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FORMULATION OF *COLLETOTRICHUM COCCODES* AS A BIOHERBICIDE

by
FADIA SAAD

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
AND RESEARCH IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
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FOREWORD

This thesis is submitted in the form of original papers suitable for journal publications. The first section is a general introduction presenting the theory and previous knowledge on this topic. The next two sections represent the body of the thesis (each is a complete manuscript). The last section is a general discussion and a synthesis of the major conclusions. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines Concerning Thesis Preparation, section 7 "Manuscripts and Authorship" which are as follows:

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Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. A.K. Watson, Department of Plant Science, Macdonald Campus

of McGill University. The first manuscript is co-authored by Dr. A.K. Watson and Dr. J.P. Smith and the second one by Dr. A.K. Watson and Dr. S. A. Sparace. Dr. J. P. Smith and Dr. S. A. Sparace were members of my Graduate Advisory Committee and each contributed to the development of the concepts in the respective manuscripts for which they are listed as co-authors. For consistency and convenience, all manuscripts follow the same format. The copies that will be sent to the respective journals, however, will follow the requirements of each journal.

CONTRIBUTION TO KNOWLEDGE

The following aspects of the research described in this thesis are original contributions to scientific knowledge of biological weed control:

(1) The formulation developed is unique for *Colletotrichum coccodes*.

(2) For the first time, the effect of various factors including germination stimulants, cutigwse, pectinase and/or cellulase inducers on formulated *c. coccodes* conidia was examined.

ABSTRACT

Ph. D. Fadia Saad Plant Science

FORMULATION OF *COLLETOTRICHUM COCCODES* AS A BIOHERBICIDE

Colletotrichum coccodes (Wallr.) Hughes, a foliar pathogen of velvetleaf, is being developed as a bioherbicide. Formulation of living organisms for use as pest control products presents unique problems. This research has achieved the development of an adequate formulation of the pathogen by using kaolin clay or talcum powder (1:2.79 wt/wt) as the fillers to dry conidia. Formulated *C. coccodes* conidia stored at 4, 30C, or at room temperature in bags permeable to oxygen remained viable and able to infect velvetleaf plants at least six months in storage. Various reported germination stimulants increased germination of formulated conidia, although not significantly, whereas increasing concentrations of cutin resulted in subsequent decreases in germination and appressoria formation of fresh as well as formulated conidia. In controlled environment experiments, 14 day-old velvetleaf seedlings were severely diseased when stearic or oleic acids were added to conidia formulated in kaolin clay or talcum powder, respectively. Combinations of germination stimulants, cutinase and/or pectinase inducers did not significantly increase germination and appressoria formation of *C. coccodes* conidia. Germination of fresh and formulated conidia increased, although not significantly, with the addition of 1% sucrose.

RESUME

Ph. D. Fadia Saad Phytologie

Colletotrichum coccodes, un pathogène foliaire de l'abutilon, est en voie de développement comme bioherbicide. La formulation des organismes vivants utilisés comme agents de biocontrôle constitue un problème unique. La présente recherche a complété le développement d'une formulation adéquate du pathogène à travers l'utilisation de la kaoline ou de la poudre de talc (1:2.79 g *C. coccodes*/g kaoline ou talc). La kaoline et le talc absorbent l'eau et permettent le séchage adéquat du champignon. Les conidies formulées de *C. coccodes*, emmagasinées à 4, 30C ou à température de la pièce dans des sacs perméables à l'oxygène, sont restées vivantes et capables d'infecter les plants d'abutilon pour au moins six mois. Plusieurs stimulants de germination reportés dans la littérature ont augmenté la germination des conidies formulées, alors que l'augmentation de la concentration de cutine a causé une diminution dans la germination et la formation d'appressoria à la fois des conidies fraîches et formulées. Les expériences dans les chambres de croissance ont montré que les plants d'abutilon étaient sérieusement atteints quand inoculés de conidies formulées dans la kaoline ou la poudre de talc auxquelles de l'acide stéarique ou oléique, respectivement, ont été ajoutés. Les combinaisons de stimulants de germination, inducteurs de cutinase, pectinase ou cellulase n'ont pas significativement afffecté la germination et la formation d'appressoria des conidies de *C. coccodes*. La germination des conidies fraîches et formulées a augmenté, non significativement, avec l'addition de 1% sucrose.

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Section II. Effect of drying and storage conditions on *Colletotrichum coccodes* conidia.

Section III. Effect of germination / penetration stimulants on germination and appressoriation of formulated *Colletotrichum coccodes* conidia.

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I. GENERAL INTRODUCTION

Wilson (1969), stated that "It is becoming more apparent in pest control that reliance on a single method of control is hazardous. Multiple methods of control give more assurance of overall success". One new weed control strategy is the use of phytopathogenic microorganisms and applying them to target weeds in a manner similar to chemical herbicides. Inoculum of a fungal plant pathogen, formulated and applied as a foliar spray that uniformly kills or suppresses weed growth is termed a mycoherbicide or bioherbicide (TeBeest and Templeton, 1985; Templeton *et al.*, 1979).

In order for bioherbicides to become effective components in integrated weed management systems, basic studies are needed. Such studies should characterize the biocontrol agents, define their interactions with the target weed(s) and the environment, and provide technology to produce, formulate, store and apply bioherbicides for maximum efficacy and reliability in the field (Quimby, 1985; Quimby and Walker, 1982).

Potential Bioherbicides

All plants, including weed species, are affected by disease and the causal agents may or may not have the potential to be developed as a bioherbicide. General requirements for the selection of potential herbicides include: 1) the pathogen must be able to produce abundant and durable inoculum in artificial culture, 2) the pathogen must be genetically stable and target specific, 3) the pathogen must be able to infect and kill the weed over a relatively wide

range of environmental conditions, 4) the pathogen must remain limited to the application site, 5) the formulation must be compatible with the existing application methods, and 6) the proposed bioherbicide product must be economical to produce, have an acceptable shelf-life, and be effective over a range of environmental conditions (Daniel *et al.*, 1973; Templeton *et al.*, 1979; Hoagland, 1990)

Key concerns in the development of any new pest control strategy are safety and efficacy (Watson and Wymore, 1990), therefore a prospective bioherbicide should (1) be safe to the user, (2) be safe to the environment, (3) be easy to produce and store, (4) be inexpensive to use, and (5) provide effective control of the target weed.

It is generally perceived that biological products are safer than chemical pesticides. However, bioherbicides are perceived as less efficacious than chemical herbicide treatments because efficacy is usually measured against the standard chemical treatment and many fungi that are evaluated as bioherbicides do not kill their host plant (Watson and Wymore, 1990; Wilson, 1969). However, in the bioherbicide approach, a weakened diseased weed is also desirable as it can be the source of secondary inoculum for spread to adjacent weeds and for infection of emerging weed seedlings (Watson and Wymore, 1990).

In spite of many reports on pathogens controlling weeds, relatively few have reached the market place as bioherbicide products. One reason proposed is that most biocontrol agents have been developed from a scientific viewpoint (Scher and Castagno, 1986). From the viewpoint of industry, a potential bioherbicide is one that 1) meets market demands, 2) is technically and economically feasible, 3) can be formulated as a stable product that can be applied with conventional spraying equipment, and 4) is cost competitive with alternative

chemical herbicides (Lisanski, 1989; Scher and Castagno, 1986).

Development of Bioherbicides

Development of a bioherbicide is often divided into three major stages: discovery, development and deployment (Templeton, 1982). The discovery phase involves the collection of diseased plant material, isolation of the causal organism, demonstration of Koch's postulates, identification of the pathogen, culturing the pathogen on artificial media and maintenance of the pathogen cultures in short-term and long-term storage. Development includes determination of the optimal conditions for spore production and disease development, determination of host range, and demonstration of weed control. During the deployment phase close collaboration and cooperation between university or public sector researchers and industry is required. The university research must provide the necessary information that will encourage industry to commit production and formulation facilities and marketing personnel for the commercialization of bioherbicide products (Templeton, 1982).

Basis for the Bioherbicide Approach

The bioherbicide approach is based on fundamental epidemiological principles of plant pathology. The impact of plant disease is determined by a tripartite interaction involving the host, the pathogen, and the environment (Van der Plank, 1968). Constraints caused by any one of these components could limit disease to endemic levels (Holcomb, 1982; Cullen and Hasan, 1988). These constraints may include plant related factors such as low plant density,

changes in susceptibility with age or resistance to the pathogen; pathogen related factors such as poor overwintering and dissemination capacities or low virulence; and environmental related factors such as unfavourable moisture or temperature conditions. A successful bioherbicide will overcome many of these constraints since, "an abundant supply of inoculum of a virulent pathogen is dispersed over a population of susceptible weeds and the application is timed and/or the bioherbicide is formulated to avoid unfavourable environmental conditions" (Watson and Wymore, 1990).

Major Constraints

Most fungal spores require exact moisture and temperature conditions for germination and host penetration. These conditions may not frequently occur under field conditions, and their absence will result in an ineffective bioherbicide application. Hoagland (1990) stressed the importance of two major problems: promoting fungal spore adhesion on the phylloplane, and providing dew (i. e. free water) for spore germination. Although many factors affect bioherbicide efficacy, moisture is the most important (Bannon *et al.*, 1990). An important means of possibly overcoming these problems of environmental variability is through effective product formulation (Jutsum, 1988). Therefore, a desirable biocontrol formulation would be the one which gives effective and consistent weed control over a range of environmental conditions (Connick *et al.*, 1990).

Formulation

Formulation involves the mixing of the active moiety with substances such as diluents

and surface active agents. The purpose of formulating is to alter the physical characteristics of the active ingredient - the fungal spore - to a more desirable form in order to enhance stability and/or biological activity, to improve mixing, flowability and spreadability, or to increase shelf-life and anticaking properties (Couch and Ignoffo, 1981). Formulation is the key factor in determining the successful marketing of a microbial pesticide and it has the following objectives : 1) to select a diluent compatible with the pathogen, 2) to supply the pathogen in an economical form, and 3) to enhance the biological stability and pathogenic activity (Devisetty, 1989). Formulation also facilitates handling and the delivery of the pathogen to its host (Smith and Berry, 1975). Connick *et al.* (1990) described a successful biocontrol formulation as one that is economically prepared, safe, stable in the environment, easily applied using conventional agricultural equipment and overcomes unfavourable environmental requirements for spore germination and infection. Accordingly, a reasonable goal, though challenging, would be a product with a shelf-life of 18 months at 40C (Connick *et al.*, 1990).

Since bioherbicides are basically living organisms, they must be carefully handled in order to maintain viability and infectivity throughout processing, storage and application. According to Kenny *et al.* (1979), the most difficult task that industry encounters is the development of a formulated product containing a fungal pathogen and Stockdale (1985) stated that "formulation has a critical effect on the efficacy of microbial insecticides". Biocontrol agents must survive and proliferate after application, and not degrade like chemical herbicides (Connick *et al.*, 1990). Soper and Ward (1981) have suggested that the proper formulation of a fungus requires a complete understanding of the life cycle and the biology of

the infective stages. Since the primary component of any formulation is the active ingredient, adequate knowledge of the physiology and the qualities of the spore is essential.

Fungal Spore

Definition

The spore is a part of the organism highly specialized for reproduction, survival, and dispersal of fungi. Normally delimited from the hypha, it is generally unicellular, although sometimes multicellular, and is characterized by a low metabolic rate, low water content, and lack of cytoplasmic movement (Bartnicki-Garcia, 1973; Smith and Berry, 1975).

Role

Since fungal cells are immediately exposed to the environment and since they lack the ability to maintain a relatively stable internal environment, they have evolved morphologic and physiologic mechanisms to survive a continuously changing environment. The mycelium is suited for ultra-rapid growth and colonization of available substrate and, as such, the mycelium is a very vulnerable phase. Sporulation is primarily a protective response to the deterioration of the physical or chemical environment. Metabolism is greatly reduced and the vegetative protoplasm is mobilized or packed, into resistant cells or spores (Bartnicki-Garcia, 1983; Smith and Berry, 1975). Conidia are specialized asexual spores of most fungi being evaluated as bioherbicides. The term spores will be used as a general reference, but in specific references to bioherbicides and specific fungi producing conidia, the term conidia will be used. Apart from their natural ecological role in propagating the species, spores and conidia are used for the maintenance of cultures of specific organisms. Cultures on agar

slants are kept immersed in mineral oil below 8C, frozen at -60 to -80C or at -196C, freeze-dried and stored at below 5C, or dried on the surface of an inert solid substrate like soil or silica gel (Smith, 1988; Chang and Elander, 1986; Dalby, 1982; Onions, 1983). Spores are also used in the facilitation of specific chemical transformations, production of low molecular weight primary and secondary metabolites and enzymes, as inoculum to produce biomass for experimental or industrial processes (Bennett, 1985; Smith and Berry, 1975), and, more recently, as the active ingredient of some bioinsecticides and essentially all bioherbicides (Boyette *et al.*, 1991).

Germination

To develop into the vegetative stages of the fungus, a spore must first germinate. This initial stage in the transformation of the spore into the thallus usually involves the formation of a germ tube. The most important and consistent feature in the process is the sudden shift in the spore from a state of very low to a high metabolic activity. This rise in both catabolic and anabolic activity depends on at least four factors: (1) the presence in the spore of innate regulatory mechanisms, (2) essential nutrients, either stored or available in the immediate environment, (3) the presence in the spores of various enzymes for respiration and synthesis, and (4) the proper physical conditions to allow the metabolic functions to proceed (Gottlieb, 1978).

Germination can be prevented by any one or a combination of limiting factors, including developmental, nutritional, and environmental factors or due to the presence of toxic compounds.

(1) Developmental (e. g. spore maturation, senescence, and dormancy) : The germinability of

a spore will depend, among other things, upon its state of maturation. Under natural conditions, spores will generally not be released until they are mature and will germinate unless a dormant state intervenes (Gottlieb, 1978). Spore dormancy is common in fungi and could be constitutive or exogenous. In constitutional dormancy, spores will not germinate even when placed under suitable environmental conditions but first require an activation process to overcome an innate property of the dormant state which may be a barrier to the penetration of nutrients, a metabolic block, or the production of a self-inhibitor. Alternatively, in exogenous dormancy, the spores are inactive because of unfavourable chemical and/or physical conditions and will quickly resume growth in a favourable environment (Bartnicki-Garcia, 1983; Smith and Berry, 1975). Teitell (1958) hypothesized that "at relative humidities just below those required for germination, the metabolic activities of the spore have changed to a point where the protection afforded by dormancy is lost, but insufficient moisture is available to complete germination, therefore the spore dies".

(2) Nutritional (e. g. the absence of water or the appropriate carbon and nitrogen sources) : The presence of liquid water or water vapour is essential for the germination of spores of most species and only few will germinate at very low humidities or in the complete absence of gaseous water. Water is unique in this process, since even deuterium oxide (heavy water) will not substitute for it (Gottlieb, 1978). Germination of *Phomopsis diospori* (Sacc.) Traverso & Spessa was 100% in water, and 0% at relative humidities below 80% (Dzimistarishvili, 1976). One of the oldest methods of controlling microorganisms in foods relies on lowering the concentration of available water for necessary biochemical reactions (Rose, 1983; Northolt and Bullerman, 1982). Food microbiologists and food scientists, have,

for more than 25 years, used the term water activity (a_w) as a useful means of characterizing the state or condition of water in foods. The a_w is defined as "the ratio of the vapour pressure of the product and that of pure water, which is also equal to the equilibrium relative humidity (ERH) divided by 100": a_w = ERH /100 (Rose, 1983)

Water activity (a_w) values estimate the proportion of "available water" in a given system - water available for biological and chemical reactions (Corry, 1987). One way of storing fungi is by lowering the water activity in the system through the use of a filler (e. g. solute). Retention of fungal spore viability is greatly affected by a_w and temperature (Beuchat, 1983), with increasing temperature decreasing viability at any given a_w (Teitell, 1958). The survival of fungal propagules seems also to be dependent on non-water constituents (Beuchat, 1979). The fillers most commonly used in biopesticide formulations are phyllosilicates (clays), because they are relatively inert, available in large quantities at low cost and are easy to handle during manufacturing and application (Ward, 1984). Selecting the proper clay diluent will increase the shelf-life and the overall compatibility of a biological insecticide by : maintaining a favourable pH, absorbing suppressive chemicals, and modifying the microclimate (Ward, 1984). It is likely that the same is true for bioherbicides.

In storage, the retention of viability of fungal spores, measured by their ability to germinate and grow when exposed to favourable environmental conditions, is greatly influenced by a_w and temperature. Conidia of *Aspergillus oryzae* (Ahlburg) Cohn were able to retain viability for 22 years when storage conditions were favourable (McCrea, 1923). At any given a_w, an increase in temperature generally results in a decrease in viability of fungal spores, while lower temperatures (above freezing) favour an extended viability (Teitell, 1958).

