and VT-medium + 10% PEG were approximatley 2%, 30%, and 60%, respectively (See Chapter 3, Figure 3-2). However, coated-submerged conidia were not more virulent than coated-aerial conidia applied in an oil carrier. The reduction in infectivity of submerged conidia relative to aerial conidia in the coated formulations, may, in part, be explained by the fact that drying and storage of the coated-spore formulations reduced the germination rate of submerged conidia but did not reduce the germination rate of aerial conidia. After drying and storage the 24-hr percent germination of submerged conidia decreased to 87%, whereas there the 24-hr percent germination of aerial conidia increased to 90%, resulting in similar 24-hr percent germination between coated-aerial conidia and coated-submerged conidia formulations (See Chapter 4, Figure 4-14 and 4-15, formulation #5).

Metarhizium anisopliae var. *acridum* appears to be less virulent to *S. americana* than *S. gregaria* on the basis of relative virulence of aerial conidia, when comparing results of these bioassays with those conducted with *S. gregaria* in Appendix III. At the

lowest dose 1 x 10^3 spores/insect, *M. anisopliae* var *acridum* aerial conidia produced no significant mortality in *S. americana* adults after 14 d of incubation, whereas, *S. gregaria* exhibited 100% mortality within 9 d of incubation (LT_{50} =7.0 d (5.5-8.9 d)). These results are in agreement with the work of Sieglaff et al. (1998) who found even lower virulence of *M. anisopliae* var *acridum* to *S. americana* than the results of our bioassays in Chapter 4. Sieglaff et al. (1998) found much lower infectivity of *M. anisopliae* var. *acridum* (IMI 330189) to *S. americana* than we found in our bioassays. After dosing *S. americana* adults with 1.2 x 10^5 spores/adult in a peanut oil, the mean mortality was only 17.6% after 7 d and 80.8% after 14 d.

Evaluation of the benefits of coating *Metarhizium anisopliae* var. *acridum* spores must consider the balance between enhanced tolerance to simulated sunlight and potential to decrease infectivity. Different variables weigh into this cost/benefit analysis for spores produced in liquid culture and aerial conidia. Spores produced in liquid culture cannot be applied in oil without first drying the spores. Since our experiments indicate that these spores do not survive the drying process without a protective coating, a coating material is a necessary step in developing an oil-based formulation. Therefore, considering the advantages that formulating with oil provides (e.g., enhanced UV-tolerance, thermal stress tolerance, infectivity and low humidity and compatibility with ULV application) combined with further enhancement of UV-tolerance by coating spores, it appears that coating spores may be highly advantageous for the formulation of spores from liquid culture. However, aerial conidia can be formulated in oil without the addition of a protective coating. Therefore, the only advantage that coating provides to aerial conidia formulations is enhanced UV-tolerance, which must be balanced with decreased infectivity. Coating aerial conidia resulted in an increased of LT_{50} following exposure to simulated sunlight by approximately four times and the final spore viability after 16-hr of exposure to simulated sunlight was an order of magnitude greater for coated-aerial conidia than for non-coated aerial conidia. However, at 1 x 10^5 spores/insect non-coated aerial conidia were more infective than coated-aerial conidia resulting in an LT_{50} that was shorter by approximately 2 days. Although the LD_{50} could not be determined from this data it is likely that the LD_{50} (14 d) of these two formulations is similar within at least an

order of magnitude, since both of the formulations produced greater than 60% mortality at a dose of 1 x 10^4 spores/insect and no significant mortality at 1 x 10^3 spores/insect. Because coating spores increased spore viability following 16-hr exposure to simulated sunlight by an order of magnitude, it may be that the residual activity of the coated-spore formulation more than compensates for any reduced infectivity.

Further experimentation with *S. gregaria* using dosing at more narrow ranges of concentration is needed to more accurately determine the LD_{50} and LD_{90} values of the three formulation types. This information would be useful in evaluating the benefit of enhanced residual activity by increasing UV-tolerance, balanced with the cost associated with reduced infectivity. Field trials will need to be conducted to determine the UV-tolerance and the infectivity of the formulations under more realistic exposure conditions. The compatibility of the formulation with ultra-low volume (ULV) application technology needs to be investigated further. Particles in these formulations are less than 80 µm, but may be too large for ULV-application which produces droplets less than 100 µm. Further improvements in the drying process and formulation components may help to reduce spore mortality resulting from drying and enhance the ability of the formulation to be broken into small particles without losing spore-viability. These experiments have demonstrated that a water-soluble coating can greatly enhance the UV-tolerance of spores and that the coated spores remain highly infective, with some reduction in infectivity. Evaluating the potential for practical application of this strategy will need to balance the benefits of enhanced environmental stability with reduction in infectivity and additional cost of the formulation process. This cost benefit analysis should include more data on the infectivity of the formulations to *S. gregaria*, the efficacy of the formulations in field trials, and potential improvements in the formulation process.

5.5. Conclusions

Coating aerial conidia with a water soluble lignin derivative (Curan 100®) in combination with skim milk and glycerol significantly enhances their tolerance to

simulated sunlight, increasing their LT_{50} values by greater than four times (3 hrs vs. 17 hrs). It is difficult to compare the efficacy of this formulation directly with other approaches reported in the literature using sunscreens or oil emulsions because the exposure times for their most promising formulations (2 to 6 hrs) were generally not long enough to generate greater than 50% loss in spore viability. However, based on spore viability reported for the longest exposure times, this water-soluble coating appears to give greater UV-protection than any of the oil-soluble sunscreens or oil emulsions that have been tested (Alves et al., 1998, Hunt et al., 1994, Moore et al., 1993). In addition, laboratory studies using sunscreens do not account for reduced protection due to the spreading of oil over hydrophobic surfaces, which may explain the reduced field performance of sunscreens that have shown promising results in the laboratory (Burgess, 1998; Moore et al., 1993; Shah et al., 1998). Spreading of oil over hydrophobic surfaces would not be expected to reduce efficacy of our formulation since the coating material would remain around the spores.

Coating aerial conidia reduced their infectivity to *S. americana* based on LT_{50} values at a dose of 1×10^5 spores/insect; however, the coated spores still remained highly virulent to *S. americana*. Although the infectivity of coated-aerial conidia and coated-submerged conidia was not significantly different on the basis of LT_{50} values at a dose of 1×10^5 spores/insect, coated-submerged conidia appeared to be less infective than coated-aerial conidia and aerial conidia resulting in lower mortality after 14 d incubation. The reduced infectivity of submerged conidia relative to aerial conidia may, in part, be explained by a reduction in germination rate that occurs during the drying process and storage of the dried coated-spore formulations.

5.7. Acknowledgments

All of the simulated sunlight experiments were conducted at the Centre de Biologie et de Gestation des Populations (CBGP) of the Institut National de la Recherche Agronomique (INRA), Montferrier-sur-Lez, France under the direction of Dr. Jacques

Fargues in collaboration with Dr. Nathalie Smits and with the assistance of Dr. Marc Rougier, Herve de Conchard, Jean-Marc Thuiller, and Bruno Serate.

Chapter 6: Major Findings and General Discussion

6.1. Summary of Major Findings

A new approach for formulating microbial biopesticides was studied that uses water-soluble UV-protective coatings and an oil carrier. This study examined effects of coating formulation on infectivity and spore-survival following exposure to simulated sunlight. Development of this formulation required information from a series of experiments. First, production of an infective a spore from in liquid culture that could survive drying was needed. This spore form then had to be dried with a coating material and processed to reduce particle size prior to suspension in an oil-carrier for application to target insects.

The two main hyphotheses tested in this study were as follows:

1) Water-soluble coatings enhance the survival of *M. anisopliae* var *acridum* spores in oil following exposure to simulated sunlight

Results support acceptance of this hypothesis

Water-soluble coatings increased the LT_{50} (95% confidence intervals) following exposure to simulated sunlight by approximately four times for aerial conidia. The LT_{50} values (95% confidence intervals) for non-coated aerial conidia and coated aerial conidia were 4.0 hr (3.1 - 5.1 hr) and 17.0 hr (12.5-23.0 hr), respectively. Water-soluble coatings also increased survival of submerged conidia. Although the LT_{50} values could not be accurately calculated for coated submerged conidia, because they did not lose more than 50% mortality following the longest exposure time (16 hr). Survival of coated-submerged conidia was similar to coated-aerial conidia. In a second experiment, percent survival of non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia following 16 hr of exposure to simulate sunlight were 6 ± 1, 62 ± 18, and 67 ± 9 hr, respectively.

2) Water-soluble coatings do not reduce the infectivity of *M. anisopliae* var *acridum* spores in oil.

Results support rejection of this hypothesis

Water-soluble coatings reduced the infectivity of aerial conidia and coated-submerged conidia were also less infective than non-coated aerial conidia. The LT_{50} values (95% confidence intervals) for non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia were 5.8 d (4.9-6.9 d), 8.2 d (7.3 - 9.3 d), and 10.4 d (8.2 - 13.3 d), respectively.

Prior to testing these two main hypotheses, a series of experiments required to examine effects of the spore production and formulation process on spore biology. The major finding from studies related to these objectives are as follows:

1) To compare the three spore-types of *M. anisopliae* var *acridum* (aerial conidia, submerged conidia, and blastospores) with respect to cell-wall characteristics, germination rate and drying stability.

   a) Submerged conidia were similar to aerial conidia with respect to spore dimensions and cell-wall thickness and these two spore types were significantly shorter than blastospores and possessed thicker cell walls.
   b) Spores from liquid culture had a higher concentration of surface carbohydrates than aerial conidia. However, submerged conidia could be distinguished from blastospores on the basis of having a lower affinity for wheat germ agglutinin, which binds n-acetyl-B-D-glucosaminyl and n-acetyl-B-D-glucosaminyl oligomers.
   c) Submerged conidia were significantly more anionic than aerial conidia and blastospores. The number of hydrophobic regions present on the surface of aerial conidia and submerged conidia were similar and both were higher than blastospores.

d) The 6-hr percent germination was highest for blastospores followed by submerged conidia and aerial conidia.
e) The drying stability on silica gel of aerial conidia and submerged conidia was similar, and both spore-types had higher drying stability than blastospores.
f) Submerged conidia were more infective to *S. americana* in an aqueous 20% molasses solution.

2) To investigate the influence of media osmolality in liquid culture on cell-wall surface characteristics, drying stability, infectivity, and spore-carbohydrate concentrations.

a) Increasing the osmolality of liquid media resulted in a change in spore-type from submerged conidia to blastospores on the basis of cell-wall characteristics. However, these blastospores had a higher survival rate after drying on silica gel than submerged conidia and were more infective to *S. americana* than submerged conidia and aerial conidia.

3) To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival in aqueous suspension.

a) The addition of sucrose, skim milk, glycerol, and a Kraft lignin (Curan 100®) to spore-coating formulations generally did not reduce percent germination over time or spore survival in aqueous suspension. Lignosulfonic acid (Ultrazine®) inhibited percent germination over time and spore survival in aqueous suspension.

4) To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival following an air-drying process.

a) Higher concentrations of sucrose reduced percent germination over time in skim milk-based formulations after drying. Sucrose generally improved survival of submerged conidia in Curan 100®-based formulations after drying, but was

generally not necessary when skim milk was present in the formulation. The final moisture content of the dry coated-spore formulation was related to the concentration of hygroscopic components in the spore-coating formulation (skim milk, sucrose, glycerol), however, spore survival after drying did not necessarily correlate with final moisture content.

5) To determine if there is an effect of spore-coating formulation on spore survival after reducing particle size of the dry coated-spore formulation.

   a) Formulation had a significant effect on spore survival after reducing the particle size of the coated-spore formulation by sieving. Higher concentrations of sucrose reduced spore survival in skim milk-based formulations after reducing particle size of the coated-spore formulation. Spore survival was generally greatest in spore-coating formulations that contained a combination of skim milk and Curan 100®.

6) To determine if there is an effect of spore-coating formulation on percent germination over time and spore survival after storing the dry coated-spore formulation at 28 C.

   a) Storage at 28 C for 6 d generally did not reduce the viability of submerged conidia or aerial conidia in dry spore-coating formulations. Germination over time was reduced for submerged conidia, but not aerial conidia, in many of the dry coated-spore formulations following 28 C-storage. The net result was increasing similarity in germination over time for submerged conidia and aerial conidia relative to values prior to drying and storage.

   b) Integrating information obtained from Objectives 3, 4 and 5 resulted in selection of a spore-coating formulation containing skim milk, Curan 100®, and glycerol (dry mass ratios of 1:0.5:0.5:0.1 for spores, skim milk, Curan 100®, and glycerol) for testing of the two main hypotheses of this study.

6.2. Summary and Conclusions

The first step in the development of this coated-spore formulation was selection of liquid-culture conditions to produce an infective spore that could survive drying. Jenkins and Prior (1993) first reported submerged conidia production in liquid culture. This study examined characteristics of submerged conidia including cell-wall characterisitics, drying stability, and infectivity. Submerged conidia were found to have similar drying stability to aerial conidia and were more infective in a water-based formulation, which may be related to their faster germination rates (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al, 1983). In addition, increasing the osmolality of submerged conidia-producing medium resulted in a shift in growth form to production of blastospores that had greater drying stability and infectivity than submerged conidia. The enhanced drying stability of these blastospores suggests a role of trehalose (Harman et al., 1991) since these spores were thin-walled and enhanced infectivity may have been related to faster germination rates.

Survival of submerged conidia and aerial conidia during air-drying with coating materials and processing of the dry material was optimized using various ratios of coating material. A series of air-drying experiments was used to select a single spore-coating formulation for further testing. This spore-coating formulation, containing a Kraft lignin (Curan 100®), skim milk and glycerol, significantly enhanced the survival of aerial conidia and submerged conidia following exposure to simulated sunlight. This approach provided greater UV-protection than other strategies that have relied on oil-soluble sunscreens in an oil carrier or water-soluble sunscreens in a water carrier (Inglis et al., 1995; Burgess, 1998; Hunt et al., 1994, Moore et al., 1993; Shah et al., 1998).

Although, the spore coating formulation reduced infectivity, it could potentially be improved in the future by further development of the formulation by increases in germination rates, hydrophobic binding with insect cuticle or water solubility of the coating material. A better understanding of changes in spore physiology during drying could improve both the germination rate and spore viability after drying and processing.

A closer look at physiological changes related to spore carbohydrate concentrations could be very useful for enhancing efficacy of formulated spores. Changes in spore-polyol concentrations during drying may effect both spore germination rate on the insect cuticle and spore germination at reduced water availability (Andersen et al., 1999; Hallsworth and Magan, 1995). Changes in spore-trehalose concentrations during drying may effect the desiccation tolerance of spores and the thermal-stress tolerance of the dry formulation **(**Crowe et al., 1984; 1988; 1990; Carpenter and Crowe, 1988 a, b; Colaco et al., 1992; Tan et al., 1995). In addition, improvements in the physical properties of the spore-coating formulation and coating process may provide a more homogenous coating reducing the need for particle size reduction.

Implementation of this approach to formulating microbial biopesticides will be strongly influenced by economic factors. The evaluation process must consider costs associated with spore production, formulation, storage, and application. Production of spores in liquid culture offers several practical and economic advantages to aerial culture (Jenkins and Goettel, 1997), however, submerged conidia cannot be readily formulated in oil (Jenkins and Thomas, 1997). This drying process allows for the oil-based formulation of submerged conidia, which has several advantages over water-based formulation, these include: 1) increased infectivity at low humidity (Bateman et al., 1993; Prior et al., 1988); 2) reduces effects of thermal stress (McClatchie et al., 1994; Hedgecock et al., 1995; Hong et al., 1997, 1998); and 3) compatibility with ultra-low volume (ULV) application technology. The cost of the coating material must be considered, and the potential for using technical grade lignin material or paper pulp industry waste products will need to be evaluated. Field testing of this material will be required to evaluate locust and grasshopper mortality under natural exposure conditions. Spore survival is typically shorter in laboratory simulated sunlight conditions, than under natural conditions, due to shading and sampling time (Ignoffo, 1992). The effect of spore coating on persistence under natural exposure conditions to solar radiation will need to be evaluated. Physical properties of the formulation, particularly hydrophobicity of the particles, will affect the potential of grasshoppers and locusts to pick-up residues of the coated-spore formulation from vegetation. Practical application with ULV-application technology will likely

require the production of smaller particle sizes, and the ability to apply using ULV or emulsifiable oil formulations will need to be tested.

This study has shown that coating spores with a water-soluble lignin derivative in combination with protective agents is an effective means for protecting spores from ultraviolet radiation. Further improvements in the spore production and coating process, spore physiological changes, and physical properties of the formulation may increase spore survival during the formulation process and enhance the infectivity of the coated spores. Field-testing will be needed to determine grasshopper and locust mortality under natural exposure conditions. Adaptation of this technology will depend on economic factors related to the cost of spore production, spore coating materials, the formulation process, storage, and application technology.

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Appendix I : Preliminary Experiments to Develop Methods for Drying Spores from Submerged Culture with UV-Protective Coatings

Freeze-drying was selected as a method for producing coated spores for initial drying experiments, based on the ability of *Beauveria bassiana* blastospores to survive freeze-drying with skim-milk coatings (Fargues et al., 1979). *Metarhizium anisopliae* var. *acridum* submerged conidia were able to survive freeze-drying with a combination of skim milk and glycerol or two lignin derivatives and glycerol. It was determined that the shelf life of this material was too short for adequate replication over time in studies on UV-tolerance and virulence, which required transport to INRA, France as described in Appendix III. Farques et al. (1979) also found that freeze-dried *B. bassiana* blastospores had relatively short shelf lives at 20 C and 30 C. It became apparent that a freeze-drying protocol we were using did not provide an effective means of producing coated submerged spores that had adequate shelf life for further experimentation or practical application. Attempting to produce a stable-coated spore by freeze-drying was abandoned for a more practical approach that used drying at ambient r.h. followed by reduced r.h. using silica gel as described in Chapter 4.

III. i. Materials and Methods

III. i. i. Preliminary Experiments on Freeze-drying Spores with UV-Protective Coatings

Spores were produced in a 1- L BioFlo III System (New Brunswick Scientific) as described in Section 4.3.1., with the exception that media was composed of 4% WBY : 4% Fructose, rather than 4% WBY : 4% Sucrose. The dissolved oxygen level was 5% of saturation and the final spore concentration after 5 d of incubation was 8.2 x 10^8 spores/mL.