The basis for survival and death of microorganisms as influenced by water activity is complex but it is the ability of the microorganisms to adapt to suboptimal environmental factors that will determine its ability to survive (Lenovich, 1987).

Molds generally grow better at a_w values considerably higher than their minimum for growth, and many grow best at a_w close to 0.99 (Corry, 1987; Beuchat, 1983). However, they are usually able to grow at lower a_w values than bacteria (Hocking, 1988; Troller, 1987; Beuchat, 1983). The minimum a_w at which growth of fungi has been observed is 0.61 to 0.62, although growth or germination at this a_w is very slow (Corry, 1987). Fungi show greatest tolerance to a low water activity when all other conditions (pH, temperature, oxygen, nutrients) are optimal (Magan and Lacey, 1984). *Alternaria citri* Ellis and Pierce in Pierce, with an optimum temperature of 30C, germinated at a_w values as low as 0.838 at this temperature, whereas at 18C/37C and 5C, higher water activities (0.876 and 0.942 respectively), were required for germination (Tomkins, 1929). However, at low a_w, growth is restricted to a narrower range of environmental conditions (Magan and Lacey, 1984). Although germination decreases with decreasing a_w at any given temperature, the presence of nutrients seems to broaden the range of a_w and temperature at which propagules will germinate and grow (Beuchat, 1983). Increased concentrations of sucrose and hence decreasing a_w resulted in an increased tolerance to heat by *Aspergillus flavus* (Link:Fr), *Byssochlamys nivea* Westling, and *Geotrichum candidum* Link (Beuchat, 1981). With the exception of *Penicillium* species, fungi generally become more sensitive to atmospheres containing oxygen and carbon dioxide as a_w and temperature are reduced (Magan and Lacey, 1984). The minimum a_w at which molds can grow is often affected by the solute which has

been used to lower a_w (Pitt and Hocking, 1977; Troller, 1987). Studies on the influence of three solutes, sodium chloride, glycerol and a glucose/fructose mixture, on six xerophilic fungi (fungi able to grow below a a_w of 0.85) demonstrated that all fungi, except for one, *Wallemia sebi* (Fr.) Arx, grew most strongly on glucose/fructose, but were partially or completely inhibited by sodium chloride (Pitt and Hocking, 1977). Tolerance to high levels of one solute does not necessarily imply the same degree of tolerance to another solute (Beuchat, 1983). At a lowered a_w, conidia might germinate but this is not necessarily followed by growth (Beuchat, 1983). Conidia of *Aspergillus flavus* germinated at a_w of 0.75 but did not grow, and eventually lost their viability; whereas at a lower a_w they remained dormant (Teitell, 1958).

Higher a_w is generally a requirement for spore formation rather than for spore germination (Beuchat, 1983). Among the most important parameters to be considered in assessing the water requirements of microorganisms are temperature, storage duration, pH, the physical structure of the substrate and the nature of added preservatives (Rose, 1983). The addition of Sorbo (64% sorbitol, Atkemic Inc., Brantford, Ontario) to conidia of *Colletotrichum coccodes* (Wallr.) Hughes before spraying, promoted disease initiation in velvetleaf (*Abutilon theophrasti* Medic.) with only three 9-hour dew periods on consecutive nights which was as effective as disease initiated following an 18-hour dew period (Wymore and Watson, 1986). A follow-up application of a viscous invert emulsion slowed evaporation of water and allowed infection and disease development of *Alternaria cassiae* Jurair & Khan to occur on sicklepod (*Cassia obtusifolia* L.), even without the presence of dew (Quimby *et al.*, 1989; Daigle *et al.*, 1990). The most recently developed invert

emulsion consists of an oil phase of 10% (w/w) paraffin wax, 3% (w/w) monoglyceride emulsifier, and 87% (w/w) paraffinic oil, to which an equal volume of deionized water was added (Connick *et al.*, 1991). Amsellem *et al.* (1990) reported that when *A. cassiae* and *A. crassa* (Sacc.) Rands conidia were applied in an invert emulsion, one conidium/droplet -which is the lowest possible threshold- was enough to cause infection to sicklepod under low or high humidity.

Some surfactants, adjuvants, or chelating agents have been reported to increase germination and/or appressoriation, probably due to the resulting high water activity. The maximum rate of germination and appressorium formation of *C. trifolii* Bain & Essary was obtained in the presence of 0.083 % Tween 20 (Miehle and Lukezic, 1972). Anthralinic acid and chelating agents were found to stimulate germination of *C. musae* (Berk. & Curt.) Von Arx conidia (Graham and Harper, 1983; Harper and Swinburne, 1980; Harper *et al.*, 1980). When the spray adjuvants Ortho X-77 and Triton BI956 were applied to grape berries, the number of germinated *Botrytis cinerea* Pers. ex Fr. conidia increased causing a concomitant increase in disease incidence (Marois *et al.*, 1987). Mycoherbicide formulations of *Alternaria cassiae* exhibited increased spore germination at a pH of 6.5 in a solution of 0.1 - 1% Tween 80, 0.02M potassium phosphate buffer and 1% dehydrated potato dextrose broth (Daigle and Cotty, 1991).

The presence of some nutrients is indispensable for germination and/or appressoria formation of fungi. L-rhamnose is the best carbon source for germination and appressorium formation of *Colletotrichum capsici* (Syd.) Butl. & Bisby (Solanki *et al.*, 1974), which failed to produce appressoria on an agar surface except in the presence of glycine (Muruganandam

et al., 1988). Conidia germination of *C. papayae* Henn (*C. gloeosporioides* (Penz.) Penz. & Sacc.) was found to increase with an increasing concentration of sucrose solution (Gupta, 1979).

(3) Environmental physical limitations (e. g. unfavourable temperature or osmotic pressure) : Similar to its actions on most physiological functions, temperature influences spore germination (Bartnicki-Garcia, 1973; Bartnicki-Garcia, 1983; Smith and Berry, 1975). Basic biochemical reactions are affected which can limit or retard the production of germ tubes. Since the reactions involved in the initiation of germination are primarily enzymatic, temperature effects on specific proteins involved in germination limit or promote the process (Gottlieb, 1978).

The optimum temperature for conidial germination of *Colletotrichum capsici* is 25C (Solanki *et al.*, 1974). Exposure of *C. trifolii* spores to temperatures above 27C decreased the number forming an appressorium. Longer periods of heat exposure than that necessary to decrease appressorium formation caused a reduction in germination (Miehle and Lukezic, 1972). Heat treatment of spores of *C. lagenarium* (Pass.) Ellis & Halst at 32C, just before the emergence of the germ tube and four hours after incubation at 24C, significantly inhibited appressorium formation (Tani *et al.*, 1977).

The optimum temperature for spore germination of *Phomopsis diospyri* (Sacc.) Traverso & Spessa was found to be 25-27C, the minimum 5C and the maximum 32-36C (Dzimistarishvili, 1976). *Phomopsis convolvulus* Ormeño has an optimal temperature for germination at 20C (L. Morin, personal communication).

(4) The presence of toxic compounds in the spore or its environment : Spore germination of

Colletotrichum gloeosporioides was generally inhibited by aromatic components (Pittervils *et al.*, 1982), whereas conidial germination of *C. graminicola* (Ces.) Wils. and *C. capsici* was found to be inhibited by phytoncides of onion (*Allium cepa* L.), garlic (*A. sativum* L.), *Azadirachta indica* Juss. and *Ocimum basilicum* L. (Gupta *et al.*, 1982). Studies indicated that the inhibition may be due to an amino-n-butyric acid, histidine, DL isoleucine and ascorbic, tartaric and maleic acids (Gupta *et al.*, 1982). Grover (1971) found that amino acids, amides and other substances present in host exudates could inhibit appressorial formation and disease development of *Gloeosporium piperatum* Ellis & Everh. in Halst on green pepper (*Capsicum annuum* L.) plants. The decreased germinability of *Colletotrichum camelliae* Massee and *P. convolvulus* at high spore densities is attributed to the presence of uncharacterized endogenous inhibitors (Wang, 1986; R. Menassa, personal communication).

Once the conditions are favourable for germination, spores will germinate. For many spores, the germination process may be divided into three distinct phases: initiation, spherical growth and germ tube emergence. Initiation of germination for some spores results from the presence of the right nutritional environment, while for others it involves overcoming the dormant state. Germinating spores initiate vegetative development by growing into large spherical cells which involves true growth and not just water imbibition. Thirdly, germ tube emergence occurs after a spore has germinated, i. e. after it has fully switched from resting to active metabolism. This process may take place while the germinating spore is already growing exponentially -a process initiated during the previous phase (Bartnicki-Garcia, 1983; Smith and Berry, 1975).

The site of germ tube emergence from the spore may be predetermined by various

morphological characteristics of the spore or may be undefined and unpredictable (Gottlieb, 1978). Usually, a rupture in the outer wall takes place during germ tube formation; but whether this rupture is caused by the physical force of protoplasm swelling or is first initiated by enzymatic degradation of the wall is not known, but spore walls are subject to enzymatic hydrolysis (Bartnicki-Garcia, 1973; Bartnicki-Garcia, 1983; Smith and Berry, 1975).

Spore protoplasm is generally dense, with small vacuoles that decrease in number, but become larger, as germination proceeds. As the germ tube forms, the contents of the spore flow into the tube.

To be able to infect, the germinated spore must penetrate the host cuticle, the pectin layer, and the cellulose wall of the epidermal cells. The cuticle is composed of cutin, a hydroxy and hydroepoxy fatty acid polymer, embedded in a mixture of nonpolar waxes which often protrude and give rise to crystals on the cuticular surface (Kolattukudy,1975; Kolattukudy *et al.*, 1985). A pectin barrier, a carbohydrate polymer that cements cells together, lies beneath the cutin layer. The major physical barrier to fungal penetration is cutin, an insoluble polymer. It is debatable whether fungal penetration is mechanical or enzymatic, but there is substantial evidence to suggest that penetration is in part at least enzymatic (Kolattukudy, 1975, 1984, 1985; Kolattukudy and Crawford, 1987; Kolattukudy and Köller, 1983; Kolattukudy *et al.*, 1985, 1987a, 1987b, 1989; Köller, 1991; McKeen, 1974; Purdy and Kolattukudy, 1973; Trail and Köller, 1990; Woloshuk and Kolattukudy, 1986). When cutinase inhibitors were included in a spore suspension of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyder & Hans or *Colletotrichum gloeosporioides*, infection of pea (*Pisum sativum* L.) stems and papaya (*Carica papaya* L.) fruits was prevented (Kolattukudy

and Crawford, 1987). Fungal spores produce small amounts of cutinase constitutively and upon landing on their host, spores are able to hydrolyse cutin immediately (Kolattukudy *et al.*, 1985). The hydrolysis products enter the fungal cells and turn on the gene responsible for cutinase production (Kolattukudy *et al.*, 1985; Kolattukudy and Crawford, 1987; Lin and Kolattukudy, 1978). This induction of cutinase is followed by the induction of polygalacturonase which attacks the pectin layer. Pectin hydrolase production was found to reach its peak at the initiation of germination (Kolattukudy *et al.*, 1985).

Pectolytic and cellulolytic enzymes of phytopathogens are thought to play a key part in the infection process (Cooper, 1984; English and Albersheim, 1969; English *et al.*, 1971; Sherwood, 1964, 1966; Tani and Nauba, 1969; Tatareau and Auriol, 1972). Various enzymes are involved in degrading pectin. *Erwinia carotovora*, a soft rot bacterium, produces pectin esterase, polygalacturonase, as well as other enzymes (Starr, 1959; Starr and Moran, 1962). Many fungi, including *Rhizoctonia solani* Kühn, *Fusarium oxysporum* Schlecht f. sp. *lycopersici* (Sacc.) Snyd. & Hans. isolate R5-6, *Botrytis cinerea*, *Aspergillus niger* V. Tiegh., *Sclerotinia rolfsii* Sacc. and *Rhizopus stolonifer* (Ehr. ex Fr.) Lind. produce polygalacturonase and most also produce pectin lyases (Sherwood, 1964, 1966; Papavizas and Ayers, 1965; Bateman, 1963; Tani and Nauba, 1969). Potassium phosphate and calcium chloride have been reported to enhance polygalacturonase activity (Boothby, 1981; Starr and Moran, 1962).

Several reports on enzyme production by *Colletotrichum* species related to pathogenicity are available. Pectinase and α-arabinosidase, β-xylosidase and cellulase, then β-glucosidase and finally α-galactosidase, are secreted by *C. lindemuthianum* (Saccardo & Magnus) Scribner (English *et al.*, 1971). Virulent isolates of *C. lindemuthianum* secrete a

significant amount of α-galactosidase when grown in isolated bean hypocotyl cell wall cultures, while avirulent isolates secrete very little (English and Albersheim, 1969). Tatareau and Auriol (1972) reported that a virulent isolate of *C. lagenarium* (Pass.) Ell. & Halst results in cell wall degradation.

After the disruption of the pectin layer, the fungus must breach the cellulose barrier with the aid of cellulases for infection to occur. Cellulose is a glucose polymer, but it is not easily hydrolysed. Efficient cellulolytic fungi have three enzymes, an endogluconase,an exo-glucanase and a β-glucosidase, while others produce one or two of them (Bateman and Basham, 1976; Nevins *et al.*, 1967). When conidia of *Neurospora crassa* Shear & Dodge were grown in the presence of some surfactants or fatty acids, C_{18} fatty acids at a concentration of 0.2% caused an increase in the amounts of exo and endoglucanase in the medium (Yazdi *et al.*, 1990). The addition of Tween 80 (0.2%) resulted in the greatest production of exoglucanase and similar concentrations of Tween 80 and Brij 35 (0.1% or 0.2%) gave similar increases in endoglucanase production (Yazdi *et al.*, 1990). Reese and Maguire (1969) studied the effects of Tween 80 and sucrose monopalmitate on yields of enzymes produced by three different of *Aspergillus* spp, five *Penicillium* spp, one basidiomycete, and *Trichoderma viride* Pers. ex Fr. Tween 80 (0.1%) was reported to increase cellulase production in various fungi by increased cell permeability, allowing the release of the enzyme (Reese and Maguire, 1969).

Enzymes play a key role in pathogenesis. They are produced in spores in small amounts constitutively and their production is induced by the presence of the substrate. A major constraint to bioherbicide reliability and efficacy under field conditions is the presence

of water (dew) for a relatively long period of time for conidia to germinate (18 hours for *C. coccodes*). This constraint could be overcome by accelerating germination by exposing conidia to "germination stimulants", or to "pectinase and cellulase inducers". Time for germination and penetration could be minimized, thereby reducing dew period duration.

Formulation types

In spite of many reports of pathogens on weeds, few pathogens have been commercially developed as marketable products. A suggested reason for the scarcity of the products is that most biocontrol agents have been developed "from a scientific viewpoint, without an industrial perspective" (Scher and Castagno, 1986). For the industry, a potential bioherbicide is one that 1) meets a market demands, 2) is technically and economically feasible, 3) is formulated as a stable product that can be applied with the conventional spraying equipment, and 4) is cost competitive with alternative chemical herbicides (Lisanski, 1989; Scher and Castagno, 1986).

Various approaches have been attempted and many different techniques have been followed in the development of commercial or experimental formulations (Boyette *et al.*, 1991). One main type of formulation predominates, the granular or solid based. The least sophisticated formulations consist of fungal propagules that are allowed to grow on an inexpensive, readily available source of nutrients (e. g. seeds) which also function as the carrier in the delivery system. These types of formulation require minimal handling since the fungus does not need harvesting and further processing. A *Sclerotinia sclerotiorum* (Lib.) de Bary formulation consisted of infested wheat kernels which were hand-broadcast in an attempt to control Canada thistle (*Cirsium arvense* (L.) Scop.) (Brosten and Sands, 1986). *Fusarium*

solani (Mart.) Sacc. f. sp. *cucurbitae* Snyder & Hans was formulated as granules of a cornmeal-sand mixture. Mycelium, microconidia, macroconidia and chlamydospores were able to grow in that medium and effective control of Texas gourd (*Cucurbita texana* (Scheele) Gray) was achieved using this formulation (Boyette *et al.*, 1985).

A more elaborated formulation is one in which the fungus is first cultured then harvested and mixed with a carrier. *Fusarium lateritium* Nees ex Fr. macroconidia were harvested after adding hydrated silica powder to the culture, dried and stored at 8C. The macroconidia remained viable for at least 1 month (Quimby, 1985). A wheat bran formulation of *Colletotrichum gloeosporioides* provided effective control of young *Hakea sericea* Schrad. seedlings when hand applied (Morris, 1989). After harvesting, conidia were resuspended in water, mixed with autoclaved wheat bran, incubated at 24C for 48 hours, and air dried. Mycelia of *Alternaria macrospora* Zimm. were added to vermiculite, exposed to light to induce sporulation, and air dried. These fungal impregnated sporulating vermiculite granules were then used to effectively control spurred anoda (*Anoda cristata* (L.) Schlechtend.) (Walker, 1981).

To overcome some of the technical limitations of the vermiculite granules, sodium alginate replaced vermiculite as the primary carrier (Walker and Connick, 1983). Kaolin clay, ground soyflour, ground oatmeal or other additives were mixed with the mycelial mass and alginate prior to pelletization (Walker and Connick, 1983). The resulting mixture was then dripped into a 0.25M solution of calcium chloride. The Ca^{2+} ions react immediately to form gel pellets and the calcium alginate matrix protects the biocontrol agent. Alginate granules are either exposed to light to induce sporulation and then air dried to produce granules with

spores or air-dried immediately after gel pelletization. These latter granules require suitable environmental conditions to induce sporulation *in situ* (Walker and Connick, 1983). This approach has been used successfully with *Alternaria cassiae* Jurair & Khan, *A. macrospora*, *Fusarium lateritium*, *F. solani* f. sp. *cucurbitae*, *Colletotrichum malvarum* (A. Braun & Casp.) Southworth, *Phyllosticta* sp. and *Sclerotinia minor* Jagger (Walker and Connick, 1983; Weidemann and Templeton, 1988a; Weidemann and Templeton, 1988b; Briere *et al.*, 1992). Nutrient amended granules of *F. solani* f. sp. *cucurbitae* were found to result in a more effective control of Texas gourd than an aqueous conidial suspensions (Weidemann and Templeton, 1988a).

"Pesta" is a relatively new formulation technology whereby fungal propagules (mycelia and/or spores) are entrapped, in a wheat gluten matrix (Connick *et al.*, 1991a). A dough formed by the mixing of the homogenized fungal biomass, flour and kaolin is passed through a pasta maker, allowed to air dry, and ground. *Alternaria cassiae*, *Alternaria crassa*, *Colletotrichum truncatum* (Schwein.) Andrus & Moore and *Fusarium lateritium* have been effectively formulated by this process (Connick *et al.*, 1991a).

Formulated (dried) spores are either applied as such, or are resuspended prior to application and applied in a manner similar to fresh spores. Since water is necessary for fungal spore germination and growth, inexpensive, readily available, and easy to handle, it is naturally the carrier of choice for bioherbicides. Problems encountered when using water as the carrier are the rapid evaporation of water limiting spore germination and thus disease incidence, and the poor wettability of weeds with a thick waxy cuticle preventing a uniform distribution of the applied bioherbicide (Quimby and Fulgham, 1986; Boyette *et al.*, 1991).

The problem of rapid evaporation was addressed by applying an invert emulsion directly after the application of the bioherbicide (Quimby and Fulgham, 1986). Sicklepod plant mortality was 88% when an invert emulsion followed the application of *Alternaria cassiae* spores as compared to 0% when the plants were only sprayed with the bioherbicide. In neither case were the plants subjected to dew (Quimby and Fulgham, 1986; Connick *et al.*, 1991b). Invert emulsions were found to be good carriers for biocontrol organisms requiring a dew period. An invert emulsion consisting of an oil phase of paraffin wax, paraffin oil and an unsaturated monoglyceride (Myverol 18-99) mixed with an equal volume of water allowed fungus proliferation without dew. The spray deposits were found to have a water content of 22.3% after 24 hours which is above the threshold required for germination of *A. cassiae* spores (Connick *et al.*, 1991b).

To achieve a more uniform wetting of the plants, surfactants have been included in the formulation (Walker, 1980; Walker and Boyette, 1985; Mitchell, 1986, 1988; Andersen and Walker, 1985). Surfactants also aid in dispersing the fungal propagules in the spray tank. Spores of *A. macrospora* Zimmerm. have been suspended in distilled water containing 0.1% Tween 80 (Walker, 1980), those of *A. cassiae* with a nonoxynol surfactant (Walker and Boyette, 1985), those of *Dichotomophthora portulaceaceae* Mehrlich & Fitzp. ex Ellis and *Gibbago trianthemae* Simmons with Tween 20 (Mitchell, 1986, 1988) and *Colletotrichum coccodes* conidia with Tween 80 (Andersen and Walker, 1985).

Certain adjuvants, usually used to decrease water evaporation and increase stability in sunlight, were found to have some positive effect on spore germination or on fungal infection (Daigle and Connick, 1990). When Sorbo (64% sorbitol, Atkemix Inc., Brandford, Ontario)

was added to *Colletotrichum coccodes*, the number of viable conidia recovered from inoculated leaves increased 20-fold (Wymore and Watson, 1986).

Alternaria cassiae (CASST™), a bioherbicide being developed against sicklepod (*Cassia obtusifolia* L.) in soybean and peanuts (*Arachis hypogaea* L.) has received considerable scientific study in the United States but has not been registered yet (Charudattan, 1991). The formulation consists of a dried active component (fungal spores) that has to be mixed with an emulsion of a paraffinic oil "adjuvant" (resuspended in water at 1% v/v) (Boyette *et al.*, 1991).

To date, three bioherbicides have been commercialized in North America (Charudattan, 1991). *Phytophthora palmivora* (Butler) Butler which infects and kills stranglervine (*Morrenia odorata* (H. & A.) Lindl., a problem weed in Florida citrus groves, has been marketed as DeVine™. Its formulation consists of a "freshmilk" liquid product with a shelf life of only about six weeks and was adopted because of the limited marketing area that permitted production upon ordering or demand (Kenny, 1986). This short-lived formulation would have certainly failed if this bioherbicide had a large market area (Kenny, 1986). *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (CGA) which controls northern jointvetch (*Aeschynomene virginica* (L.)B. S. P.) in rice (*Oryza sativa* L.) and soybean (*Glycine max* (L.) Merr., is marketed as COLLEGO™. COLLEGO™ is formulated as an active component (dried CGA spores) and a rehydrating agent used to wet the spores and enhance germination (Bowers, 1982, 1986). The third product, BioMal™ is the first bioherbicide to be marketed in Canada. It is a wettable powder prepared by drying a conidial suspension of *C. gloeosporioides* f. sp. *malvae* mixed with kaolin (1:3 v/v) which, upon rehydration, controls

round leaf mallow (*Malva pusilla* Sm.) (Boyette *et al.*, 1991).

Velvetleaf bioherbicide

Colletotrichum coccodes, a fungal pathogen, has been determined as a potential bioherbicide for velvetleaf control (Gotlieb *et al.*, 1987; Poirier, 1984; Wymore *et al.*, 1988). In an original study, Poirier (1984) evaluated its possible use as a bioherbicide and determined the etiology of the disease, the general conditions for disease development, and the host range of the pathogen. *C. coccodes* causes severe necrosis and can significantly reduce the growth of velvetleaf (Poirier, 1984; Wymore *et al.*, 1988). Attempts to reduce the length of the dew period required for the pathogen to infect velvetleaf were reported by Wymore and Watson (1986). All previous research dealt with fresh conidial suspensions of *C. coccodes*. Further research was needed to determine the possibility and feasibility of effectively formulating the fungus. Formulation would allow storage of the bioherbicide until subsequent use, and might address the problem of dew requirement.

The main objectives of this project were to evaluate various systems to "dry" virulent conidia, to determine the viability and infectivity of formulated *C. coccodes* conidia under various conditions of storage, and to maximize disease expression on velvetleaf seedlings through the addition of germination stimulants, cutinase and or pectinase inducers to formulated conidia to achieve efficient weed control.

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II. Effect of drying and storage conditions on *Colletotrichum coccodes* conidia

INTRODUCTION

Velvetleaf (*Abutilon theophrasti* Medic.) is an aggressive, annual weed producing thousands of long-lived seeds and is extremely difficult to control (Mitich, 1991; Warwick and Black, 1988). Velvetleaf control must be achieved through different strategies in an integrated approach since reliance on herbicides has resulted in velvetleaf spread and development of resistant biotypes (Mitich, 1991; Warwick and Black, 1988). One new control strategy is to apply bioherbicides. A bioherbicide (mycoherbicide) is inoculum of a plant pathogen, formulated and applied, in the inundative strategy, as sprays that uniformly kill or suppress weed growth (TeBeest and Templeton, 1985; Templeton *et al.*, 1979). Some of the basic studies needed for the successful integration of bioherbicides into weed management systems are ones that characterize the biocontrol agents, define their interaction with the target weed, the environment and other components of the production system, and provide technologies to produce, store and apply bioherbicides for maximum efficacy and reliability in the field (Quimby, 1985; Quimby and Walker, 1982).

Colletotrichum coccodes (Wallr.) Hughes, a fungal pathogen, is being developed as a bioherbicide for velvetleaf control (Poirier, 1984; Wymore *et al.*, 1988), but the final product form in which conidia could be presented to the market and used by producers has not yet been determined. Hoagland (1990) stressed the importance of a feasible economical production and an acceptable shelf-life of potential bioherbicide products, and according to Watson and Wymore (1990), one of the criteria a prospective bioherbicide should meet is the

ease of production and storage. Biopesticides should have a "reasonable" shelf-life (1 to 2 years) when stored under ambient conditions without the need of any special handling (Lisanski, 1989; Baker and Henis 1990). Connick *et al.* (1990), stated as "a reasonable goal, but one difficult to reach, the making of a product that has a minimum stability of 18 months at 40C". A suitable bioherbicide formulation would be expected to have an extended shelf-life (a minimum of six months may be adequate) and once used under field conditions, provide acceptable levels of weed control. Since the active ingredient of most bioherbicides is the fungal spore, adequate knowledge of the qualities of the spore is essential for the development of effective bioherbicide products.

Spores are highly specialized for reproduction, survival and dispersal. A spore is generally delimited from the hypha, unicellular or multicellular, and is characterized by a low metabolic rate, low water content and lack of cytoplasmic movement (Bartnicki-Garcia, 1973; Smith and Berry, 1975). When used as the active ingredient of a biopesticide, conidia must have low metabolic activity while in storage and be able to resume normal activity when applied as a pest control (Connick *et al.*, 1990). If fungal propagules are to be used as biocontrol agents, once removed from storage they must be able to germinate and proliferate, therefore toxic compounds cannot be used to prevent conidial germination in storage. Conidial exogenous dormancy should be imposed to inhibit germination during storage; however, the process by which it is imposed should be harmless to conidia, since normal metabolic activities are required upon use of the product. One way of achieving enforced dormancy could be by decreasing the availability of water, i. e. lowering the water activity, a well established concept and practise in the food industry and one which has major

applications in biopesticide studies.

In the food industry, the most important factor determining whether or not a mold ("any profuse or woolly fungus growth on decaying or damp matter or on surfaces of plant tissues" (Agrios, 1988)) will grow, and at what rate, is the relative humidity surrounding the product (Seiler, 1976). When a product is stored under conditions where no loss or gain of moisture occurs, relative humidity is equal to Equilibrium Relative Humidity (ERH). The term water activity (a_w) is ERH divided by 100 (Rose, 1983; Seiler, 1976). Water activity measurements estimate the amount of water available for biological and chemical reactions in a system (Corry, 1987). Food scientists are interested in lowering water activity in food to levels that would inhibit fungal growth, thus preserving food. A parallel logic is applicable to biopesticide development. To be able to store spores/conidia as biopesticides, available water should be reduced to prevent germination during storage. However, the usual drying procedures used in food industry for yeasts or bacteria are not generally suitable for all filamentous fungi. Freezing might damage cellular membranes, freeze-drying is not adequate for a number of species (Smith, 1969), spray-drying reports on filamentous fungi could not be found and air or oven drying might give spores/conidia enough time to germinate before water evaporates. If the water activity of the biopesticide is reduced rapidly to levels sufficient to inhibit the germination of fungal propagules, while maintaining viability and infectivity for subsequent efficacious use, the preservation goal would be achieved.

Most fungi formulated as bioherbicides have been part of a granular or solid based formulation. The simplest formulations consist of fungal propagules that are allowed to grow on an inexpensive, readily available source of nutrients (e. g. seeds) which also function as

the carrier in the delivery system e. g. wheat kernels or a commeal-sand mixture (Brosten and Sands, 1986; Boyette *et al.*, 1985). Some fungi are grown, harvested, and mixed with a carrier like hydrated silica, wheat bran or vermiculite (Quimby, 1985; Morris, 1989; Walker, 1981), while other formulations consist of granules in which fungal propagules are entrapped in alginate granules or wheat gluten matrix (Walker and Connick, 1983; Connick *et al.*, 1991). Alginate granules are either exposed to light to induce sporulation and then air dried to produce granules with spores or air-dried immediately after gel pelletization. The latter granules require suitable environmental conditions to induce sporulation *in situ* (Walker and Connick, 1983).

When the formulated (dried) spores are not applied as such, water is the carrier of choice for bioherbicides. Problems encountered when using water as the carrier are the rapid evaporation of water limiting spore germination and thus disease incidence, and the poor wettability of weeds with a thick waxy cuticle preventing an equal distribution of the applied bioherbicide (Quimby and Fulgham, 1986; Boyette *et al.*, 1991). More uniform wetting of the plants has been achieved with the aid of various surfactants (Walker, 1980; Walker and Boyette, 1985; Mitchell, 1986, 1988; Andersen and Walker, 1985), whereas the problem of rapid evaporation has been addressed by applying an invert emulsion directly after the application of the bioherbicide (Quimby and Fulgham, 1986; Connick *et al.*, 1991).

Of the three commercialized bioherbicides, DeVine™ formulation consists of a "freshmilk" liquid product of *Phytophthora palmivora* with a shelf life of only about six weeks and was adopted because of the limited marketing area that permitted production upon ordering or demand (Kenny, 1986). The formulation of COLLEGO™ consists of an active

component (dried *Colletotrichum gloeosporioides* f. sp. *aeschynomene* spores) and a rehydrating agent used to wet the spores and enhance germination (Bowers, 1982, 1986). The formulation of the third product, BioMal™, is a wettable powder prepared by drying a conidial suspension of *C. gloeosporioides* f. sp. *malvae* mixed with kaolin (1:3 v/v) (Boyette *et al.*, 1991).

Many of the techniques used to formulate commercial products are usually subject to patents and/or trade secrets, with the latter being unavailable for testing in independent laboratories. Moreover, since every fungus has its own requirements and different abilities to withstand different adverse conditions, no one recipe can be followed in bioherbicide development. The objectives of these studies were to determine the "best" way to store *C. coccodes* conidia while maintaining their viability and infectivity (i.e. to develop a formulation of *C. coccodes* conidia), and to determine the effect of different storage conditions on the viability/infectivity of formulated *C. coccodes* conidia.

MATERIALS AND METHODS

1. Inoculum production and harvesting of *C. coccodes*

Small pieces of agar with mycelium from the stock culture (single conidium isolates of *C. coccodes* grown on potato dextrose agar (PDA) (dehydrated, Difco Laboratories, Detroit, MI) slants in small vials and maintained under mineral oil at 4C were transferred to fresh PDA in 9 cm petri dishes. Plates were sealed with Parafilm® (American Can Co.,

Greenwish, CT) and incubated at 24C without light for 7 to 21 days. Using a #2 cork borer, agar plugs were removed from the mycelial periphery of the colony and transferred to 250 ml Erlenmeyer flasks containing 100 ml of V-8 liquid medium. This medium consisted of 10 g sucrose, 10 g potassium nitrate, 2.5 g magnesium sulfate, 5 g potassium dihydrogen ortho phosphate, 0.02 g ferric chloride and 150 ml V-8 juice per litre, and was autoclaved for 17 minutes at 100 kPa and 120C. Inoculated flasks were placed on a rotary shaker at 250 rpm. After one week, conidia were harvested by pouring the contents of the flasks through four layers of cheese cloth in a funnel. The filtered conidia suspension was centrifuged at 250 rpm for 8 minutes, the pellets washed with distilled water and centrifuged a second time under the same conditions and then the pellets were resuspended in distilled water. Inoculum density was determined with the aid of a haemacytometer, and the desired quantities were subsequently processed.

2. Effect of water activity on *C. coccodes*

To determine the effect of water activity (a_w) on mycelial growth of *C. coccodes*, PDA plates, to which different amounts of glycerol (Bacto-glycerol, Difco laboratories, Detroit, MI) or polyethylene glycol (PEG) (Sigma Chemical Co, St Louis, MO) were added (3 replicates/treatment) were inoculated with agar plugs from *C. coccodes* cultured on PDA at 24C in the dark for 7 days. The media (treatments) used were : PDA, PDA + 0.1% glycerol, PDA + 0.2% glycerol, PDA + 0.4% glycerol, PDA + 10% glycerol, PDA + 0.3% PEG, PDA + 0.9% PEG and PDA + 2.7% PEG. PDA + PEG media had their pH adjusted with 1M KOH to have the same pH as PDA. Plates were incubated at 24C in dark in an incubator

(Model 1-35D, Johns Scientific Inc., Lachine, Québec) in a completely randomized design. There were three replicates for each treatment and the experiment was repeated twice. Water activity measurements were recorded initially (before inoculation) with a water activity meter. Colony diameter was measured over time.