Spores were washed three times by centrifugation at 3900-4000 RPM (2800 x G) for 5 minutes (Fisher Scientific Marathon 6K centrifuge) in 50-mL sterile centrifuge

tubes and re-suspension in 0.05% Tween 80 in sterile distilled water. The washed spores were divided into three equal portions. The following suspension media were added to the three portions of washed spores: 1) 10% skim milk, 1% glycerol, in sterile distilled water; 2) 10% Curan 100®, 1% glycerol in sterile distilled water; or 3) 10% Ultrazine®, 1% glycerol in sterile distilled water. Curan 100® is a Kraft alkali lignin and Ultrazine® is a sodium salt of a lignosulfonic acid (Aldrich # 37-095-9 and # 37-097-5, respectively). Suspension media (20 mL) was added to 20 mL of spore pellet. The estimated dry weight ratios were 1 : 0.3 : 0.03; spores : coating material : glycerol. Spores were suspended in the suspension media by vortexing and then immediately frozen at –80 C. The spore suspensions were freeze-dried. The maximum vacuum pressure obtained during freeze was 100mTorr. Initial sample temperatures were -80 C at the start of freeze-drying, but the freeze drier did not provide for the cooling of samples during the freeze-drying process or the monitoring of sample temperatures.

After freeze-drying, the percent germination was determined for each treatment as described in 4.3.2., without the use of benomyl The freeze-dried material was shipped to INRA, France for further processing, UV-tolerance testing, and virulence to *S. gregaria* (see Appendix 5.6). To reduce the particle size of the coated spores, the three freeze-dried treatments (skim milk; Curan 100®; Ultrazine®) were gently ground against the surface of a sieve (126 mesh) using a rubber spatula. In each case, the entire sample was able to pass the sieve without leaving residual material. The viability of this material was determined by checking germination as described in Section 4.3.2., without the use of benomyl. Freeze-dried formulations were qualitatively much easier to break into particles that would pass the sieve than air-dried formulations described in the body of this chapter.

Spores were to be suspended in 70% diesel fuel : 30% peanut oil for the purposes of UV-tolerance testing and virulence testing (Appendix III). However, the coating materials are not soluble in 70% diesel fuel : 30 % peanut oil. Therefore, the number of spores in a suspension of 70% diesel fuel : 30% peanut oil was estimated based on the number of spore/g of dry formulation, which was obtained by suspending a specific mass of formulation in water and determining the spore concentration. The concentration of

particles per mL of 70% diesel fuel : 30% peanut oil was also determined for each formulation at a corresponding spore concentration of 5 x 10^7 spores/mL. This is only an estimate of the particle concentration because hemacytometer counting is not recommended for such large particles.

This experiment was repeated twice. In the second experiment (Freeze-Drying Experiment 2) material was sieved before shipment. Additional test groups were added to the third experiment (Freeze-Drying Experiment 3), that used increased the mass of coating material (1 : 1 : 0.1; spores : coating material : glycerol (dry mass ratios)). In addition, material from the third replication of this experiment was purged with nitrogen gas and packaged in dry ice prior to shipment to INRA, Montipillier, France. This material was not sieved before shipment.

I. i. ii. Preliminary Experiments on Effects of Non-reducing Disaccharides on Desiccation Tolerance and Comparing Spore Survival during Freeze-drying to Air-drying

Experiments were conducted to study the effects of non-reducing disaccharides on desiccation tolerance and compare the ability of spores to survive freeze-drying versus air-drying. The first experiment (Sugar Freeze-drying Experiment 1) examined the effect of two non-reducing disaccharides (sucrose and trehalose) on desiccation tolerance spores when combined with skim milk, Ultrazine® and Curan 100® during freeze-drying. The second experiment (Freeze-drying/Air-Drying Experiment 1) evaluated the effect of various sucrose concentrations in combination with varying levels of skim milk, Ultrazine®, Curan 100® and glycerol on survival during freeze-drying and air-drying.

For the first experiment (Sugar Freeze-drying Experiment 1), spores were produced in a 1- L BioFlo III System (New Brunswick Scientific) as described in Section 4.3.1. The dissolved oxygen level was maintained at 5% of saturation and the final spore concentration after 6 d of incubation was **?** (need to get this from Heather's notebook) spores/mL. These spores were stored in 20% glycerol at -20 C rather than -80 C, which was standard for all other experiments in this dissertation. For the second experiment

(Freeze-drying/Air-Drying Experiment 1), spores were produced in a 1-L BioFlow III System as described in 4.3.1. The dissolved oxygen level was maintained at 50% of saturation and the final concentration after 6 d of incubation was 1.3 x 10^9 spores/mL. These spores were used in this drying experiment on the final day of the 6-d incubation and were not first stored in a frozen state.

Spores were washed three times by centrifugation at 3900-4000 RPM (2800 x G) for 5 minutes (Fisher Scientific Marathon 6K centrifuge) in 50 mL sterile centrifuge tubes and re-suspending sterile distilled water. The final pellet was resuspended in suspension media described in Tables I-i and I-ii for Sugar Freeze-drying Experiment 1 and Freeze-drying/Air-Drying Experiment 1, respectively. The percent germination of spores in each of the suspension media was tested for 24-hr incubation on 2% malt as described in Section 4.3.2 without the use of benomyl. Individual components of the suspension media were autoclaved at 114 C to avoid denaturing of the skim milk products and reaction between coating products at higher temperatures (Humber, 1997).

Table I-i. Coating Materials for Submerged Conidia During Freeze-drying (Sugar Freeze-drying Experiment 1).

Test Group	Dry Mass Ratios of Spores and Coating Material[1]
1	1 : 1 : 0.1 Spores : Skim Milk : Glycerol
2	1 : 1 : 0.1 Spores : Ultrazine® : Glycerol
3	1 : 1 : 0.1 Spores : Curan 100® : Glycerol
4	1 : 1 : 1 : 0.1 Spores : Skim Milk : Sucrose : Glycerol
5	1 : 1 : 1 : 0.1 Spores : Ultrazine® : Sucrose : Glycerol
6	1 : 1 : 1 : 0.1 Spores : Curan 100® : Sucrose : Glycerol
7	1 : 1 : 1 : 0.1 Spores : Skim Milk : Trehalose : Glycerol
8	1 : 1 : 1 : 0.1 Spores : Ultrazine® : Trehalose : Glycerol
9	1 : 1 : 1 : 0.1 Spores : Curan 100 ®: Trehalose : Glycerol

[1] Suspension media were as 10% dilutions of the coating materials (e.g. the suspension media in Test Group 1 was 10% Skim Milk : 1% Glycerol)

For freeze-drying, 0.7 mL samples of spore suspension from each treatment were placed into 15, 1.5 mL microcentrifuge tubes per treatment. These samples were capped with sterile cotton. Samples were maintained at 4 C for 3 hr and then transferred to -13

C for 0.5 hr to approximate a cooling rate of 3 C/min (Berny and Hennebert, 1991). They were then immediately transferred to dry ice.

Table I-ii. Coating Materials for Submerged Conidia During Freeze-drying and Air-drying (Freeze-drying/Air-Drying Experiment 1).

Test Group	Dry Mass Ratios of Spores and Coating Material[1]	
1	1 : 1 : 1 : 0.1	Spores : Skim Milk : Sucrose : Glycerol
2	1 : 1 : :1 : 1 : 0.1	Spores : Skim Milk : Ultrazine® : Sucrose : Glycerol
3	1 : 1 : 1 : 1 : 0.1	Spores : Skim Milk : Curan 100® : Sucrose : Glycerol
4	1 : 1.5 : 1.5 : 0.1	Spores : Skim Milk : Sucrose : Glycerol
5	1 : 0.5 : 0.5 : 0.1	Spores : Skim Milk : Sucrose : Glycerol
6	1 : 0.5 : 0.5 : 0.5 : 0.1	Spores : Skim Milk : Ultrazine® : Sucrose : Glycerol
7	1 : 0.5 : 0.5 : 0.5 : 0.1	Spores : Skim Milk : Curan 100 ®: Sucrose : Glycerol
8	1 : 0.75 : 0.75 : 0.1	Spores : Skim Milk : Sucrose : Glycerol
9	1 : 0.25 : 0.25 : 0.1	Spores : Skim Milk : Sucrose : Glycerol
10	1 : 0.25 : 0.25 : 0.25 : 0.1	Spores : Skim Milk : Ultrazine® : Sucrose : Glycerol
11	1 : 0.25 : 0.25 : 0.25 : 0.1	Spores : Skim Milk : Curan 100® : Sucrose : Glycerol
12	1 : 0.375 : 0.375 : 0.1	Spores : Skim Milk : Sucrose : Glycerol

[1] Suspension media were as 10% dilutions of the coating materials. For example the suspension media in Test Group 1 was 10% Skim Milk : 10% Sucrose : 1% Glycerol.

A modification was made to the freeze drier that allowed for samples to be placed into a -10 C to-13 C freezing chamber during the freeze-drying process. Samples were transferred directly from dry ice to the freezing chamber. Samples were dried under 40 to 50 mTorr vacuum. The temperature of the condenser was -60 C. The percent moisture for three replicate samples (approximately 0.1 g) of each test group was determined after freeze-drying by determining change in mass after heating for 24 hr at 100 C. The percent germination of freeze-dried spores was tested for three replicate samples after 24 hr incubation on 2% malt as described in Section 4.3.2., without the use of benomyl. Three replicate samples of freeze-dried spores from each test group were stored at 28 C for 2 weeks. Following 2 weeks of incubation, the percent germination was determined for the three replicate samples after 24-hr incubation on 2% malt as described in Section 4.3.2., without the use of benomyl.