3. Methods to formulate *C. coccodes* conidia

a) Freezing

Frozen conidia of *C. coccodes* were obtained from the Biotechnology Research Institute (BRI). After harvesting and before freezing, conidia were mixed with 1% starch (soluble potato powder, Baker Chemical Co., Phillipsburg, NJ), 1% sucrose (crystals, Mallinckrodt AR®, Mallinckrodt Canada Inc., Pointe Claire, Qué.), 0.2% Tween 20 (polyoxyehylene-sorbitan-monolaureate, Sigma Chemical Co., St Louis, MO), or 1% mannitol (D-mannitol, crystalline, Sigma Chemical Co., St Louis, MO) (2 replicates /treatment), then stored in a freezer (Danby/Ignis, The Danby Corporation, Montréal, Qué.) at -12C for two weeks. Conidia were allowed to thaw at room temperature; concentrations were determined with the aid of a haemacytometer, adjusted to 10^9 conidia/m^2 and used in viability/infectivity tests.

b) Freeze-drying

After harvest, conidia were freeze-dried alone ("plain") or after mixing with 0.2% Tween 20, 1% mannitol, 1% sucrose or 1% starch ("other") (2 replicates/treatment). "Plain" freeze-dried conidia were resuspended in water (10^9 conidia/m^2), 1% glucose (D-glucose, BDH Inc., Toronto, ON), 0.2% Tween 20, or 1% glucose+0.2 % Tween 20, and allowed to

rehydrate for 20 minutes on a rotary shaker at 200 rpm. The "other" freeze-dried conidia were resuspended in water ($10^9/m^2$), 1% sucrose or 0.2% Tween 20, and allowed to rehydrate 2 * 20 minutes on a rotary shaker at 200 rpm (with a still pause of 20 minutes in between). Freeze-dried conidia were tested immediately after processing and after 2 weeks of storage in a freezer at -12C.

c) Oven-drying

One or 3 ml of a 10^9 conidia/m^2 suspension were poured into small (5.1 cm) or large (9.2 cm diameter) glass petri plates, and the plates were then placed in an oven (Precision Thelco Model 4, Precision Scientific Co, Chicago, IL) at 30C. Each treatment was replicated twice. Forty five and ninety days later, oven-dried conidia were resuspended in water and tested for viability and infectivity.

d) Spray-drying

Suspensions of known concentrations of *C. coccodes* conidia were subjected to the following many different spray-drying conditions to identify conditions that keep the conidia viable and infective. They were mixed with many different concentrations of talcum powder (Fisher Scientific Ltd., Montreal, Qué.) or kaolin clay (hydrated aluminium silicate, Sigma® Chemical Co., St. Louis, MO) before or after spray-drying, spray-dried without any additive but with low inlet and outlet temperatures and flow rates, or with high temperatures and high flow rates, using a Mini Spray Drier (Büchi/Brinkmann, Model 190, Brinkmann Instruments Co., Westbury, NY). Each treatment was replicated twice. Spray-dried conidia were either

rehydrated directly in water for viability/infectivity tests or were put in moist chambers prior to resuspension to ensure a slow rehydration.

4. Effect of various fillers on drying of *C. coccodes* conidia

Pellets or concentrated suspensions of *C. coccodes* were mixed with different amounts of sucrose (crystals, Mallinckrodt AR®, Mallinckrodt Canada Inc., Pointe Claire, Qué), arabic gum (from acacia tree, Sigma® Chemical Co., St. Louis, MO), casamino acids (Difco, Difco laboratories, Detroit, MI), skim milk (instant skim milk powder, Carnation, Carnation Inc., Toronto, ON), glycerol, casein (from bovine milk, Sigma® Chemical Co., St. Louis, MO), L-tryptophan (L-α-amino 3-indole propionic acid, Sigma® Chemical Co., St. Louis, MO), soluble starch (Mallinckrodt Canada Inc., Pointe Claire, Qué.), kaolin clay, or talcum powder. Seventeen of these preparations were stored in an oven at 30C, the remaining 24 preparations were kept on a shelf at room temperature. These preparations were resuspended in distilled water (10^9 conidia/m^2), put on a shaker for 20 minutes to ensure conidial rehydration, and used in viability/infectivity tests.

5. Final selected preparations

With respect to the above drying procedures, only the use of certain fillers proved to be effective in preserving the viability and infectivity of *C. coccodes* conidia. These fillers were, in decreasing order of effectiveness, kaolin clay, talcum powder and soluble starch. Accordingly, the following formulations were prepared and used in all subsequent experiments :

Prep. I = 1 : 2.78 (g *C. coccodes* -Cc- : g kaolin clay)

Prep. II= 1: 3.34 (g Cc : g kaolin clay)

Prep. III= 1: 3.07 (g Cc : g kaolin clay)

Prep. IV= 1: 2.79 (g Cc : g talcum powder)

Prep. V= 1: 3.98 (g Cc : g talcum powder)

Prep. VI= 1 : 4.98 (g Cc : g talcum powder)

Prep. VII= 1: 2.54 (g Cc : g soluble starch)

Prep. VIII= 1 : 3.78 (g Cc : g soluble starch)

Prep. IX= 1 : 5.04 (g Cc : g soluble starch)

a) Effect of temperature on viability/infectivity of *C. coccodes*. Conidia were produced and processed as described previously, and stored in glass petri plates. Six plates of the 9 formulations were placed in a plastic bag (polyethylene, 16.5x39.5 cm^2) and put in an oven at 30 or 40C (Thelco Precision Scientific Co, Chicago, IL) or in a fridge at 4C (Danby/IGNIS, Montréal, Qué.). Viability/infectivity tests were conducted every 24 hours for a week, after a 2 week period, and every month for six months.

b) Effect of bags permeable to oxygen on viability/infectivity of *C. coccodes*. After mixing conidia with kaolin, talcum or starch, conidia were stored in glass petri dishes in bags permeable to oxygen (Film E 50S, Low Density Polyethylene/Ethylene Vinyl Acetate, OTR-Oxygen Transmission Rate = 4000 $cc/m^2/day$ at 23C and 0% RH, Winpak Packaging, Winnipeg). Headspace gas composition of the bags was measured on a monthly basis for six

months. Gas samples were withdrawn from the bags with a 0.5 ml gas-tight Pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, LA) through the attached silicone seals. Headspace gas was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector and using Porapack Q (80-100 mesh) and Molecular Sieve 5A (80-100 mesh) columns in series. The carrier gas consisted of helium which flow rate was 20 ml/min. The column oven was set at 80C. The injector and the detector were set at 100C. Peaks were recorded and analysed with a Hewlett-Packard integrator (model 3390A, Hewlett-Packard Co., Avondale, PA). Water activity measurements were taken initially and every month for a period of six months with a water activity meter (Decagon (CX-1) meter, Decagon Devices Inc., Pullman, WA). Weight of the preparations was recorded using a Sartorius balance (L610D, Sartorius GmbH, Goettingen, Germany) subsequent to water activity measurements; and this was followed by bioassays to determine if conidia in preparations were still viable and able to infect.

6. Testing for viability / infectivity

a) Tests conducted. Processed and "fresh" (control) conidial suspensions were: 1) sprayed on PDA plates (3 plates / treatment), 2) placed in moist chambers (2 slides / plate, 2 drops of 0.02 ml each / slide), 3) used to inoculate excised leaves (3 leaves/moist chamber, 2 drops of 0.02 ml on leaf midvein) or 4) used to inoculate whole velvetleaf plants (3 plants / treatment).

b) Seed germination and velvetleaf growth. To prepare plant material for infectivity evaluation, seeds were placed on moist filter paper (Whatman®, #1) in glass petri dishes

placed at 5C for 24 hours then incubated at 30C for 24 hrs. Four germinated seeds were sown in potting medium (Pro-Mix Bx®, Premier Brand, Inc., New York, NY) in 10 cm plastic pots. Seedlings were grown in controlled environment chambers (Conviron®, Model E-15, Controlled Environments, Winnipeg, Man.) (24/18 C day/night temperature, 14-hr photoperiod, 400 $\mu Em^{-2}\ s^{-1}$) and thinned to three seedlings per pot before use.

c) Sprayer / application specifications. Conidia were resuspended in 10 ml of distilled water and sprayed on 14 day-old velvetleaf plants at a rate of 10^9 conidia/m^2. Three pots (3 plants/pot) and one PDA plate were sprayed per treatment. Sprayed velvetleaf plants were incubated in a dark dew chamber at 24C for 18 hours and subsequently transferred to a growth cabinet at the original conditions.

d) "Fresh conidia" production. Conidia used as controls were prepared fresh for each experiment as discussed above (Inoculum production and harvesting of *C. coccodes*). After determination of the inoculum density of the obtained suspension, the desired volume of the suspension was diluted in a specific volume of distilled water in order to apply the equivalent of 10^9 conidia/m^2 onto potted plants in the spray chamber.

e) Data collection. PDA plates and moist chambers were examined 24 hours after inoculation for germ tube formation, the excised leaves were examined six days after inoculation and whole plants 3 to 14 days after inoculation, and compared to excised leaves or whole plants inoculated with fresh conidia or water as controls. When the number of lesions was low, lesions were counted; otherwise disease ratings of 0 (no lesions) to 10 (>90% of the sprayed area consisting of lesions) were visually assigned to velvetleaf plants.

7) Data analysis

A completely randomized design with three replicates/treatment was used in all experiments. Quantitative experiments were performed two times and data were pooled because of the homogeneity of variances. Differences between treatments were established with Duncan's multiple range test (α =0.05) (Steel and Torrie, 1980). Disease ratings obtained from experiments run over time were analyzed at the 6th month by the Krusgal-Wallis 1-way ANOVA by ranks and multiple comparisons were conducted. These data were also regressed over time according to Theil's method for estimating slope parameters, and point estimates of slope coefficients β were calculated for each formulation (Daniel, 1990). Water activities of the formulations within the bags were regressed nonlinearly over time, and water activity was quadratically regressed over weight loss.

RESULTS AND DISCUSSION

Determining the effect of water activity on *Colletotrichum coccodes*

The water activity of PDA did not decrease noticeably unless glycerol concentrations reached 10 or 20% in the medium (Table 1). Growth of *C. coccodes* was therefore minimally affected by the addition of PEG or low concentrations of glycerol. Colony diameter after 12 or 14 days was significantly lower on PDA to which 2.7% PEG was added (Table 1a); but eventhough *C. coccodes* growth was first retarded as the result of the addition of 0.1, 0.2, or 0.4% glycerol, after 21 days there was no significant difference between

Table 1. Water activity of PDA and augmented PDA and average colony diameter of *C. coccodes* when plated on PDA, PDA to which 0.3%, 0.9% or 2.7% PEG are added (A), when plated on PDA and PDA to which 0.1, 0.2 or 0.3% glycerol was added (B)and on PDA to which 10 or 20% glycerol was added (C).

(A)

Time (days)	Average colony diameter (cm) when plated on PDA (a_w=0.989)	PDA+0.3% PEG (a_w=0.988)	PDA+0.9% PEG (a_w=0.987)	PDA+2.7% PEG (a_w=0.986)
6	3.37 a[1]	3.17 bc	3.27 ab	3.08 c
9	4.43 a	4.05 b	4.33 a	4.25 ab
12	6.22 a	5.98 a	5.9 a	5.43 b
14	6.95 a	6.75 a	6.8 a	6.03 b

(B)

Time (days)	Average colony diameter (cm) when plated on PDA (a_w=0.989)	PDA+0.1% glycerol (a_w=0.988)	PDA+0.2% glycerol (a_w=0.987)	PDA+0.4% glycerol (a_w=0.983)
3	2.41 a	1.78 c	1.96 b	1.65 d
7	4.53 a	4.45 ab	4.37 ab	4.18 b
15	8.4 a	8.02 b	8.07 b	7.68 c
21	8.5 a	8.5 a	8.5 a	8.5 a

(C)

Time (days)	Average colony diameter (cm) when plated on PDA (a_w=0.989)	PDA+10% glycerol (a_w=0.948)	PDA+20% glycerol (a_w=0.913)
3	2.48 a	0 b	0 b
11	5.95 a	0.67 b	0 c
19	8.11 a	1.5 b	0 c
25	8.5 a	2.81 b	0 c

[1] : numbers in the same row followed by the same letter are not significantly different according to Duncan's multiple range test.

treatments (Table 1b). At a specific a_w, one solute may be more inhibitory to an organism than another (Troller, 1987). In this experiment PDA+0.3% PEG and PDA+0.1% glycerol had the same a_w (as did PDA+0.9% PEG and PDA+0.2% glycerol) but a difference was observed in *C. coccodes* growth. The addition of 10% glycerol to PDA (a_w= 0.948) decreased *C. coccodes* growth by about 70%, and 20% glycerol (a_w= 0.913) completely inhibited *C. coccodes* growth (Table 1c). A water activity of 0.948 is apparently too low for *C. coccodes* growth. Most molds have an optimum a_w for growth near 1.0, but the minimum at which growth (at a very slow rate) of fungi has been observed is 0.61-0.62 (Corry, 1987). *C. coccodes* did not grow at all when the a_w of the medium was 0.913. These results indicate that *C. coccodes* needs a high availability of water to resume normal metabolic activity, or that *C. coccodes* lacks the capacity to build an internal osmotic potential that would balance the external osmotic potential which would allow it to resume growth.

Freezing

Conidia which were frozen did not germinate on PDA nor did they form lesions on excised velvetleaf leaves. During freezing, the water activity of a product is reduced. Water separates in the form of ice leaving an increasingly concentrated solution (Christian, 1963). Although the viability of many microorganisms is preserved for 1-2 years through freezing, it is known that this method is not suitable for long term storage of many microorganisms (Chang and Elander, 1986). In the case of *C. coccodes*, freezing, a slow process that results in ice crystal formation, seems to have damaged cell membranes, resulting in their death.

Freeze-drying

No germination was observed when freeze-dried conidia were rehydrated in moist chambers. A few conidia formed colonies on PDA, while others caused lesions on plants (Table 2). However, the overall number of viable and infective conidia after freeze-drying was very low. All fresh conidia treatments (positive controls) showed germ tube and appressorium formation when incubated for 24 hours in moist chambers at room temperature. Furthermore, all PDA plates and velvetleaf plants inoculated with fresh conidia showed colony and lesion formation, respectively.

Freeze-drying relies on the fact that very rapid freezing may not damage fungal spores and that the addition of a non-electrolyte, such as glycerol or sugar, to conidia prior to freeze-drying decreases mortality during dehydration (Brown, 1976). The non-electrolyte may act directly by substituting as a "solvating" molecule for water removed by dehydration, preventing protein inactivation and preserving cell viability (Lenovich, 1987). However, a number of species cannot be lyophilized successfully and one reason could be their thin cell walls (Smith, 1969). The thickness of *C. coccodes* cell walls has not been compared relative to fungi which have been successfully lyophilized. Perhaps *C. coccodes* cells are thin walled possibly explaining why freeze-drying was not satisfactory.

Oven drying

When tested 45 and 90 days after storage, oven dried conidia were still viable and able to infect, but to a lesser extent than fresh conidia (Table 3). Fresh conidia resulted in a large number of lesions that were difficult to count, whereas oven dried conidia resulted in a

Table 2. Viability and infectivity of freeze-dried *Colletotrichum coccodes* conidia.

Preparation	Number of colonies on a PDA plate	Number of lesions on plants ($\bar{X}$ ± SD)
FD[1] conidia+0.2%Tween 20[2]+water[3]	0	0
FD conidia+0.2%Tween 20[2]+1% sucrose[3]	0	0
FD conidia+0.2%Tween 20[2]+ 0.2% Tween 20[3]	0	1.3 ± 2.31
FD conidia+1% mannitol[2]+water[3]	0	1.0 ± 1.73
FD conidia+1% mannitol[2]+1% sucrose[3]	3	0
FD conidia+1%mannitol[2]+0.2%Tween 20[3]	7	0
FD conidia+1% sucrose[2]+ water[3]	0	2.3 ± 2.52
FD conidia+1% sucrose[2]+1% sucrose[3]	0	1.0 ± 1.73
FD conidia+1% sucrose[2]+0.2% Tween 20[3]	0	7.3 ± 12.7
FD conidia+1% starch[2]+water[3]	0	2.3 ± 2.52
FD conidia+1% starch[2]+1% sucrose[3]	0	1.0 ± 1.73
FD conidia+1% starch[2]+0.2% Tween 20[3]	7	0
Fresh conidia	+[4]	+, +, +
Fresh conidia + 1% sucrose	+	+, +, +
Fresh conidia + 0.2% Tween 20	+	+, +, +
Water	0	0

FD[1] =freeze-dried. [2] : the compound has been mixed with conidia prior to freeze-drying. [3] : the compound was used as a resuspending agent for freeze-dried preparations. [4]+ : colonies on PDA or lesions on velvetleaf were too numerous to be counted.