For air-drying (Freeze-drying/Air-Drying Experiment 1), 0.7 mL of suspended spores from each of the 12 treatments was placed into three replicate 2.5 cm diameter

stainless steel dishes. These dishes were placed into a plastic container with a continuous flow of air that passed through a 500 mL Erlenmyer flask containing silica gel followed by a 0.2-µm air filter. The first Air-Drying Experiment was side experiment to Freeze-drying/Air-Drying Experiment 1. As a result some important parameters, such as relative humidity of the drying environment and % moisture of the dry product were not measured as they were in subsequent Air-Drying Experiments. Air dried samples were left at room temperature for 10 d before rehydrating to test for % germination as described in Section 4.3.2., without the use of benomyl.

I.ii. Results and Discussion

I.ii. i. Preliminary Experiments on Freeze-Drying Spores with UV-Protective Coatings

The viability of spores after specific steps in the formulation process and the final concentration of spores (spores/g of freeze-drying material) for the first of the freeze-drying experiments (Freeze-drying Experiment 1) are presented in Table I-iii. The viability after sieving represents loss in viability during the shipment to INRA, France and from the sieving process.

Table I-iii. Viability[1] of Coated Spores After Freeze-drying and Sieving (Freeze-drying Experiment 1)

Treatment (Coating) [2]	Viability After Freeze-drying (% germination)	Viability After Sieving (% germination)
Skim Milk	91	88
Curan 100®	83	71
Ultrazine®	86	63
No Protection	13	N/A

[1] Viability based on percentage of spores germination on 2% malt agar after 24-hr incubation.
[2] The dry mass ratios of treatments were all 1:0.3:0.03; spores : coating material : glycerol.

The concentration of spores in the freeze-dried material and an estimate of the number of spores corresponding to each particle in the freeze-dried formulation are provided in Table I-iv.

Table I-iv. Concentrations of Spores within a Coating Matrix Based on Dry Weight and Number of Spores per Sieved Particle.

Treatment (Coating) [1]	Spore Concentration In freeze-dried Powder (spores x 10^9/g)	Particle Concentration in Oil Formulation (Particles / mL) at 5 x 10^7 spores/mL	Average Spore Concentration (Spores / Particle)
Skim Milk	4.9	4.6 x 10^6	11
Curan 100®	7.0	6.7 x 10^6	7
Ultrazine®	6.3	6.8 x 10^6	7

[1] The dry mass ratios of treatments were all 1:0.3:0.03; spores : coating material : glycerol.

This material was used for UV-tolerance experiments and bioassays against *S. gregaria* (Appendix III), which were to be repeated over time to provide adequate replication. However, after two months storage at 4 C, viability of this material declined to between 1/500 and 1/1000 of its initial viability. The freeze-drying experiment was repeated twice in an effort to produce additional material for replication of UV-tolerance assays and *S. gregaria* bioassays, but neither of these replications of the experiment resulted in material that had adequate viability upon arrival at INRA. The viability of this material after freeze-drying and upon arrival at INRA is presented in Table I-v.

The ability of spores to survive freeze-drying was inconsistent among the three freeze-drying experiments and the shelf life of freeze-dried material was short. The short shelf lives may have been related to incomplete removal of water during the freeze-drying process. It is typical for freeze-dried fungi to have high viability after freeze-drying when insufficient water is removed, but rapid deterioration occurs (Smith, 1994). The moisture content of samples was not measured for these three experiments because the amount of material was limited and a minimum of 1 g of material was needed to accurately measure percent moisture.

Table I-v. Viability[1] of Coated Spores After Freeze-drying and Sieving (Freeze-Drying Experiments 2 and 3)

Treatment (Coating) [2]	Viability After Freeze-drying (% germination)	Viability After Sieving (% germination)	Viability After Shipment (% germination) [2]
Skim Milk - Expt. 2 A	65	47	< 1%
Curan 100® - Expt. 2 A	51	48	< 1%
Ultrazine® - Expt. 2 A	47	47	< 1%
Skim Milk - Expt. 3 A	44	N/A	N/A
Curan 100® - Expt. 3 A	30	N/A	N/A
Ultrazine® - Expt. 3 A	30	N/A	N/A
Skim Milk - Expt. 3 B	76	N/A	< 20%
Curan 100® - Expt. 3 B	62	N/A	< 20%
Ultrazine® - Expt. 3 B	56	N/A	< 20%

[1] Viability based on percentage of spores germination on 2% malt agar after 24-hr incubation.
[2] The dry mass ratios for 'A' treatments were 1:0.3:0.03; spores : coating material : glycerol and for 'B' treatments were 1 : 1 : 0.1 spores :coating material : glycerol.
[2] Viability after shipment from Experiment 2 was based on CFU counts performed at INRA.

I.ii.ii. Preliminary Experiments on Effects of Non-reducing Disaccharides on Desiccation Tolerance and Comparing Spore Survival during Freeze-drying to Air-drying

The moisture contents and survival of spores after freeze-drying and after storage for 2 weeks at 28 C as a dry product for Sugar Freeze-drying Experiment 1 are presented in Table I-vi. Skim milk demonstrated greater protection during freeze-drying than either of the two lignin derivatives regardless of the addition of non-reducing dissacharides (α = 0.05). Both sugars (sucrose and trehalose) improved the ability of spores to survive freeze-drying with either skim milk, Curan 100®, or Ultrazine® (α = 0.05). Sucrose provided better protection than trehalose when combined with milk or Curan 100®, but this effect was not significant when combined with Ultrazine® (α = 0.05). There was a significant decrease in viability after storage for 2 weeks at 28 C in all of the samples

other than those that had a combination of skim milk and sugar as a protective coating ($\alpha = 0.05$).

Table I-vi. Percent Moisture and Change in Viability[1] Before Drying, After Drying and After Storage of the Dry Spores for 2 Weeks at 28 C (Sugar Freeze-drying Experiment 1)

Test Group[2]	% Moisture After Drying	Viability Before Drying	Viability After Drying	Viability After 2 wks @ 28 C
1) Milk	11.8 ± 0.1 a[3]	62	18 ± 4 aef; α[4]	7 ± 4 acd; β
2) Curan 100®	4.7 ± 0.2 b	56	6 ± 1 b; α	4 ± 1 a; β
3) Ultrazine®	4.3 ± 0.1 c	61	8 ± 1 b; α	3 ± 0.4 a; β
4) Milk + Suc.	11.8 ± 0.3 a	72	40 ± 9 c; α	29 ± 5 b; α
5) Curan 100® + Suc.	5.8 ± 0.3 d	69	24 ± 5 ad; α	7 ± 1 c; β
6) Ultra. + Suc.	6.2 ± 0.2 d	75	26 ± 6 adf; α	11 ± 1 d; β
7) Milk + Tre.	7.5 ± 0.3 e	77	32 ± 10 d; α	39 ± 7 b; α
8) Curan 100® + Tre.	4.6 ± 0.3 bc	78	13 ± 3 ef; α	4 ± 2 a; β
9) Ultra. + Tre.	3.8 ± 0.2 c	71	17 ± 2 f; α	12 ± 3 d; β

[1] Viability determined by percent germination after 24-hr incubation on 2% malt agar.

[2] The coating material are described in greater detail in Table 5. Suc = sucrose, Ultra = Ultrazine®, Tre = trehalose.

[3] Values followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations). Values presented as means ± standard deviations.

[4] Values for viability after drying followed by different Greek letters are significantly different than values for viability after 2 wk incubation at 28 C at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

The moisture contents of freeze-dried samples and survival of spores after freeze-drying and air-drying for Freeze-drying/Air-Drying Experiment 1 are presented in Table I-vii. The freeze-drying process did not appear to be as efficient at removing water as in the previous experiment. The source of this difference is not known, but may relate to the inability of the freeze-drying system to maintain an accurate sample temperature. The survival of air-dried spores was dramatically higher than that of freeze-dried spores, although this could not be statistically tested because not all of the freeze-dried samples

were tested for viability. There was generally little difference among survival of air-dried spores using different protective coatings. Although viability may have been be statistically differentiated among the coating treatments, the differences were of little biological significance.

The data from preliminary drying experiments was used to justify a further investigation of air-drying methods for coating spores from liquid culture with UV-protective materials. Some of the changes in freeze-drying methods over the course of these experiments, which were intended to improve the stability of the freeze-dried

Table I-vii. Percent Moisture and Change in Viability[1] After Freeze-Drying and Air-drying (Freeze-drying/Air-Drying Experiment 1)

Test Group[2]	% Moisture After Freeze-drying	Viability After Freeze-drying[3]	Viability After Air-drying[4]
1) 1:1:1 SP:SM:Suc	24 ± 0.2	6	88 ± 2 a
2) 1:1:1:1 SP:SM:Ult:Suc	20 ± 0.3	2	82 ± 6 ab
3) 1:1:1:1 SP:SM:Cur:Suc	20 ± 0.2	4	89 ± 2 a
4) 1:1.5:1.5 SP:SM:Suc	25 ± 0.1	22	83 ± 1 b
5) 1:0.5:0.5 SP:SM:Suc	22 ± 0.3	0.5	89 ± 4 a
6) 1:0.5:0.5:0.5 SP:SM:Ult:Suc	17 ± 0.2	13	84 ± 8 abc
7) 1:0.5:0.5:0.5 SP:SM:Cur:Suc	18 ± 0.3	8	88 ± 3 ab
8) 1:0.75:0.75 SP:SM:Suc	22 ± 0.2	3	82 ± 6 abc
9) 1:0.25:0.25 SP:SM:Suc	20 ± 0.1	1	82 ± 3 b
10) 1:0.25:0.25:0.25 SP:SM:Ult:Suc	15 ± 0.7	2	76 ± 4 c
11) 1:0.25:0.25:0.25 SP:SM:Cur:Suc	16 ± 0.04	0	80 ± 4 bc
12) 1:0.38:0.38 SP:SM:Suc	19 ± 0.2	0.5	88 ± 6 ab

[1] Viability determined by percent germination after 24 hr incubation on 2% malt agar. For freeze-dried samples, viability in the remaining two replicates was not determined because viability was so low as compared to air dried samples.

[2] The coating material corresponding to each test group expressed as dry mass ratios (sp=spores; sm=skim milk; ult=ultrazin; cur=Curan 100®; suc=sucrose); each contained 0.1 glycerol. These formulations are described in greater detail in Table 6.

[3] Values followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

[4] Values for viability after drying followed by different Greek letters are significantly different than values for viability after 2 wk incubation at 28 C at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

product, may have acted to reduce spore viability. The freeze-drying equipment that was available for these experiments did not allow for the maintenance of sample temperature during the freeze-drying process. Samples freeze-dried in initial freeze-drying experiments may have partially thawed during freeze-drying resulting in high initial viability but poor storage characteristics (Smith 1984). In an attempt to ensure that samples remained frozen throughout the freeze-drying process an addition to the freeze-drying chamber was constructed that allowed samples to be maintained at -10 to -13 C. This temperature, however, may have been close to the freezing point of the cells cytoplasm, resulting in additional freeze/thaw damage (Smith 1984). The source of the difficulty with freeze-drying was not pursued further pursued because air-drying showed promise as a practical method for coating spores and was a more practical method for coating spores on an industrial scale (Hong et al., 2000).

Appendix II: Using Benomyl to Improve the Accuracy of Viability Determination Based on Percentage of Germinating Spores.

Spores with faster germination rates may have a greater potential for infection by reducing potential for desiccation, effects of other microorganisms, or loss during molting while on the insect cuticle (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnely, 1985; Hassan et al., 1983). One difficulty with evaluating the rate of germination, particularly over relatively long time intervals, is that the growth from spores with faster germination rates may obscure spores that have not yet germinated (Goettel and Inglis, 1997; Milner et al., 1991). This may lead to an overestimate of percent germination or the inability to practically assess percent germination due to interwoven hyphae. Additionally, I found that spores with fast germination rates may produce secondary spores during the incubation period. This seems to be particularly problematic for spores from submerged culture which typically have much faster germination rates that aerial conidia. This can lead to an underestimate of percent germination because the secondarily produced spores may be counted as non-germinated. Milner et al., (1991) developed a method for using benomyl (Benlate®) in germination media, which inhibits spindle formation during mitosis preventing nuclear division; allowing spores to germinate but inhibiting further hyphal development.

II.i. Materials and Methods

Milner et al. (1991) described liquid media for testing percent germination. This media contained 0.1% yeast extract; 0.1% chloramphenicol; 0.01% Tween 80; and 0.001 to 0.005% Benlate® (WP). I wanted to adapt the use of benomyl to techniques for measuring percent germination already being used in our laboratory. Benlate® (50 WP) was added to 2% malt agar in the following concentrations 0%, 0.001%; 0.01% and 0.1%. A suspension of blastospores (60 µL) from Adamek's media were spread on four replicate 60 mm Petri dishes of each treatment. These plates were incubated for 16 hr

and 22 hr at which time they were killed with 20% formalin by spreading the formalin solution over the surface of the plate with a glass rod.

To further examine the effects of benomyl germination counts, initial germination of spores in various suspension media from Air-Drying Experiment 1 (see Section 4.3.3.) was tested on 2% malt agar and 2% malt agar plus 0.001% benomyl. Germination was stopped after 24-hr with 20% formalin.

II.ii. Results and Discussion

The percent germination of blastospores on 2% malt agar containing varying levels of benomyl after 16- and 22-hr incubation is presented in Table II-i. In the absence of benomyl, blastospores produced secondary spores between 16- and 22-hr incubation times leading to a significant underestimate of spore viability at the 22-hr incubation time ($\alpha = 0.05$). This underestimate may have been accentuated by the using a glass rod to spread formalin over the surface of the plate causing the release of secondary spores from sporogenous hyphae (data not shown). The method of spreading formalin over the surface of the plate with a glass rod was abandoned for a method that distributed formalin by tilting the plate. Higher benomyl concentrations (0.01 and 0.1%) inhibited germination of spores ($\alpha = 0.05$).

Table II.i. Percent Germination of Blastospores After 16 hr and 22 hr Incubation on 2% Malt Agar Plus Increasing Concentrations of Benomyl

Treatment	% Germination (16 hr Incubation)	% Germination (22 hr Incubation)
2% Malt (No Benomyl)	94 ± 4 a[1], α[2]	63 ± 5 a, β
2% Malt + 0.001% Benomyl	94 ± 2 a, α	96 ± 1 b, α
2% Malt + 0.01% Benomyl	73 ± 3 b, α	86 ± 3 c, α
2% Malt + 0.1% Benomyl	4 ± 2 c, α	N/A

[1] Values followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations). Values are presented as means ± standard deviations

[2] Values followed by different Greek letters are significantly different than values within the same row at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

The percent germination of spores in various media before drying from Air-Drying Experiment 3 after 24-hr incubation on 2% malt and 2% malt + 0.001% benomyl is presented in Table II-ii. Incubation on 2% malt without benomyl resulted in an underestimate of viability because of secondary spore production in all but treatments (Treatments 5 and 6) ($\alpha = 0.05$). The germination rates of spores in these suspension media may have been delayed resulting in a decreased production of secondary spores after 24-hr of incubation ($\alpha = 0.05$) (see Section 4.3.3.).

Table II-ii. Percent Germination of Spores in Various Suspension Media from Air-Drying Experiment 1 Incubated for 24 hr on 2% Malt or 2% Malt + 0.001% Benomyl

Treatment[1]	% Germination (24 hr) 2% Malt	% Germination (24 hr) 2% Malt + Benomyl
0.5 : 0.5; SM : Suc	61 ± 8 a[2]	88 ± 2 b
0.5 : 0.25; SM : Suc	65 ± 7 a	90 ± 1 b
0.5 : 0.125; SM : Suc	52 ± 2 a	91 ± 2 b
0.5 ; SM	66 ± 4 a	91 ± 2 b
Glycerol Only	73 ± 10 a	79 ± 1 a
0.5 : 0.5 : 0.5; SM : Ult : Suc	61 ± 7 a	63 ± 16 a
0.5 : 0.5 : 0.5 ; SM : Cur : Suc	57 ± 2 a	89 ± 2 b

[1] The mass/volume ratios (g of coating material per L of distilled water) were 100 times the dry mass ratio relative to 1 part spores presented above (e.g. the dry mass ratios of spores, skim milk, sucrose and glycerol for Test Group 1 were 1: 0.5 : 0.5 : 0.1 x 100 = suspension media containing 100 g/L spores : 50 g/L skim milk : 50 g/L sucrose : 10 g/L glycerol).

[2] Values followed by different lower-case letters are significantly different than values within the same column at the $\alpha = 0.05$ level (Wilcoxon two-sample test for non-paired ranked observations).

Appendix III. Effects of Coating Spores with Water-Soluble Lignin Derivatives during Freeze-drying on the Tolerance of Spores to Simulated Sunlight and Infectivity to *Schistocerca gregaria*

Abstract

Experiments were conducted to determine if coating *M. anisopliae* var. *acridum* submerged conidia with two lignin derivatives and skim milk during a freeze-drying process affected their UV-tolerance and infectivity to *S. gregaria*. Coating submerged conidia with either of two lignin derivatives or skim milk resulted in a spore formulation that appeared to have greater UV-tolerance than aerial conidia. These coated formulations remained virulent to *Schistocerca gregaria* when suspended in an oil formulation. The shelf life of the freeze-dried formulation was too short to replicate the simulated sunlight experiment and bioassay over time. Attempts to produce additional freeze-dried coated spores did not produce material with an adequate shelf life for replication of the simulated sunlight experiments or bioassays. Because of the short shelf life of freeze-dried formulations this method of drying was abandoned for an air-drying process described in Chapter 4.

III. i. Materials and Methods

III. i. ii Production of Dry Coated-Spore Formulations

The production of coated aerial conidia and submerged conidia is described in Appendix I: Preliminary Experiments to Develop Methods for Drying Spores from Submerged Culture with UV-Protective Coatings. For UV-tolerance testing and infectivity to *S. gregaria*, the final sieved product from the first freeze-drying experiment, including sumberged conidia coated in Curan 100®, Ultrazine®, and skim milk each at approximately 33% of the dry weight of spores and each containing 10% glycerol relative to the dry weight of spores.

The formulated material was produced at Virginia Tech and simulated sunlight experiments and *S. gregaria* bioassays were conducted with the assistance of collaborators at the Centre de Biologie et de Gestation des Populations (CBGP), Institut Nationale de Recherche Agronomique (INRA), Monferrier-sur-Lez, France. I traveled to this research center to conduct these experiments. For subsequent experiments using simulated sunlight described in Chapter 5, formulated material was produced at Virginia Tech and shipped to INRA along with 2% malt Petri plates containing benomyl for germination counts. Simulated sunlight exposure was conducted by researchers at INRA and germinated plates were shipped back to Virginia Tech for evaluating percent germination.