Table 3. Infectivity of oven dried *Colletotrichum coccodes* conidia (A) after 45 days in oven at 30C, (B) after 90 days in oven at 30C.

(A) After 45 days of storage at 30C

Preparation	Number of lesions 8 days after inoculation (X ± SD)	# lesions 11 days after inoculation (X ± SD)	# lesions 14 days after inoculation (X ± SD)
3 ml in 9.2 mm diam. dish	3.0 ± 1.73 5.3 ± 0.58	4.0 ± 2.65 5.7 ± 1.53	5.3 ± 0.58 6.0 ± 1.0
3 ml in 5.1 mm diam. dish	9.3 ± 1.15 9.0 ± 1.00	7.3 ± 0.58 8.0 ± 2.00	9.0 ± 1.73 7.7 ± 0.58
1 ml in 9.2 mm diam. dish	4.3 ± 0.58 4.3 ± 0.58	5.6 ± 0.58 5.3 ± 0.58	3.7 ± 1.53 2.7 ± 2.08
1 ml in 5.1 mm diam. dish	2.0 ± 1.73 3.0 ± 1.00	1.0 ± 0.00 2.3 ± 2.31	2.3 ± 1.53 2.3 ± 2.31
Fresh conidia	+, +, + +, +, +	+, +, + +, +, +	+, +, + +, +, +

(B) After 90 days of storage at 30C

Preparation	# lesions 7 days after inoculation (X ± SD)
3 ml in 9.2mm diam. dish	1.7 ± 1.53 1.7 ± 1.53
3 ml in 5.1mm diam. dish	0.7 ± 0.58 1.7 ± 0.58
1 ml in 9.2mm diam. dish	4.3 ± 0.58 4.3 ± 0.58
1 ml in 5.1mm diam. dish	5.3 ± 0.58 3.3 ± 1.53
Fresh conidia	+, +, + +, +, +

+ : lesions were very numerous and difficult to count.

"countable" lesion number. Viability decreased after 45 days, independently of the quantity dried and the size of the dishes used. After 90 days of storage, infectivity of the 3ml preparations decreased and no lesions were observed on some plants (Table 3). The optimum temperature for *C. coccodes* growth has been determined to be 24C (Poirier, 1984; Wymore *et al.*, 1988), but the progression of the disease on velvetleaf was similar at both 24 and 30C (Poirier, 1984). This might suggest that conidia were able to germinate before water evaporates, resulting in a reduced infectivity when compared to fresh conidia. Another problem encountered with oven dried conidia was that they were hard and brittle and resuspending them in water was difficult.

Spray-drying

When conidia were spray-dried at low/high temperatures and under low/high flow rates, or mixed with kaolin clay or talcum powder prior to spray-drying, germination occurred when plated directly on PDA after drying. However, spray-dried conidia did not incite disease when used to inoculate velvetleaf seedlings. *C. coccodes* conidia were apparently adversely affected by the harsh conditions of spray-drying, and although they remained viable, they were not able to infect.

When conidia were spray-dried "normally" (high temperatures, regular flow rates) with or without additives, they did not germinate on PDA. Even when placed in moist chambers to permit a slow rehydration before resuspension, conidia still failed to germinate. Spray-drying has been used in drying bacteria or yeasts (Peppler, 1979), but no reports on its use with filamentous fungi could be found.

Adding fillers

Oven dried conidia with whatever filler, lost viability after a few weeks (Table 4). Arabic gum and sucrose preparations were brittle and not easy to handle. When mixed with one or more amino acids *C. coccodes* seemed to loose viability. Few or no lesions were observed on plants inoculated with these preparations. The glycerol preparation seemed to have decreased the a_w of the preparation to a point where the fungus was not able to survive. Skim milk preparations did not show a high degree of conidial survival and particle size was such that the nozzle was clogged when these preparations were sprayed on velvetleaf plants. Mortality of *C. coccodes* in protein/amino acids preparations may have been caused by the denaturation that occurred when these amino acids were autoclaved; and the same might apply to preparations with skim milk. Of all the fillers tested, kaolin clay, talcum powder (Fig. 1A) and starch were the most promising (Table 4, Figure 1B, C). Some of the preparations with kaolin clay or talcum powder kept the conidia viable and able to infect in a manner similar to fresh conidia, for a period of at least six months, when stored at room temperature (Figure 2 A, B, C, D). When tested after 14 months of storage, preparations with kaolin clay produced lesions on 14 day-old velvetleaf seedlings (Figure 1D).

Table 4. Infectivity of formulated *Colletotrichum coccodes* conidia in various fillers on whole velvetleaf plants or excised velvetleaf leaves (at 2 weeks) after storage at room temperature or at 30C.

Preparation	1wk	2wks	1mo	2mo	3mo	4mo	5 mo	6 mo
*[1] 1.04g Cc pellet + 0.008g starch	+++[2]	+++	- - -	- - -				
* 1.00g Cc pellet[3] + 0.145g starch	+++	+++	- - +	- - -	- - -			
* 1.003g Cc pellet[3] + 0.156g skim milk	+++	+++	- - +	- - -	- - -			
1.007g Cc pellet[3] + 1.584 starch	+++	+++	- ++	- - -	- - -			
3.018g Cc[4] + 5.604g sucrose	+++	+++	+++	- - +	- - -	- - -		
3.003g Cc[4] + 1.173g skim milk	- - +	+ - -	- - -	- - -				
3.014g Cc[4] + 3.089g skim milk	+++	+++	- ++	- ++	- ++	- ++	- -+	- - -
3.006g Cc[4] + 3.827g starch	+++	+++	+++	- ++	- - -	- - -		
* 3.008g Cc[4] + 4.561g sucrose	- ++	- ++	- - -	- - -				
* 3.006g Cc[4] + 0.377g arabic gum	- + -	- - -	- - +	- - -	- - -			
* 3.011g Cc[4] + 1.833g casamino acids	+++	+++	+++	+++	- ++	- - -	- - -	
* 3.024g Cc[4] + 1.006g skim milk	+++	- ++	- ++	- ++	- - -	- - -		
* 3.017g Cc[4] + 2.051g skim milk	+++	+++	- ++	- ++	- - -	- - -		
* 3.027g Cc[4] + 3.526g starch	+++	+++	- ++	- ++	- ++	- - -	- - -	
* 3.016g Cc[4] + 3.093g starch	+++	+++	+++	+++	- ++	- - -	- - -	
* 0.911g Cc[4]	+++	- ++	df[7]					
* 3.983g Cc[5] + 2.944g kaolin	- ++	- ++	- ++	- - +	- - -	- - -		
* 3.935g Cc[5] + 2.642g talcum powder	+++	- ++	- ++	- - -	- - -			

Table 4 (cont'd)

* 3.941g Cc^5 + 9.651g glycerol	- ++	- ++	- - -	- - -				
* 3.901g Cc^5 + 2.477g L-tryptophan	+++	- - +	- - -	- - -				
* 3.957g Cc^5 + 0.831g L-tryptophan +0.57g leucine + 3.222g asparagine + 0.508g threonine	+++	- - +	- - -	- - -				
3.99?g Cc^5 + 2.927g kaolin	+++	- ++	+++	- - +	- - -	- -+	- -+	- -+
3.959g Cc^5 + 3.936g kaolin	+++	+++	+++	- ++	- - -	- -+	- - -	- -+
3.977g Cc^5+ 5.078g talcum powder	+++	+++	- ++	- ++	- - +	- - +	- - +	- ++
3.962g Cc^5 + 3.039g casein	- ++	+++	- - +	- - -	- - -			
3.879g Cc^5 + 4.788g starch	- ++	- ++	- - +	- - -	- - -			
5.535g Cc^6 + 12.07g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++
5.535g Cc^6 + 21.105g starch	+++	+++	+++	+++	+++	+++	- ++	- - +
6.693g Cc^6 + 15.557g skim milk	+++	+++	+++	+++	- ++	- -+	- - +	- - -
3.94g Cc^7 + 10.56g kaolin	+++	+++	+++	+++	+++	+++	+++	+++
3.903 Cc^7 + 13.127g kaolin	+++	+++	+++	+++	+++	+++	+++	+++
3.93g Cc^7 + 11.17g kaolin	+++	+++	+++	+++	+++	+++	+++	+++
3.977 Cc^7 + 13.191g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++
3.955g Cc^7 + 14.553g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++
3.931g Cc^7 + 11.652g starch	+++	+++	+++	+++	+++	+++	- ++	- - +
3.918g Cc^7 + 13.652g starch	+++	+++	+++	+++	+++	+++	- ++	- - +

Table 4 (cont'd)

9.889g Cc[7] (5 replicates)	+++	+++	- ++	df				
2.500g Cc[8] +12.5g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++
2.000g Cc[8] + 8.0g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++
2.024g Cc + 5.684g talcum powder	+++	+++	+++	+++	+++	+++	+++	+++

[1] preparations stored at 30C are indicated with *. [2] + or - : presence or absence of lesions in each replicate. [3] : *C. coccodes* (Cc) concentration = 1.4875×10^8 conidia/ml; [4] : Cc concentration = 8.25×10^9 conidia/ml; [5] : Cc concentration = 3.125×10^9 conidia/ml; [6] : Cc concentration = 1.997×10^9 conidia/ml; [7] : Cc concentration = 1.369×10^9 conidia/ml; [8] : Cc concentration = 2.904×10^7 conidia/ml. df[9] : difficult to resuspend.

Figure 1. (A) Preparations of conidia of *Colletotrichum coccodes* formulated in talcum powder, (B, C) Leaves of velvetleaf plants sprayed with conidia formulated in kaolin clay (B, C), talcum powder (B), or soluble starch (B) and stored for 4 months at room temperature, (D) Velvetleaf seedlings inoculated with a 14 month-old preparation of *C. coccodes* conidia in kaolin clay.

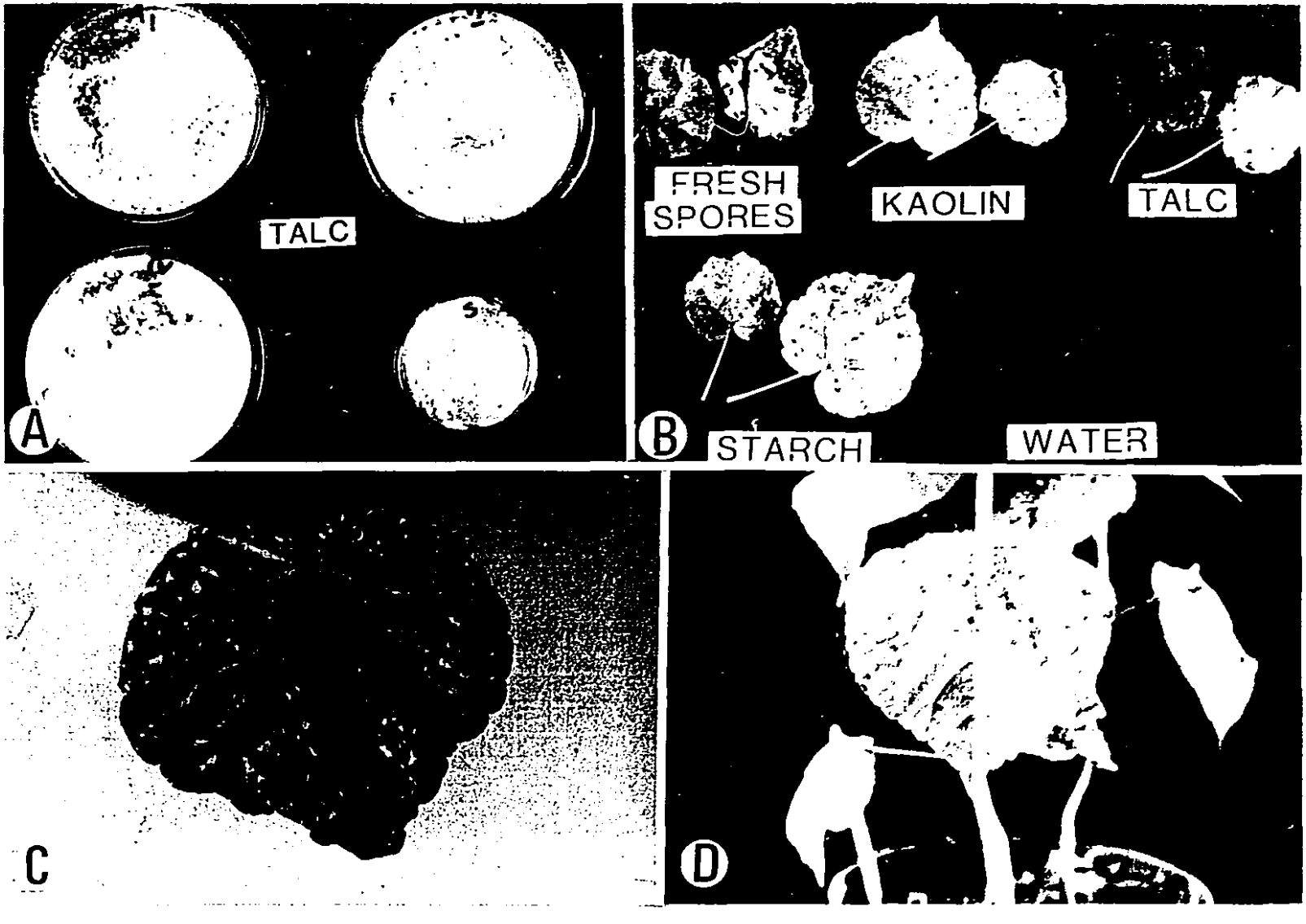
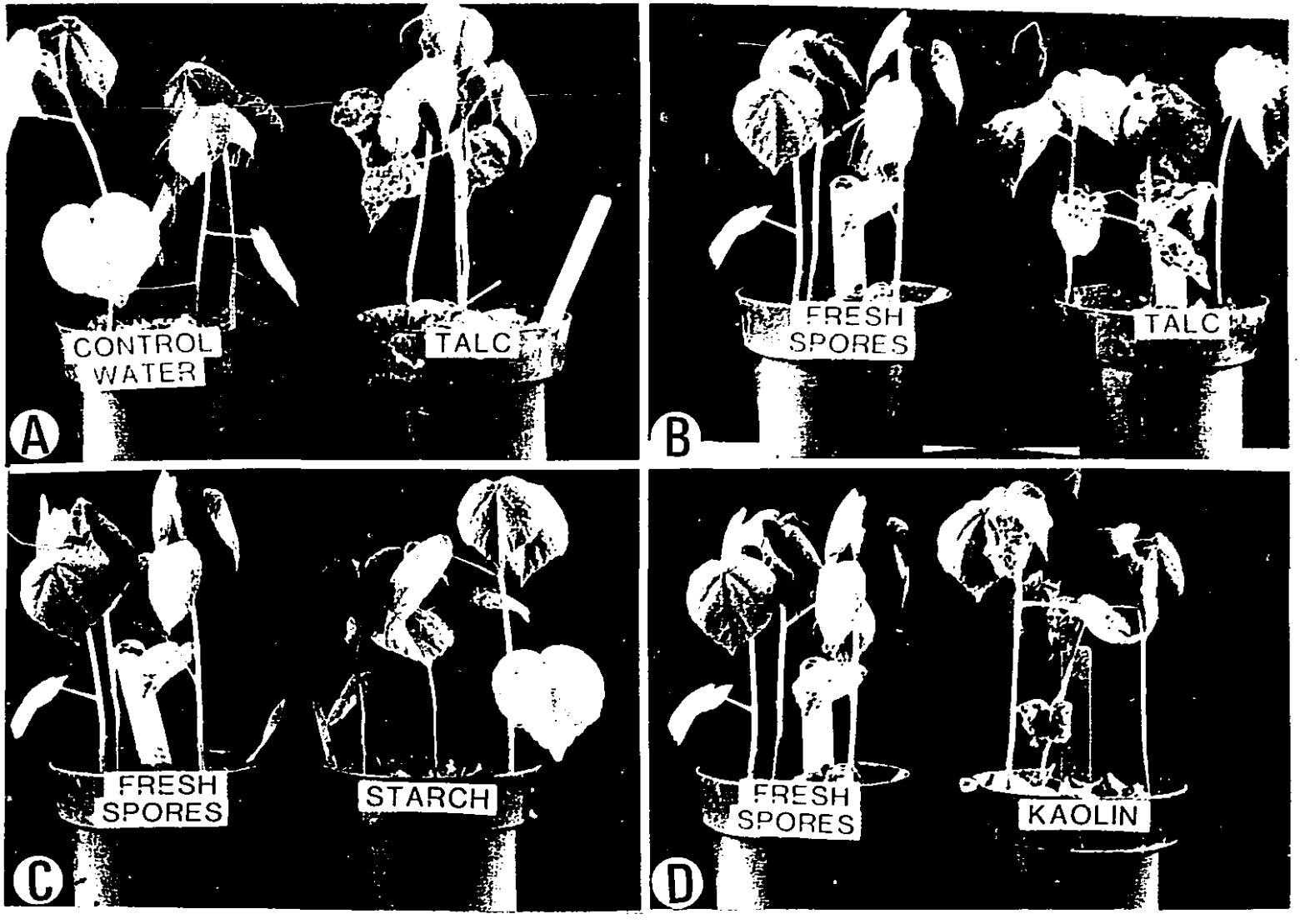

TALC
A
FRESH SPORES
KAOLIN
TALC
B
STARCH
WATER
C
D

Figure 2. Fourteen day-old velvetleaf plants sprayed with conidia of *Colletotrichum coccodes* formulated in talcum powder (A, B), soluble starch (C), or kaolin clay (D) and stored for 6 months at room temperature compared to velvetleaf seedlings sprayed with water (A), or fresh conidia (B, C, D).


CONTROL
WATER
TALC
A
FRESH
SPORES
TALC
B
FRESH
SPORES
STARCH
C
FRESH
SPORES
KAOLIN
D

Effect of temperature on viability/infectivity of *C. coccodes* conidia

C. coccodes conidia in the final nine selected preparations were still viable and able to infect after 6 months when stored at 4 or 30C, whereas after 6 months conidia stored at 40C did not incite disease on velvetleaf seedlings (Table 5). Multiple comparison analysis of disease ratings at the sixth month showed that Prep. VII at 4C, and Prep. V and VI at 30C performed best and were significantly different from preparations stored at 40C (Table 5). However, variable results of Prep. VII stored at 4C were obtained over the 6 month period (Table 5). Conidia stored at 40C were subjected to conditions that lead to a decrease in infectivity after only 2 weeks of storage (Table 5). Estimates of the slope β of the population regression line describing the linear relationship between the disease ratings and time, calculated for all preparations, demonstrated that formulated *C. coccodes* conidia stored at 4 or 30C lost infectivity slowly (0.051 to 0.294) especially when formulated in kaolin clay or talcum powder (0.051 to 0.192) (Table 6). However, the rate of decline in infectivity was greater for conidia at 40C (0.318 to 0.401) irrespective of the filler used (Table 6). The higher rate of decline in infectivity lead eventually (after 6 months) to a greater loss in infectivity of preparations stored at 40C as compared to preparations stored at 4 or 30C. At any given temperature (4, 30 or 40C), conidia formulated in soluble starch (Prep. VII, VIII and IX) generally resulted in lower disease ratings than did conidia formulated in kaolin clay or talcum powder. This coincides with previous observations when formulated conidia were stored at room temperature (section Adding fillers).

Table 5. Infectivity of various preparations of *Colletotrichum coccodes* conidia stored at three different temperatures and then sprayed on velvetleaf.

	Prep.[1]	Disease rating[2]												
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs	2 wks	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
at 4C	I	+	+	9.3	9.6	+	+	8.6	9.6	8.3	6.3	4.6	8.6	7.6 ab
	II	+	+	9.6	10.0	+	+	9.0	8.3	9.6	6.6	5.0	4.3	6.6 ab
	III	+	+	10.0	9.6	+	+	8.0	9.0	9.6	7.3	5.6	3.0	6.3 ab
	IV	+	+	10.0	9.6	+	+	6.0	10.0	9.3	8.3	9.3	8.0	8.0 ab
	V	+	+	8.3	8.6	+	+	5.6	9.0	7.0	10.0	8.6	4.0	7.0 ab
	VI	+	+	9.3	8.3	+	+	6.6	10.0	8.6	9.3	8.0	5.6	7.6 ab
	VII	+	+	9.0	9.6	+	+	5.0	8.6	5.3	4.6	1.0	10.0	10.0 a
	VIII	+	+	9.3	9.3	+	+	5.3	1.6	4.3	4.3	6.0	8.0	7.0 ab
	IX	+	+	8.6	9.0	+	+	1.3	1.6	3.0	1.3	4.0	7.0	2.0 ab
at 30C	I	+	+	9.0	9.3	+	+	10.0	10.0	8.3	6.3	5.6	9	9.3 ab
	II	+	+	8.3	10.0	+	+	9.6	9.6	7.3	6.6	5.3	6	6.3 ab
	III	+	+	9.0	10.0	+	+	10.0	10.0	7.0	6.0	5.3	6.3	8 ab
	IV	+	+	8.3	9.6	+	+	6.6	10.0	7.6	8.6	6	5.3	6.3 ab
	V	+	+	8.6	8.6	+	+	7.6	10.0	8.0	9.3	5.6	4.3	10 a
	VI	+	+	9.0	9.3	+	+	7.0	10.0	7.3	8.6	5.6	3.6	10 a
	VII	+	+	9.0	9.3	+	+	4.6	6.6	3.6	5.3	6.6	4.3	3.8 ab
	VIII	+	+	8.6	8.6	+	+	4.0	3.0	1.3	0.3	6	4	1 ab
	IX	+	+	8.3	7.6	+	+	2.3	1.6	1.6	0.0	5.3	2.6	0.3 ab
at 40C	I	8.5	8.0	7.5	+	+	+	3.3	6.0	0.6	0.3	1.6	2.0	0.0 b
	II	9.0	7.5	8.5	+	+	+	1.0	2.6	1.6	0.6	0.6	2.3	0.0 b
	III	8.0	8.5	8.0	+	+	+	1.6	3.6	0.6	0.0	1.0	2.0	0.0 b
	IV	8.0	7.5	8.5	+	+	+	0.3	0.3	0.3	0.3	0.6	1.6	0.0 b
	V	8.5	8.5	7.5	+	+	+	0.3	0.6	4.3	0.6	0.0	1.0	0.0 b
	VI	8.0	8.5	7.5	+	+	+	2.6	1.6	6.0	0.3	0.0	0.0	0.0 b
	VII	7.0	7.5	8.0	+	+	+	0.3	0.0	0.3	0.0	0.0	0.6	0.0 b
	VIII	7.0	7.5	7.0	+	+	+	0.3	1.0	0.0	0.0	1.0	0.0	0.0 b
	IX	7.5	8.0	8.0	+	+	+	0.3	0.3	0.0	0.0	0.0	1.0	0.0 b

[1] Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC), Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC, Prep. IV=1 ml Cc:2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP, Prep. VII=1 ml Cc:2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

[2] each value is the average of three replicates, a + sign represents the presence of lesions on excised leaves, whereas the numerical values are a visual estimate of disease with 0 being no lesions and 10>90% of the sprayed area is covered with lesions.

[3] Means followed by the same letter(s) are not significantly different according to Krusgal-Wallis multiple comparison test.

Table 6. Estimates of the ß parameter (comparing decrease in infectivity of *Colletotrichum coccodes* conidia on velvetleaf over time) of disease ratings of preparations at 4, 30, or 40C or at room temperature in bags permeable to oxygen.

Prep.[1] at 4C	ß estimate	Prep. at 40C	ß estimate	Prep. at 30C	ß estimate	Prep. in bags (23C)	ß estimate
I	-0.085	I	-0.318	I	0.043	I	-0.258
II	-0.192	II	-0.389	II	-0.079	II	-0.241
III	-0.185	III	-0.354	III	-0.105	III	-0.030
IV	-0.037	IV	-0.380	IV	-0.065	IV	-1.368
V	-0.047	V	-0.344	V	-0.051	V	-0.343
VI	-0.080	VI	-0.325	VI	-0.091	VI	-0.060
VII	0.103	VII	-0.328	VII	-0.197	VII	-0.249
VIII	-0.086	VIII	-0.319	VIII	-0.296	VIII	-1.578
IX	-0.216	IX	-0.401	IX	-0.294	IX	-1.055

[1] Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC), Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC, Prep. IV=1 ml Cc:2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP, Prep. VII=1 ml Cc:2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

Had water been available, conidia would have probably germinated and resumed growth but since water was bound to fillers, growth was inhibited. Conidia in conidial pellets or suspensions stored at 4C would germinate in a two three days period and eventually die probably because of the lack of oxygen. However, when a filler was added, this was prevented and the preparations stored for at least six months. Forty degrees was too high a temperature for *C. coccodes* and might have had acted directly on conidia by causing an irrecoverable conidial damage or indirectly by reducing a_w drastically. As Teitell (1958) reported, at a given a_w any increase in temperature would result in a decrease in spore viability, whereas lower temperatures (above freezing) favour longevity.

Effect of bags permeable to oxygen on viability and infectivity of formulated *C. coccodes* conidia

Moisture loss or decrease in a_w was greatest during the first month and then remained constant throughout the storage period (Fig. 3, 4, 5). A priori it is expected that water would evaporate within the first month (or even first few days) resulting in a decreased a_w and an increased weight loss of the preparations in the first month. Once water evaporated, weight loss stabilized (Fig. 6).

The rate at which conidia lost infectivity over time depended on the preparation and varied from 0.03 to 1.578 (Table 6). Conidia mixed with talcum powder at a ratio of 1:2.79 or with soluble starch at ratios of 1:3.78 and 1:5.04 were the ones that showed the highest rates, and therefore were the worst preparations. These data were supported by the analysis of disease ratings at the 6th month, with the worst preparations being one with talcum powder

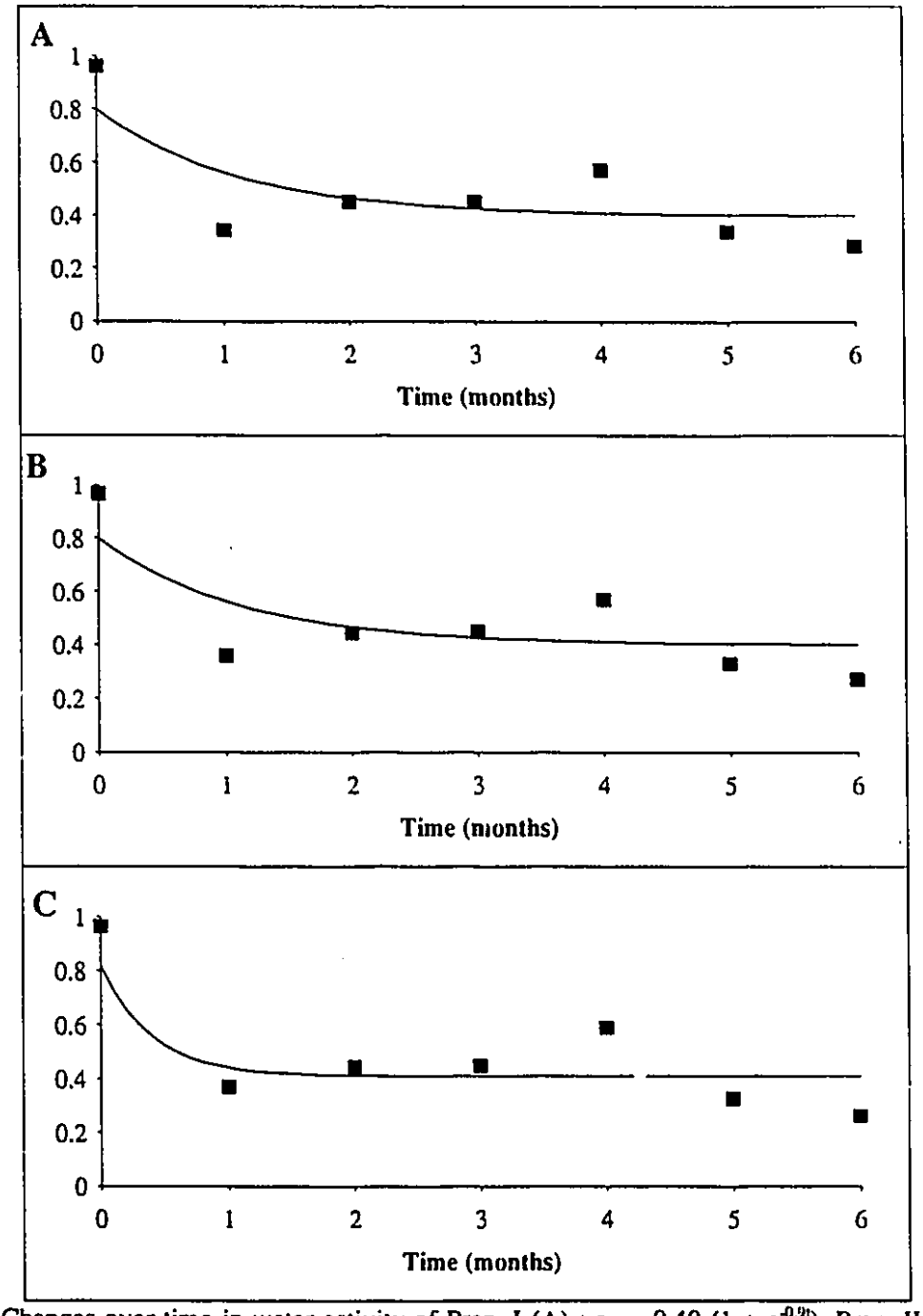


Figure 3. Changes over time in water activity of Prep. I (A) : $a_w = 0.40\ (1 + e^{-0.9t})$, Prep. II (B) : $a_w = 0.40\ (1 + e^{-0.9t})$, and Prep. III (C) : $a_w = 0.41\ (1 + e^{-2.57t})$, when stored at room temperature in bags permeable to oxygen; where Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC); Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC.

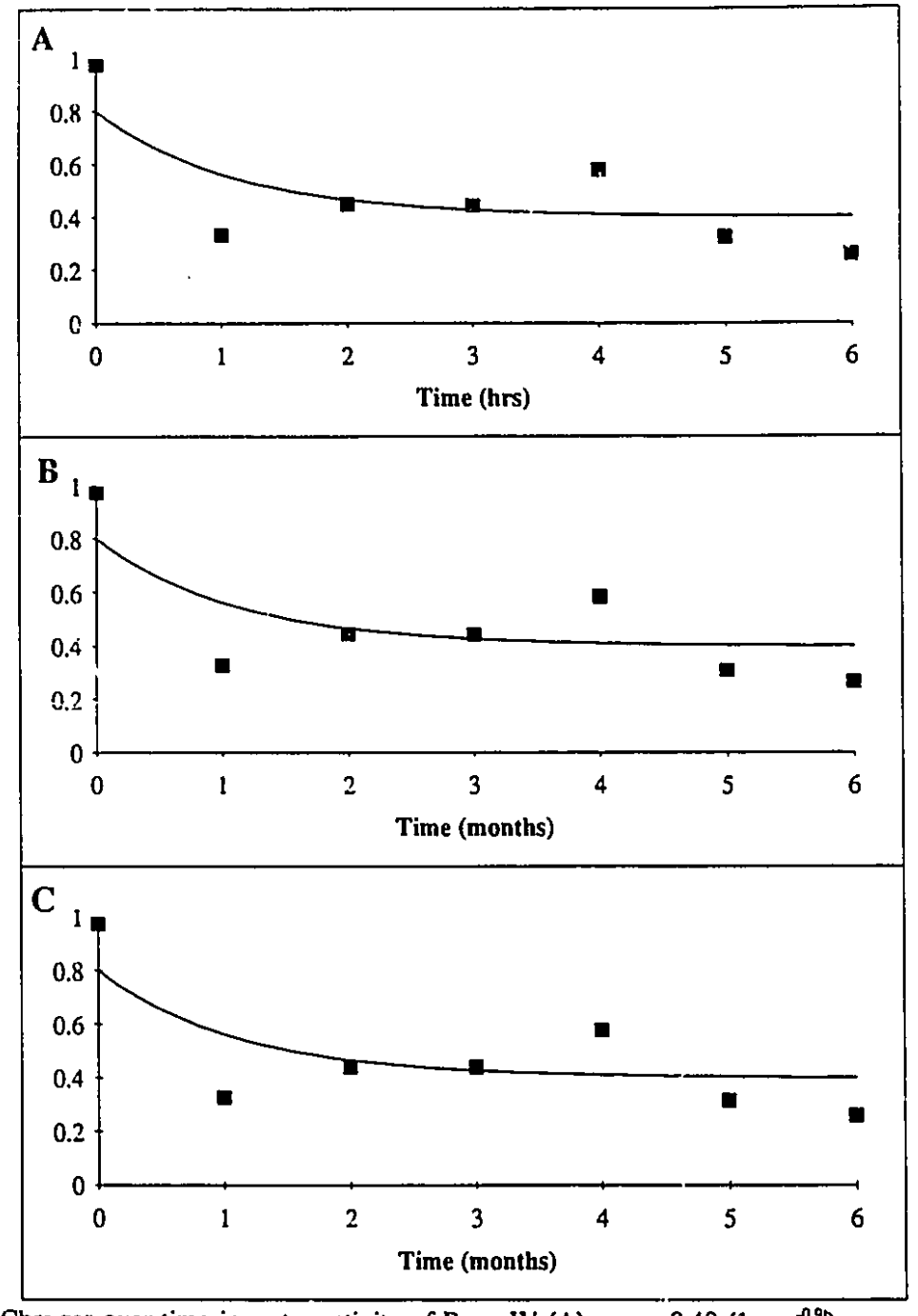


Figure 4. Changes over time in water activity of Prep. IV (A) : $a_w = 0.40\ (1 + e^{-0.9t})$, Prep. V (B) : $a_w = 0.40\ (1 + e^{-0.9t})$, and Prep. VI (C) : $a_w = 0.40\ (1 + e^{-0.9t})$, when stored at room temperature in bags permeable to oxygen; where Prep. IV=1 ml *Colletotrichum coccodes* (Cc):2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP.