III. i. ii. Exposure to Simulated Sunlight

Irradiation experiments consisted of depositing non-coated aerial conidia and coated-submerged conidia formulations onto membrane filters and exposing them to broad wavelengths greater than 295nm, at increasing increasing time intervals. Non-coated aerial conidia from 2- to 3- week old cultures on Paris medium and the three coated-submerged conidia formulations were suspended in 70% diesel fuel:30% peanut oil at a concentration of 1×10^7 spores/mL. The concentration of non-coated aerial conidia was determined using a hemacytometer. The spore concentration for the freeze-dried material was based on the mass of freeze-dried material and a predetermined spore concentration (spores/g of freeze-dried material). For each treatment, 10 mL of 1×10^7 spores/mL were deposited onto a 0.45 µm membrane filter (ME25 Schleicher and Schuell, Dassel, Germany). Deposition onto the filter was done using a Millipore vacuum filtration system (Millipore 10-047-04, 10-047-02). The total area of the inoculum deposit using this system was 10 cm^2. Six membrane filters were prepared for each treatment. Irradiation tests were conducted in a controlled environment chamber using an artificial sunlight device. The artificial sunlight device consisted of two 400 W high pressure metallic halogen lamps (HQI-TS, OSRAM, F67120, Molsheim, France), which emit a continuous spectrum from 270 to 1100 nm. A long pass glass filter

(WG295-Schott Glaswerke, Mainz, Germany) was used to block shorter wavelengths (under 295 nm) to simulate natural sunlight. One filter membrane containing spores from each treatment type was exposed to UV irradiance of 0.585 W m^{-2} for 0, 4, 8, 12 or 16 hr, which correspond to UV irradiances of 0, 4.32, 8.46, 12.78, and 17.10 kJ m^{-2}, respectively. Membrane filters covered with a metal plate to block radiation were used as controls for each of the different formulations and were placed in the controlled environmental chamber under simulated sunlight for 16 hr. During the exposure period, the surface temperature of the membrane filters was regulated at 25 ± 1 °C and the air humidity ranged from 40 to 50% r.h.

After exposure, spores were removed from the filter by placing the membrane in 10 mL of sterile distilled water (with approximately 15 µL of Tween 80) in 25-mL flasks and then shaking the flasks for 5 minutes at 700 oscillations min^{-1} (10 cm vertical travel) on a mechanical shaker. For the samples that contained lignin, it was necessary to first moisten the filter paper with water to reduce sample loss resulting from the lignin dissipating as a powder. Suspensions (30 µL) of approximately 1 x 10^6 spores/mL were plated onto Paris media and percent germination after 24- and 48-hr incubation at 25 °C was determined for 200 spores examined at 320 x magnification. A single germination plate was observed for each treatment/exposure time combination. The germination of controls (0-hr exposure) and treatments exposed for 4 hr of UVB was examined after 24 hr; whereas the germination of all other treatments was examined after 48 hr of incubation. This was done because benomyl was not being used for germination tests at the time that this experiment was conducted. For the 0-hr and 4-hr treatments, there was too much hyphal growth following 48 hr of incubation making it difficult to determine germination percentages. However, since UV-exposure is known to delay the germination rate of *Metarhizium* spp. this makes it difficult to include the data obtained for the 4-hr exposure period with longer exposure times when determining LT_{50} values.

In addition to percent germination, colony forming units (CFUs) were determined for suspensions of irradiated spores on four replicate Petri plates containing Paris medium using a spiral plating method (Gilchrist et al., 1973). The spore suspension used for CFU counts was approximately 3.3 x 10^5 spores/mL before the spiral plating. After 4 d of

incubation in the dark at 25° ± 1°C, colonies were enumerated on three consecutive days to ensure that CFU's accounted for delayed growth.

Two criteria were used to examine the viability of *M. anisopliae var. acridum* propagules after exposure to simulated sunlight, percent germination and colony forming units (CFU's). Viability estimates based on CFU's are concentration dependent and, therefore, may be affected by percent recovery of spores from the membrane filter and clumping of spores in the suspension. Viability estimates based on percent germination use direct observation and, therefore, independent of these factors. This will be discussed further in Appendix V.

III. i. iii. Infectivity to *Schistocerca gregaria*

The source of coated spore formulations including (Ultrazine®-coated, Curan 100®-coated and skim milk-coated submerged conidia) was the same as for simulated sunlight experiments. The inoculum concentration of submerged spores was determined by weighing a mass of freeze-dried material having a known concentration of spores per gram. Aerial conidia were harvested from 20-day old plate cultures on Paris medium. All inoculum suspensions were made in 70% diesel fuel : 30% peanut oil as a carrier. Controls insects were exposed to the carrier only.

A laboratory colony of *S. gregaria* was maintained at 28/35 °C (12/12 h photoperiod and thermoperiod) in cages provided with a 60 watt incandescent light bulb during the elevated thermoperiod, which provided a vertical heat gradient. Nymphs and adults were reared on a diet of wheat leaves and bran.

The percent germination of each inoculum was measured by observing 200 spores at a concentration of approximately 1×10^6 spores/mL in sterile distilled water on Paris medium after 24 hr of incubation at 24 °C. Each formulation was tested at four doses corresponding to 1×10^3, 1×10^4, 1×10^5, and 1×10^6 viable spores/insect.

Bioassays were performed on approximately 8-d old *S. gregaria* adults. Lots of 10 adults with a sex ratio approximately 1:1 were treated using topical application of 2 µL of formulated inoculum with a micropipette directly under the pronotal shield. Treatment

groups of 10 adults were contained in wire cages (27 cm x 19 cm x 13 cm) and maintained at 28 °C, 43% R. H. and a light regime of 12:12 (L:D) in individual controlled-humidity chambers. Relative humidity was maintained at 43% in these chambers by a custom-made device that pumped air over a saturated K_2CO_3, $2H_2O$ solution into the chambers. Relative humidity was monitored within each test chamber with a probe attached to a data logger (21X Micrologger, Campbell Scientific, Shepshed, Leicestershire, U.K.).

Locust mortality was determined daily and cages were replenished with fresh wheat seedlings. Cages were also provided with a constant supply of fresh wheat bran. This experiment was repeated twice using 10 insects/dose for a total of 20 insects per dose. The relative infectivity of each of the formulations was compared on the basis of LT_{50} values for each of the dose levels.

III. ii. Results

III. ii. i. Exposure to Simulated Sunlight

The percent germination for aerial conidia and three coated-submerged conidia formulation following exposure to increasing exposure times to simulated sunlight is presented in Table III-i. Initial percent germination reflects viability loss during the formulation process. Values for percent germination corrected for initial viability is presented in parentheses in Table III-i. The LT_{50} values for exposure simulated sunlight could not be accurately determined because the 4-hr exposure time used a shorter germination time (24 hr) than the longer exposure times (48 hr). The 24-hr incubation time likely resulted in an underestimate of viability of spores exposed to 4-hr of simulated sunlight because exposure to simulated sunlight resulted in delayed germination. However, because benomyl was not added to the Paris medium, which was used for percent germination, excessive hyphal growth by a proportion of the spore population after 48-hr incubation made it was impossible to accurately determine percent germination at this time.

Table III-i. Percent Germination[1] of Aerial Conidia and Three Coated-Submerged Conidia Formulations Following Exposure to Increasing Time Intervals of Simulated Sunlight.

Formulation	Percent Germination following Exposure (Hr) to Simulated Sunlight					
	0 hr (Control)	4 hr	8 hr	12 hr	16 hr	16 hr (control)[3]
Non-Coated Aerial Conidia	95 (100)[2]	44 (46)	7 (8)	3 (3)	0 (0)	92 (97)
Milk-Coated Submerged Conidia	88 (100)	33 (38)	55 (62)	13 (15)	11 (11)	74 (84)
Curan 100®-Coated Submerged Conidia	77 (100)	35 (50)	42 (59)	32 (45)	18 (25)	53 (75)
Ultrazine®-Coated Submerged Conidia	63 (100)	41 (65)	43 (68)	25 (40)	18 (29)	50 (80)

[1] Percent germination for 0 and 4 hr of exposure and 16 hr control was based on 24-hr incubation and for longer exposure times (8, 12, and 16 hr) was based on 48-hr incubation.

[2] Numbers provided in parentheses represent % germination corrected for initial viability (Corrected % germination = % germination at 4, 8, 12, or 16 hr of exposure ÷ proportion germinating in the control (0 hr of exposure))

[3] The 16-hr (Control) represents germination of spores that on filter membranes that were covered with a metal plate to eliminate exposure to simulated sunlight and left in the simulated sunlight incubation chamber for 16 hr.

The change in viability based on colony forming units (CFU) for aerial conidia and three coated-submerged conidia formulations following exposure to increasing exposure times to simulated sunlight is presented in Table III-ii. Submerged conidia in each of the coating treatments appeared to have greater UV-tolerance than aerial conidia. Curan 100®- coated spores appeared to have the highest UV-tolerance followed by Ultrazine®-coated spores and milk-coated spores. Ultrazine® was the only formulation that lost viability in the 16-hr covered control. This treatment also demonstrated a rapid decline in spore viability from 12- to 16-hr of UV exposure. None of these comparisons were statistically analyzed because this data could not be replicated over time due to the short shelf life of freeze-dried formulations. The four replicate plates referenced in the

footnote to Table III-ii represent four plates taken from the same membrane filter and, therefore, are not independent measurements that could be used for statistical analysis.