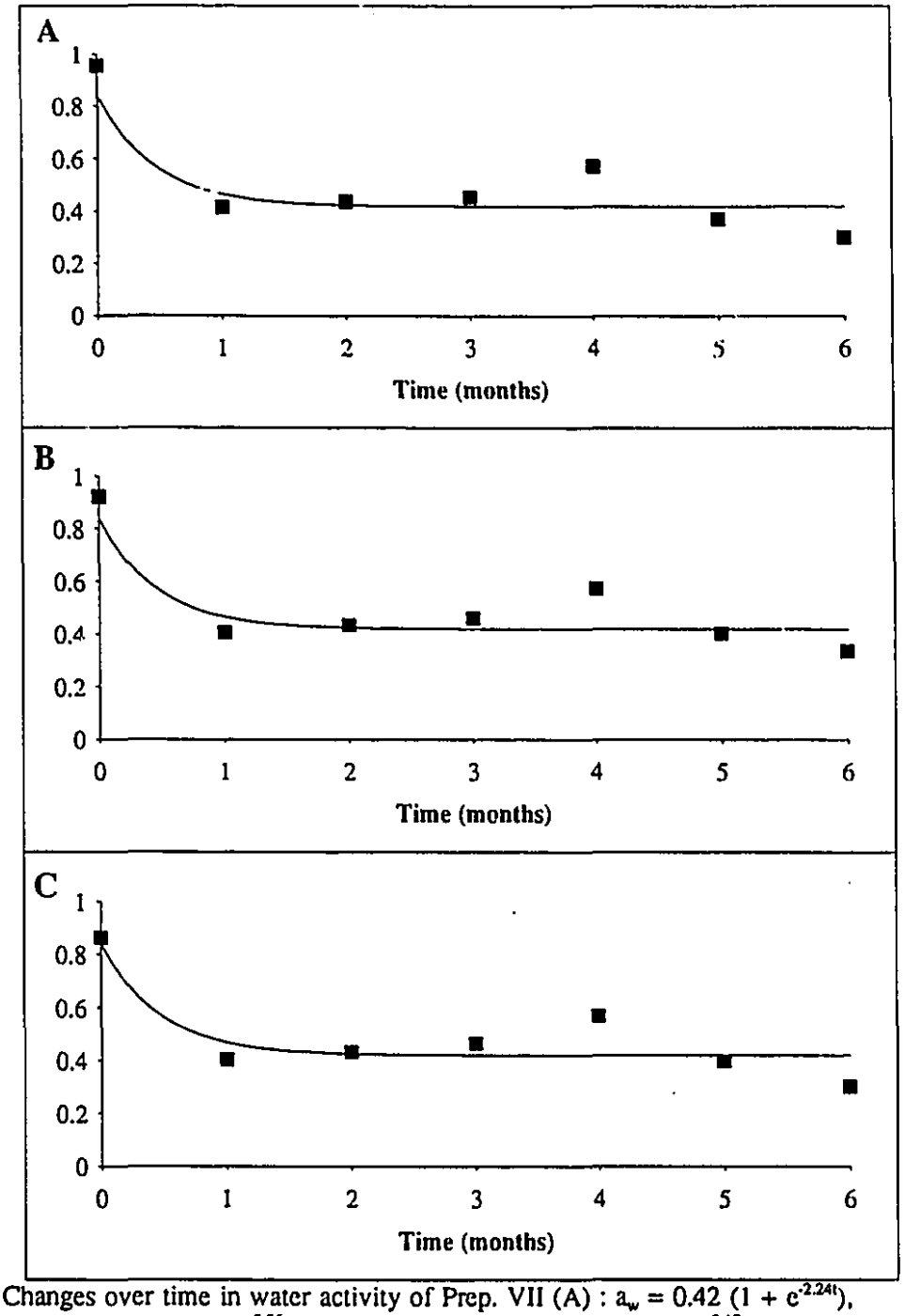


Figure 5. Changes over time in water activity of Prep. VII (A) : $a_w = 0.42\ (1 + e^{-2.24t})$, Prep. VIII (B) : $a_w = 0.42\ (1 + e^{-2.35t})$, and Prep. IX (C) : $a_w = 0.42\ (1 + e^{-3.17t})$, when stored at room temperature in bags permeable to oxygen; where Prep. VII=1 ml *Colletotrichum coccodes* (Cc):2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

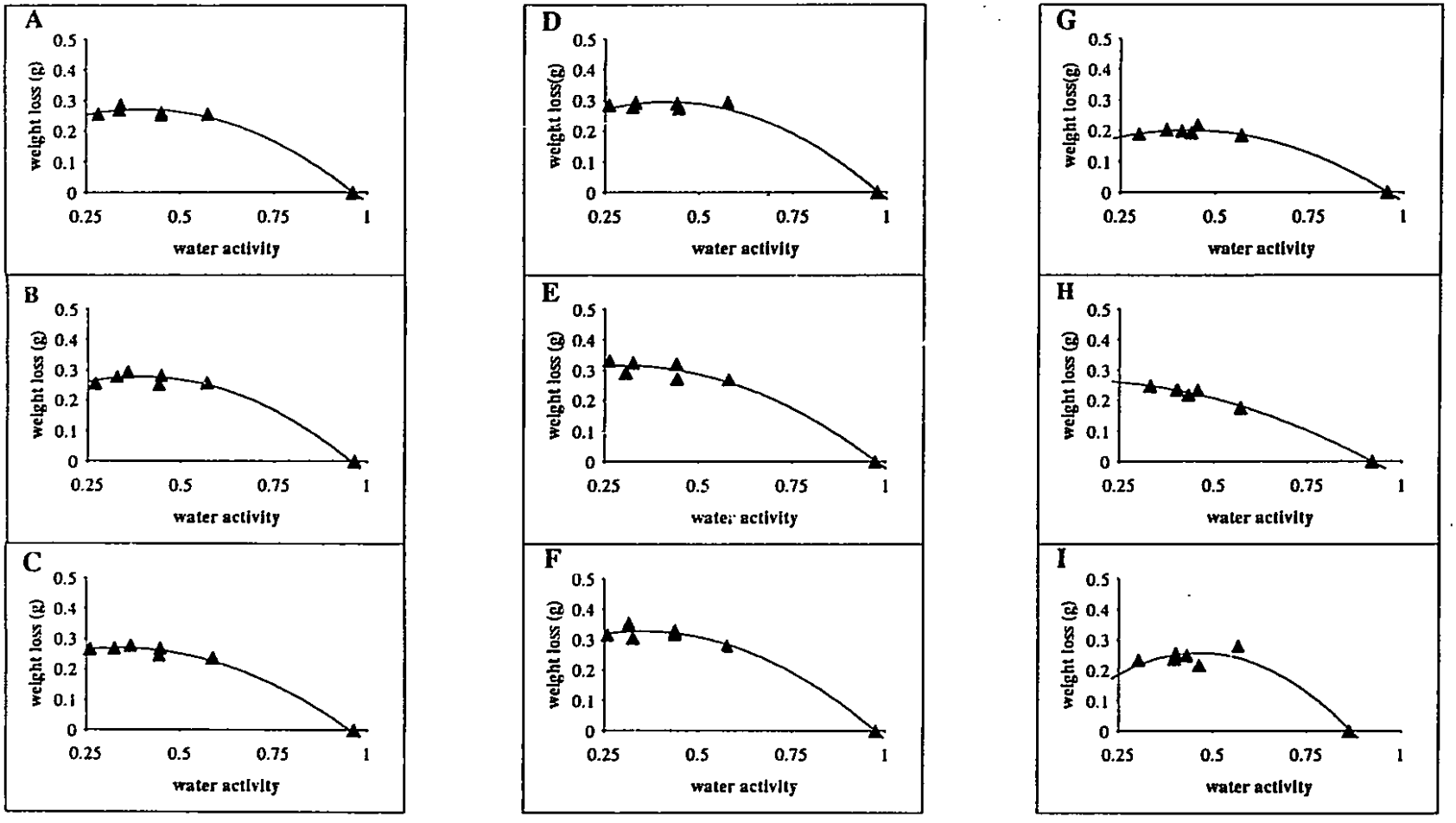


Figure 6. Changes in weight loss (wtls) of preparations in relation to water activity (a_w). (A): Prep. I, wtls=0.135 + 0.683 a_w - 0.856a_w^2, R^2 = 0.987; (B): Prep. II, wtls=0.136 + 0.704 a_w - 0.874 a_w^2, R^2 = 0.983; (C): Prep. III, wtls=0.183 + 0.499 a_w - 0.715 a_w^2, R^2 = 0.991 ; (D): Prep. IV, wtls=0.135 + 0.770 a_w - 0.929 a_w^2, R^2 = 0.981; (E): Prep. V, wtls=0.259 + 0.393 a_w - 0.676 a_w^2, R^2 = 0.971; (F): Prep. VI, wtls=0.222 + 0.599 a_w - 0.851 a_w^2, R^2 = 0.985; (G): Prep. VII, wtls=0.068 + 0.621 a_w - 0.724 a_w^2, R^2 = 0.988; (H): Prep. VIII, wtls=0.261 + 0.095 a_w - 0.411 a_w^2, R^2 = 0.992; (I): Prep. IX, wtls=-0.079 + 1.449 a_w-1.565 a_w^2, R^2 = 0.930; Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC), Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC, Prep. IV=1 ml Cc:2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP, Prep. VII=1 ml Cc:2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

(Prep. IV, 1:2.79) and one with soluble starch (Prep. VIII, 1:3.78) (Table 7). However, these were only significantly different from a preparation with kaolin clay (Prep. III, 1:3.07) (Table 7). Preparations with the highest amounts of soluble starch had, from the beginning, a low water activity (0.922 and 0.863), which might explain their relative poor performance over time. In addition, it is known that although two different fillers might result in a similar a_w, they might very well have different effects on fungal spores or conidia (Pitt and Hocking,1977; Troller, 1987). Soluble starch appears to have a negative effect on *C. coccodes* conidia over time. Starch may have stimulated the germination of conidia prior to water evaporation, which resulted in a poor survival rate over the subsequent storage period.

Within the storage bags oxygen and nitrogen concentrations were in similar proportions to air. Although conidia were respiring, carbon dioxide was not produced in detectable proportions (carbon dioxide in the air was not detected either). It would appear that conidia were in a dormant state since respiration was occurring at a non-detectable low rate. Exogenous dormancy has then been successfully forced onto conidia of *C. coccodes*. They had a low metabolic activity in storage, while being able to function in a normal way when brought into favourable conditions.

Table 7. Disease ratings on velvetleaf sprayed with preparations of *Colletotrichum coccodes* conidia stored in bags permeable to oxygen over a six month period.

Preparations[1]	Disease ratings[2]					
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
I	8.6	10.0	9.3	8.3	8.0	8.0 ab[3]
II	10.0	10.0	9.3	9.3	9.6	8.6 ab
III	10.0	9.6	10.0	9.6	9.3	10.0 a
IV	9.6	10.0	6.0	2.6	5.3	3.6 b
V	9.3	10.0	8.0	9.3	8.0	7.3 ab
VI	9.3	9.3	8.3	8.3	8.6	8.3 ab
VII	9.6	10.0	3.6	9.6	9.0	8.0 ab
VIII	8.3	10.0	4.6	8.3	8.6	7.0 ab
IX	7.3	10.0	0.6	6.0	6.3	5.0 b

[1] Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC), Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC, Prep. IV=1 ml Cc:2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP, Prep. VII=1 ml Cc:2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

[2] Disease ratings based on a visual scale where 0 = no lesions and 10>90% of the sprayed area.

[3] Means followed by the same letter(s) are not significantly different according to Krusgal-Wallis multiple comparison test.ea is covered with lesions, and values are means of three replicates.

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CONNECTING TEXT

An effective formulation, one that preserves viability and infectivity of conidia, is one of the final aspects to be evaluated in assessing the potential of an organism to be used as a bioherbicide, and is the key factor for the successful marketing of a microbial pesticide (Devisetty, 1989).

More research was required to enhance infectivity of formulated *C. coccodes* conidia. The presence of free water is the major limiting factor of most bioherbicides under natural conditions. Accelerating germination and penetration of velvetleaf by *C. coccodes* conidia could help overcome this constraint. Several experiments were designed to evaluate the impact of germination stimulants, cutinase, pectinase and / or cellulase induced on disease incidence and severity.

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III. Effect of germination / penetration stimulants on germination and appressoriation of formulated *Colletotrichum coccodes* conidia

INTRODUCTION

Velvetleaf (*Abutilon theophrasti* Medic.) is an extremely difficult weed to control because it reproduces by producing thousands of seeds of variable dormancy periods (Warwick and Black, 1988). Because velvetleaf germinates throughout the growing season and causes severe crop losses especially in corn (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), soybean (*Glycine max* (L.) Merr.), and cotton (*Gossypium hirsutum* L.), diverse strategies are needed to ensure effective control of this troublesome weed (Spencer, 1984; Warwick and Black, 1988). One new strategy is to apply phytopathogenic microorganisms to target weeds as bioherbicides (mycoherbicides) in a manner similar to chemical herbicides. A fungal pathogen, *Colletotrichum coccodes* (Wallr.) Hughes was found to be effective in controlling velvetleaf (Gotlieb *et al.*, 1987; Poirier, 1984). The optimum conditions for infection and disease development have been established under controlled environment by Wymore *et al.*, 1988. A minimum of 18 hr of free water on the velvetleaf foliage at 24C was necessary for extensive disease development by *C. coccodes* on plants inoculated with $1*10^9$ conidia/m^2 (Wymore *et al.*, 1988; Wymore and Watson, 1986).

Disease constraints and bioherbicide limitations.

Plant disease is a tripartite interaction involving the host, the pathogen, and the

environment (Van der Plank, 1968). Any constraint on these components would limit disease to endemic levels (Holcomb, 1982; Cullen and Hasan, 1988). These constraints include plant related factors such as low plant densities, changes in susceptibility with age or resistance to the pathogen; pathogen related factors such as poor overwintering and dissemination capacities or low virulence; and environment related factors such as unfavourable moisture and temperature conditions. The use of a bioherbicide overcomes many of these restraints. In effect, "an abundant supply of inoculum of a virulent pathogen is dispersed over a population of susceptible weeds and the application is timed and/or the bioherbicide formulated to avoid unfavourable environmental conditions" (Watson and Wymore, 1990). Often, however, the exact moisture and temperature conditions required by most fungal spores for germination and host penetration are not consistently present naturally under field conditions, often resulting in an ineffective bioherbicide application. When sprayed in the field, *C. coccodes* results in inconsistent levels of velvetleaf mortality due to its specific environmental requirements (Armour, I. , personal communication). Environmental conditions represent a problem since nearly all foliar pathogens require a film of free moisture on the plant surface at early stages of infection (Colhoun, 1973). Improved formulation and application would help decrease bioherbicide failure in the field (Watsum, 1988).

Various surfactants, adjuvants or chelating agents increase germination and/or appressoria formation of fungi, probably due to the increased amounts of available water when these compounds are present. Tween 20, anthralinic acid/chelating agents were found to be germination stimulants of *C. trifolii* Bain & Essary and *C. musae* (Berk. & Curt.) Von Arx respectively (Miehle and Lukezic, 1973; Graham and Harper, 1983; Harper and

Swinburne, 1980; Harper *et al.*, 1981). Similarly, various nutrients, for example L-rhamnose and sucrose increase germination and appressorium formation in many *Colletotrichum* spp. and other fungal plant pathogens (Gupta, 1979; Solanki *et al.*, 1974).

To be able to infect, the germinated spore must penetrate the host cuticle (composed of mostly cutin), the pectin layer, and the cellulose wall of the epidermal cells. The penetration of many plant pathogens is enzymatic, through the secretion of cutinases, pectinases and cellulases (Kolattukudy *et al.*, 1985; Köller, 1991;English and Albersheim, 1969; English *et al.*, 1971; Yazdi *et al.*, 1990); and potassium phosphate and calcium chloride have been reported to enhance polygalacturonase activity (Boothby, 1981; Starr and Moran, 1962).

Formulation, the blending of an active moiety with diluents and surface active agents, facilitates handling and carrying the pathogen to its host (Smith and Berry, 1975). It also helps to overcome unfavourable environmental requirements for spore germination and infection. After developing a system to store viable *C. coccodes* conidia with an extended shelf-life (refer to previous chapter), work was conducted to determine if germination stimulants, cutin concentrations, or fatty acids could increase germination and/or penetration and subsequent infection of velvetleaf. The acceleration or augmentation of conidia germination or penetration should help to decrease the dew period requirement and would broaden the environmental range in which *C. coccodes* could act as an effective bioherbicide.