Table III-ii. Colony Forming Units (CFU) of Aerial Conidia and Three Coated-Submerged Conidia (S. C.) Formulations Before and After Exposure to Increasing Time Intervals of Simulated Sunlight.

	Viability in CFU x 10^6 / cm^2 Hours of UVB Exposure					
Treatment	0 hr	4 hr	8 hr	12 hr	16 hr	16 hr (control)
Non-coated Aerial Conidia	4.2[1]	0.62	0.15	0.083	0.026	4.9
Skim Milk-Coated S. C.	4.5	1.7	0.91	0.34	0.088	4.4
Curan 100®-Coated S. C.	1.9	1.8	1.2	0.56	0.11	2.1
Ultrazine®-Coated S. C.	3.3	1.5	1.5	0.53	0.02	2.4

[1] **The number of colony forming units is based on four replicate plates. Each exposure time / formulation treatment combination was performed once.**

The three coated-submerged conidia formulations appeared to have greater tolerance to simulated sunlight than non-coated aerial condia. The two lignin-coated formulations appeared to provided greater UVB protection than skim milk coating. Statistical comparisons have not been made because this experiment has not been able to be replicated due to the shorter shelf life of freeze-dried formulations. In order to quantify the amount of protection provided by coating, it will be necessary to expose uncoated spores produced by submerged liquid fermentation. This was not possible because submerged conidia could not be suspended in the oil formulation without first drying the spores, and the spores could not survive drying without a protective coating.

III. ii. ii. Infectivity to *Schistocerca gregaria*

Mortality over time (Abbott's) for *S. gregaria* adults exposed by direct dosing to four doses each of aerial conidia and three coated-submerged conidia formulations is presented in Figure III-i. The LT_{50} values that could be accurately determined from mortality data using probit analysis are presented in Table III-iii. Because of the rapid increase in mortality over relatively short time intervals, there were few measurements between 0 and 100% mortality that could be used for estimating LT_{50} values. LD_{50} values could not be accurately determined because only a single dose resulted in less than 100% mortality in each of the coated-spore formulations and all doses resulted in 100% mortality for aerial conidia. Each of the coated-submerged conidia treatments were highly virulent to *S. gregaria* adults resulting in 100% mortality in all of the treatments but the lowest dose, with an apparent reduced infectivity at the lowest dose resulting in delayed mortality relative to aerial conidia. The infectivity of the coated submerged conidia treatments appeared to be lower than that of aerial conidia at the lowest dose (1×10^3 spores/insect). However, because of the short shelf life of the freeze-dried formulations, this bioassay could not be repeated more than two times to provide adequate replication for statistically comparing final mortality at the 1×10^3 spore/insect dose.

On the basis of relative infectivity of aerial conidia, *M. anisopliae* var. *acridum* appears to be more virulent to *S. gregaria* than *S. americana* when comparing results of these bioassays with conducted with *S. americana* in Chapter 5. At the lowest dose 1×10^3 spores/insect, *M. anisopliae* var *acridum* aerial conidia produced no significant mortality in *S. americana* adults after 14 d of incubation, whereas, *S. gregaria* exhibited 100% mortality within 9 d of incubation. These results are in agreement with the work of Sieglaff et al. (1998) who found even lower infectivity of *M. anisopliae* var *acridum* to *S. americana* than the results of our bioassays in Chapter 4.

Table III-iii. LT_{50} Values for Non-coated Aerial Conidia and Three Coated-Submerged Conidia (S. C.)Formulations against *Schistocerca gregaria* Adults using Direct Dosing at Four Spore Concentrations

Formulation	1×10^3 spores/insect LT_{50} (95% C.I.)	1×10^4 spores/insect LT_{50} (95% C.I.)	1×10^5 spores/insect LT_{50} (95% C.I.)	1×10^6 spores/insect LT_{50} (95% C.I.)
Non-coated Aerial Conidia	7.0 (5.5-8.9) [1]	***	***	***
Skim Milk-Coated S. C.	7.7 (6.8-8.8)	***	***	***
Ultrazine®-Coated S. C.	***[2]	6.2 (5.5-7.0)	***	***
Curan 100®-Coated S. C.	***	6.2 (5.4-7.0)	***	***

[1] Numbers are presented as LT_{50} values followed by 95% confidence intervals in parentheses.

[2] Treatments designated with *** indicate that the LT_{50} could not accurately be determined because there were not enough values that were between 0 and 100% mortality to accurately estimate an LT_{50} or those that were between 0 and 100% could not be statistically distinguished from a slope of 0.

II. iii. Conclusions

Results from simulated sunlight experiments suggest that coated submerged conidia had greater tolerance to simulated sunlight than aerial conidia, and the highest UV-tolerance appeared to be in the Curan 100®-coated spores. Each of the coated-submerged conidia treatments were highly virulent to *S. gregaria* adults with an apparent decline in infectivity at the lowest dose (1×10^3 spores/insect). *Metarhizium anisopliae* var *acridum* aerial conidia appeared to be more virulent to *S. gregaria* than *S. americana* on the basis of comparison with bioassays conducted in Chapter 5. Statistical comparison of this data was not conducted because of the lack of replication over time and the inability to produce freeze-dried material that had a long enough shelf life to allow for transport of the material to INRA and replication of the experiment over time at INRA.

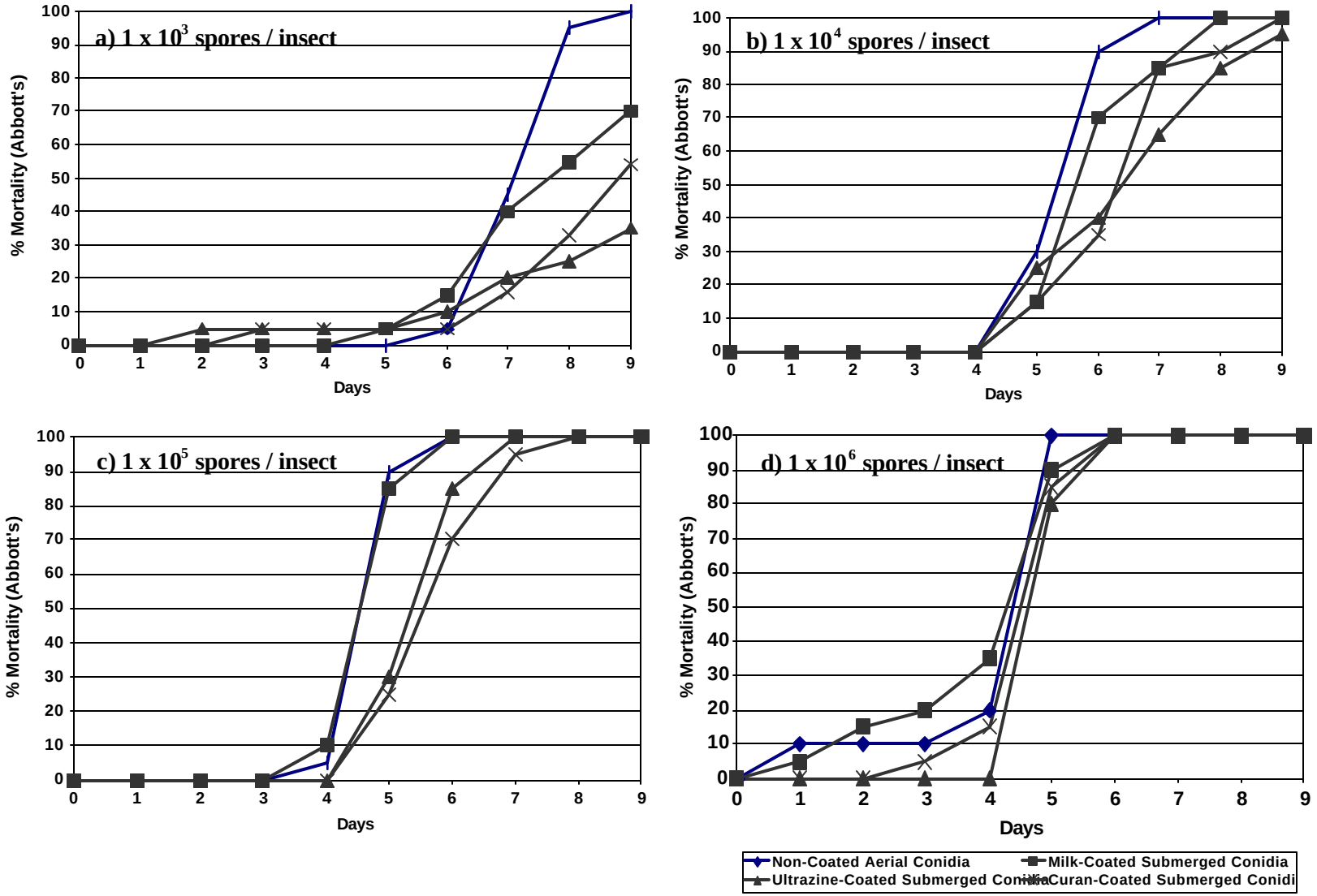

Figure III-i. Average adult *Schistocerca gregaria* mortality over time for aerial conidia and three coated-submerged conidia formulations of *Metarhizium anisopliae* var. *acridum* at three doses [a) 1 x 10^3 viable spores/insect; b) 1 x 10^4 viable spores / insect; c) 1 x 10^5 viable spores/insect; and d) 1 x 10^6 viable spores/insect. Mortality is corrected for control mortality by Abbott's formula. Each data point represents two replicate cages of ten insects

Appendix IV: Comparison of Percent Germination Verses Colony Forming Units for Evaluating Survival of Aerial and Submerged Conidia following Exposure to Simulated Sunlight.

Abstract

Two criteria were used to examine the viability of *M. anisopliae var. acridum* propagules following exposure to simulated sunlight in experiments described in Appendix III and Chapter 5, percent germination and colony forming units (CFU). Viability estimates based on CFU are concentration dependent and, therefore, may be affected by percent recovery of spores from the membrane filter and clustering of spores in the suspension. Viability estimates based on percent germination use direct observation of the spores and, therefore, independent of these concentration factors. While conducting percent germination counts in the two simulated-sunlight experiments (UV-Experiments 1 and 2) described in Chapter 5, the clustering of non-coated aerial conidia appeared to increase with increasing exposure times to simulated sunlight. The clustering of spores in each of the formulations (non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia) at each of the simulated sunlight exposure times was quantitated to explain differences in using percent germination and CFU's as methods for estimating viability and provide guidance for future work with formulated material in simulated-sunlight experiments.

IV. i. Materials and Methods

Methods used for testing the tolerance of three formulations (non-coated aerial conidia, coated aerial conidia and coated submerged conidia) to simulated sunlight are described in detail in Chapter 5. Aerial conidia and submerged conidia were coated with a dry mass ratio of 1 : 0.5 : 0.5 : 0.1 spores : Curan 100® : skim milk : glycerol. In order to quantitate the potential effect of spore clustering on viability estimates, an estimate of the mean number of spores per cluster was obtained. This estimate was obtained by counting the number of spores in the first 26 potential CFU's (ranging from a single spore

to a cluster of spores) that were randomly encountered while observing transects of the 2% malt Petri plates during percent germination counts.

IV. ii. Results and Discussion

The mean number of spores per cluster for each of the three formulations (non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia) over increasing simulated sunlight exposure time during UV-Experiment 1 from Chapter 5 is presented in Figure IV-i. The average number of spores per cluster increased for the non-coated aerial conidia over increasing exposure time. At the final exposure time (16 hr), viability estimates based on CFU counts could have underestimated spore viability by as much as 50% due to spore clustering. However, for the purposes of the first UV-experiment, this effect likely did not greatly influence results of CFU experiments relative to percent germination counts since the loss in viability was almost 100% at the longest exposure time. Spores in the coated-spore formulations did not appear to become clustered with increasing exposure time. It may be that the formulation acts to separate spores, eliminating the potential for spores to adhere to one another.

The clustering of spores in non-coated aerial conidia, coated-aerial conidia, and coated-submerged conidia formulations from the second UV-experiment (UV-Experiment 2) of Chapter 5 when suspended in water, suspended in oil, and following exposure to 16 hr of simulated sunlight is presented in Table IV-i. The initial suspension of non-coated aerial conidia in oil had clusters of aerial conidia. The difference between this non-coated aerial conidia suspension and the non-coated aerial conidia suspension used in UV-Experiment 1 that caused clustering in the second experiment is not known The UV-tolerance of non-coated aerial conidia in the second simulated sunlight experiment appeared to be higher than in the first. It may be that clustering of aerial conidia in the second simulated sunlight experiment resulting in the shading of a portion of the non-coated aerial conidia resulting in increased tolerance to simulated sunlight. Following exposure to 16-hr of simulated sunlight, the clusters of non-coated aerial conidia were too large to accurately estimate the number of spores per cluster. Coated

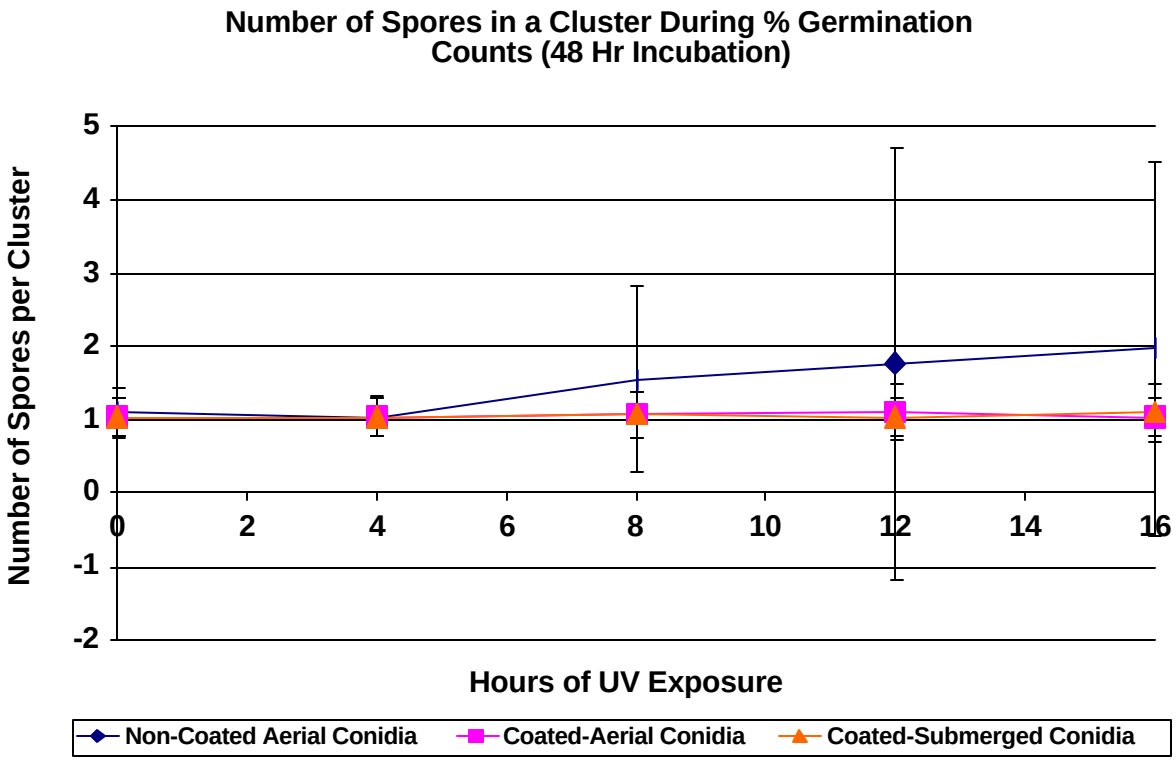

Figure IV-i. Number of Spores in individual clusters that could form a single colony-forming unit during percent germination counts of spores exposed to simulated sunlight. Values presented as means ± standard deviation.

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Table IV-i. Number of Spores in Individual Clusters that Could Form a Single Colony-Forming Unit for Spores in Three Formulations Suspended in Water, Oil, or Following Exposure to 16-hr of Simulated Sunlight in an Oil Suspension

Formulation	Number of Spores per Cluster (Means ± Standard Deviations)		
	Initial Suspension (In Water)	Initial Suspension (In Oil Carrier)	16-hr Exposure to Simulated Sunlight
Non-coated Aerial Conidia	1.1 ± 0.3	3.2 ± 5.0	Too high to accurately estimate
Coated Aerial Conidia	1.1 ± 0.3	1.1 ± 0.4	1.2 ± 0.5
Coated Submerged Conidia	1.1 ± 0.4	1.1 ± 0.4	1.0 ± 0.3

aerial conidia and coated submerged conidia did not form clusters in any of the treatments.

IV. iii. Conclusions

The clustering of non-coated aerial conidia in oil based formulations may cause difficulties with using CFU to estimate viability in simulated sunlight experiments because this measurement is concentration-dependant and because clustering may result in shading of a portion of the population. Coated spores did not form clusters when spread onto Petri plates for percent germination counts after being suspended in oil or following exposure to simulated sunlight in the oil formulaiton. The coating material may have prevented spores from adhering to one another in the oil formulation. The mechanism by which simulated sunlight caused clustering of the aerial conidia is not known, and it is not known if this effect occurs in aqueous suspensions. For future simulated sunlight experiments using oil-based formulations, this data indicates that percent germination counts should be used as a basis for estimating viability.

Vita

Jarrod Ethan Leland was born on September 27, 1971 in Montague, Massachusetts to Laurence and Deborah Leland. Jarrod Graduated from Elkins High School in Elkins, West Virginia in May 1989. Jarrod attended Virginia Polytechnic Institute and State University in Blacksburg, Virginia where he received his Bachelor of Science degree in Biochemistry in 1993. He continued his studies at Virginia Polytechnic Institute and State University to complete his Masters of Science in Entomology in December, 1998 with a thesis entitled Evaluating the Hazard of Land Applying Composted Diazinon Using Earthworm Bioassays. He received his Ph.D. in Entomology from Virginia Polytechnic Institute in December, 2001 with a dissertation entitled Environmental-Stress Tolerant Formulations of *Metarhizium anisopliae* var. *acridum* for Control of African Desert Locust (*Schistocerca gergaria*). Jarrod currently lives with his wife, Myra, in Stoneville, Mississippi where he works as a research entomologist for USDA-ARS, Southern Insect Management Research Unit as an invertebrate pathologist.