The objective of this study was to determine the effect of twenty germination stimulants, eight concentrations of cutin, three "pectinase inducers" and five fatty acids / surfactants on germination, appressoriation and infectivity of *C. coccodes* conidia.

MATERIALS AND METHODS

Inoculum production and storage

Small pieces of agar with mycelium from the stock culture (single conidium isolation of *C. coccodes* grown on potato dextrose agar (PDA) (dehydrated, Difco Laboratories, Detroit, MI) slants in small vials and maintained under mineral oil at 4C were transferred to fresh PDA in 9 cm petri dishes. Plates were sealed with Parafilm® (American Can Co., Greenwish, CT) and incubated at 24C without light for 7 to 21 days. Using a #2 cork borer, agar plugs were removed from the mycelial periphery of the colony and transferred to 250 ml flasks containing 100 ml of V-8 liquid medium (10 g sucrose, 10 g potassium nitrate, 2.5 g magnesium sulfate, 5 g potassium dihydrogen ortho phosphate, 0.02 g ferric chloride and 150 ml V-8 juice per litre), which had been autoclaved for 17 minutes at 100 kPa and 120C. Inoculated flasks were placed on a rotary shaker at 250 rpm. After one week, conidia were harvested by pouring the contents of the flasks through four layers of cheese cloth in a funnel. The filtered conidia suspension was centrifuged at 250 rpm for 8 minutes, the pellets washed and centrifuged a second time under the same conditions and then the pellets were resuspended in distilled water. Inoculum density was determined with the aid of a haemacytometer, and the desired quantity mixed with kaolin clay (hydrated aluminium silicate, Sigma® Chemical Co., St. Louis, MO), talcum powder (Fisher Scientific Ltd., Montreal, Qué.) or soluble starch (Mallinkrodt Canada Inc., Pointe Claire, Qué.) in ratios of

1:2.79, 1:2.79 and 1:2.52 (wt/wt) respectively. Dry preparations were stored at room temperature. When used in later experiments, an amount containing 10^9 conidia was first resuspended in 10 ml of water and shaken on a rotary shaker for one hour to ensure rehydration of conidia.

Effect of germination stimulants on germination and appressoria formation of *C. coccodes* on dialysis membranes

Fifteen cm pieces of dialysis membranes (Dialysis tubing, Baxter Diagnotics Corporation, Canlab Division, Pointe Claire, Qué.) were soaked in distilled water for 2 minutes, cut into 1*1 cm^2 squares and placed in 100 ml distilled water in 250 ml flasks (100-125 membranes/flask). The flasks containing the dialysis membranes were autoclaved for 30 minutes (100 kPa and 120C). The water was then decanted and the flasks refilled with distilled water, hand shaken, and the water was decanted again, the flasks were refilled with 100 ml distilled water and autoclaved again for 20 minutes. Membranes were then placed on moist filter paper (Whatman® #1, Whatman International Ltd, Maidstone, England) in glass petri plates (25 membranes/plate) and the plates were autoclaved for 18 minutes. Open petri dishes were placed in a spray chamber (Research Instrument Manufacturing Co., Ltd., Guelph, Ont.) and sprayed with resuspended spore formulations at a rate of $1*10^8$ conidia/m^2, using a full cone nozzle (TG 0.7), 200 kPa air pressure, a speed of 0.85 kpH and a spray volume of 500 L/ha). Sprayed membranes were transferred (12 membranes / plate) and incubated at room temperature under light conditions on the following media : Water Agar (WA) as a control, WA to which $2*10^{-2}$ M, 10^{-2} M, or 10^{-3} M glycine (amino acetic acid, Sigma®

Chemical Co., St. Louis, MO), $2*10^{-2}$ M, 10^{-2} M, or 10^{-3} M asparagine (Fisher Scientific Ltd., Montreal, Qué.), $5*10^{-4}$ M or $5*10^{-5}$ M ethylene diamine tetraacetic acid (EDTA) (ferric sodium salt, Sigma® Chemical Co., St. Louis, MO), 10^{-2} M, 10^{-3} M or 10^{-4} M anthralinic acid (o-amino benzoic acid, Sigma® Chemical Co., St. Louis, MO), 0.2% Triton B 1956 (modified phthalic glycerol alkyd resin, 77% a.i. in ethylene dichloride, Sigma® Chemical Co., St. Louis, MO), 10%, 1%, or 0.1% sucrose (Mallinkrodt Canada Inc., Pointe Claire, Qué.), $2*10^{-2}$ M, 10^{-2} M, or 10^{-3} M α-L-rhamnose (6 deoxy-L-mannose, Sigma® Chemical Co., St. Louis, MO) , 1% or 0.1% Sorbo (64% sorbitol, Atkemix Inc., Brantford, Ont.) were added. Every hour for 10 hours, one membrane per medium was selected randomly and placed on a glass slide in a drop of lactophenol cotton blue, covered with a cover slip, and conidia germination/appressoriation assessed by counting 100 conidia and recording the ones that have germinated and appressoriated.

In further experiments the best three germination stimulants were added to formulated *C. coccodes* conidia, resuspended in 10 ml of distilled water and sprayed, alone or in combination with cutin and/or pectinase inducers, on 14 day-old velvetleaf plants at a rate of 10^9 conidia/m^2. Velvetleaf seed dormancy was broken by placing seeds on moist filter paper (Whatman®, #1) in glass petri dishes placed at 5C for 24 hours then incubated at 30C for 24 hrs. Four germinated seeds were sown in potting medium (Pro-Mix Bx®, Premier Brand, Inc., New York, NY) in 10 cm plastic pots. Seedlings were grown in controlled environment chambers (Conviron®, Model E-15, Controlled Environments, Winnipeg, Man.) (24/18 C day/night temperature, 14-hr photoperiod, 400 $\mu Em^{-2}\ s^{-1}$) and thinned to three seedlings per pot before use.

Effect of cutin on germination and appressoriation of formulated *C. coccodes* spores on dialysis membranes

Cutin was obtained from apple peels by modifying Kolattukudy's method (Walton and Kolattukudy, 1972). After the overnight mixing of peels with chloroform:methanol (2:1) (Mallinkrodt Canada Inc., Pointe Claire, Qué.; BDH Inc., Toronto, Ont.), peels were air dried, frozen at -85C (Bio-Freezer®, Forma Scientific, Marietta, OH), then lyophilized (Freeze-dry-5, Labconco®, Labconco Corporation, Kansas City, MO) for 48 hours and ground first with a blender (Eberback 8580, Canadawide Scientific, Ottawa, Ont.) and then further ground with a mortar and pestle. Cutin thus prepared was stored at -85C and used in subsequent experiments and in media preparation. Dialysis membranes were prepared and inoculated as described above, and transferred onto petri plates containing PDA, PDA + 0.25 g/L cutin, PDA + 2.5 g/L cutin, PDA + 5.0 g/L cutin, PDA + 10.0 g/L cutin, PDA + 20.0 g/L cutin, PDA + 30.0 g/L cutin or PDA + 40.0 g/L cutin.

Effect of the "best" germination stimulants/"best" cutin concentration/three reported pectinase inducers alone or in combination on disease incidence and development

Velvetleaf plants were prepared for bioassays as described above. Fourteen day-old plants were inoculated with formulated conidia resuspended in 10 ml of water to which 1% sucrose, 10^{-2} M rhamnose, 10^{-3} M asparagine, 0.1% Sorbo, 0.25g/L cutin, 0.01g polygalacturonic acid (PGA) (Sigma Chemical Co., St Louis, MO), 0.1M potassium phosphate (Mallinckrodt Canada Inc., Pointe Claire, Québec), or 0.001M calcium chloride (Baker Chemical Co., Phillipsburg, NJ) were added, alone or in combination. Due to the space

constraints in a growth cabinet, only two (rhamnose and Sorbo) of the four germination stimulants were used in combination with cutin and the three reported "pectinase inducers". The suspensions were allowed to shake on a rotary shaker for 1 hour before spraying at a rate of 10^9 conidia/m^2. Sprayed velvetleaf plants were then incubated in a dark dew chamber at 24C (Percival®, Model E-54UDL, Boone, IA) for 18 hours and subsequently transferred to a growth cabinet at the original conditions. Disease incidence was evaluated daily and disease severity after seven and ten days following inoculation using a rating system based on a scale from 0 to 10 where 0 = no visible symptoms and 10 = > 90 % of the inoculated area was necrotic. Disease ratings were evaluated for each pot and results were pooled and averaged for the three replicates (pots).

Effect of fatty acids / surfactants on disease incidence and development

Velvetleaf plants were prepared for bioassays as described above. Fourteen day-old plants were inoculated with formulated conidia resuspended in 10 ml of distilled water to which 0.02% of Brij 35 (polyoxyethylene 23 lauryl ether, Sigma® Chemical Co., St. Louis, MO), linoleic acid (cis-9, cis-12-octadecadienoic acid, Sigma® Chemical Co., St. Louis, MO), oleic acid (cis-9-octadecenoic acid, Sigma® Chemical Co., St. Louis, MO), stearic acid (octadecanoic acid, Sigma® Chemical Co., St. Louis, MO), or Tween 80 (polyoxyehylene sorbitan mono-oleate, Sigma® Chemical Co., St. Louis, MO) were added. The suspensions were allowed to shake on a rotary shaker for 1 hour before spraying at a rate of 10^9 conidia/m^2. As above, sprayed velvetleaf plants were incubated in a dark dew chamber at 24C for 18 hours and subsequently transferred to a growth cabinet at the original conditions.

Disease incidence and severity were evaluated as above.

Data analysis

A completely randomized design with three replicates/treatment was used in all experiments. All experiments were performed three times. Data were analyzed as a complete randomized block design with time being the blocks. Differences between treatments were established with Duncan's multiple range test (α =0.05) (Steel and Torrie, 1980). Regression analysis was used on selected treatments to illustrate effect over time. Disease ratings were compared by the Friedmann 2-way analysis of variance by ranks followed by a multiple comparison procedure to evaluate differences between treatment means (Daniel, 1990).

RESULTS AND DISCUSSION

Effect of germination stimulants on spore germination and appressoria formation

Formulated spores performed differently depending on the filler, germination stimulant, or both. When incubated on WA, germination and appressoriation proceeded at rates comparable to those of fresh conidia, and conidia in kaolin clay or talcum powder had higher germination rates than fresh conidia (Fig. 1A). Conidia formulated in kaolin clay germinated earlier than fresh or other formulated conidia, indicating that the 1-hour rehydration period provided favourable conditions for germination to commence (e. g. swelling), even before incubation on the different media. Although all conidia showed a delay in appressoria

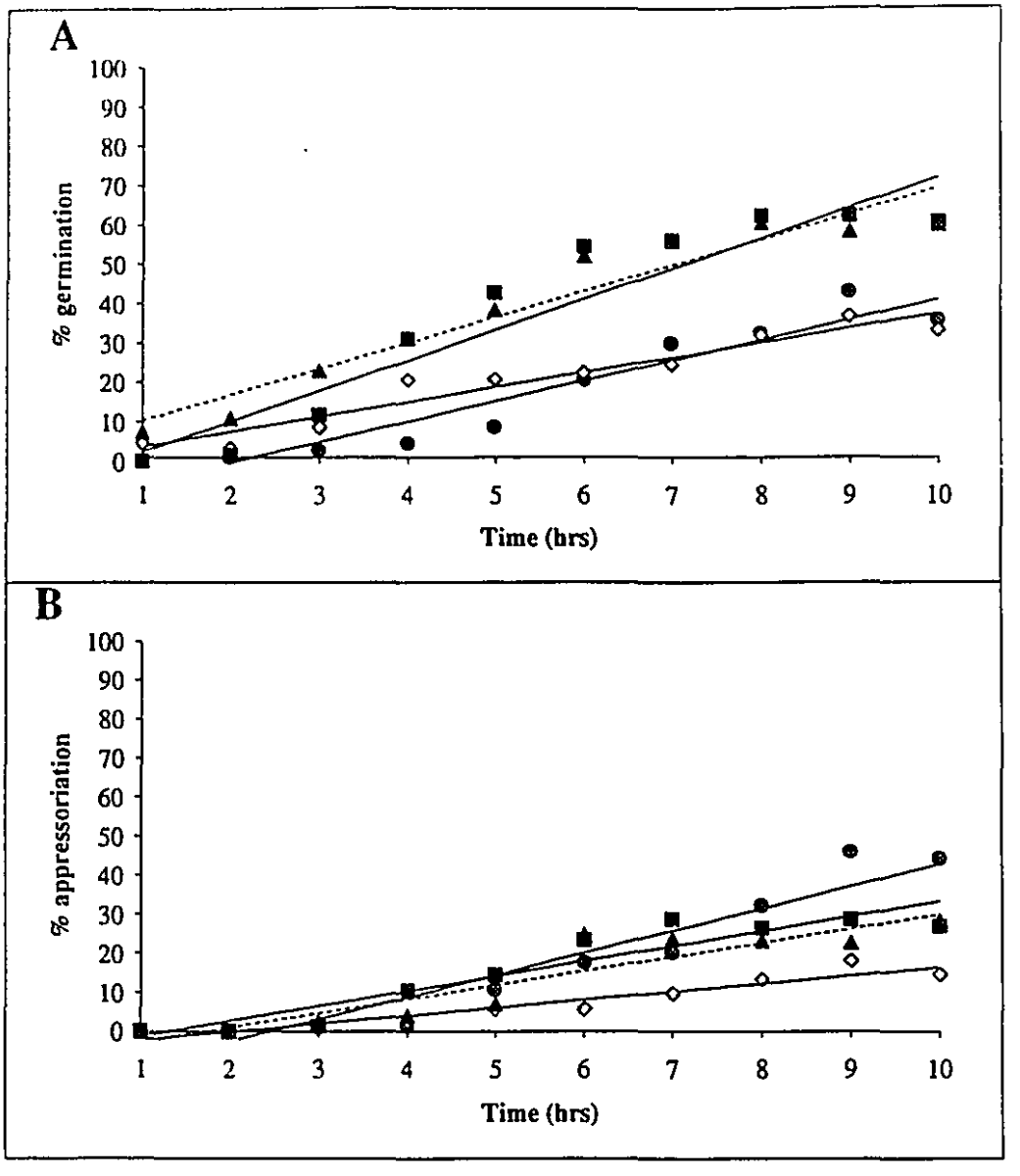


Figure 1. Germination (A) and appressoria formation (B) of fresh and formulated conidia of *Colletotrichum coccodes* over a 10hr incubation on water agar;

where (A) : Talcum powder, y= -5.35 + 7.92x (R^2=0.89)
Kaolin clay, y= 3.78 + 6.54x (R^2=0.92)
Soluble starch, y= 0.07 + 3.73x (R^2=0.92)
Fresh conidia, y= -10.53 + 5.14x (R^2=0.91)

and (B) : Talcum powder, y= -5.06 + 3.84x (R^2=0.89)
Kaolin clay, y= -6.27 + 3.62x (R^2=0.85)
Soluble starch, y= -4.26 + 2.06x (R^2=0.91)
Fresh conidia, y= -13.99 + 5.68x (R^2=0.91)

formation, fresh conidia had higher appressoriation rates (Fig. 1B). All germinated fresh conidia formed appressoria, whereas only a fraction of germinated formulated conidia did. Formulated conidia may have exhausted some of the essential components (through respiration in storage), and therefore were not able to appressoriate. Conidia with the shortest lag period prior to appressoria formation were those formulated in talcum powder. Rapid appressoria formation is an advantage as it implies that conidia would penetrate the host plant faster and therefore would be less exposed to unfavourable environmental conditions. When germination stimulants were added to WA, fresh conidia responded similarly to that reported in the literature (Graham and Harper, 1983; Gupta, 1979; Harper *et al.*, 1980; Miehle and Lukezic, Muruganandam *et al.*,1988). Most additives increased germination and appressoriation with the only one, 0.2% Triton, that decreased germination significantly. High concentrations of anthralinic acid in the medium also resulted in lower germination, but germination was not significantly different from the control (Fig. 2A). Formulated conidia followed the same pattern. The additives stimulatory to fresh conidia did not have a significant effect on formulated conidia, and the similar ones were inhibitory, with the addition of $5*10^{-5}$M EDTA in the case of conidia in kaolin clay (Fig. 2B, C, D). When analyzed after 3, 6 and 10 hours, germination of conidia formulated in kaolin clay or talcum powder and incubated on WA to which 10^{-2}M asparagine, 10^{-3}M rhamnose, or 0.1% Sorbo were added, was not significantly different from germination of fresh conidia. Germination of conidia formulated in soluble starch was significantly different from germination of fresh conidia after 10 hours of incubation on WA to which 10^{-2}M asparagine or 0.1% Sorbo were added. To achieve 50% germination on these media, fresh conidia required 5 to 6 hours,

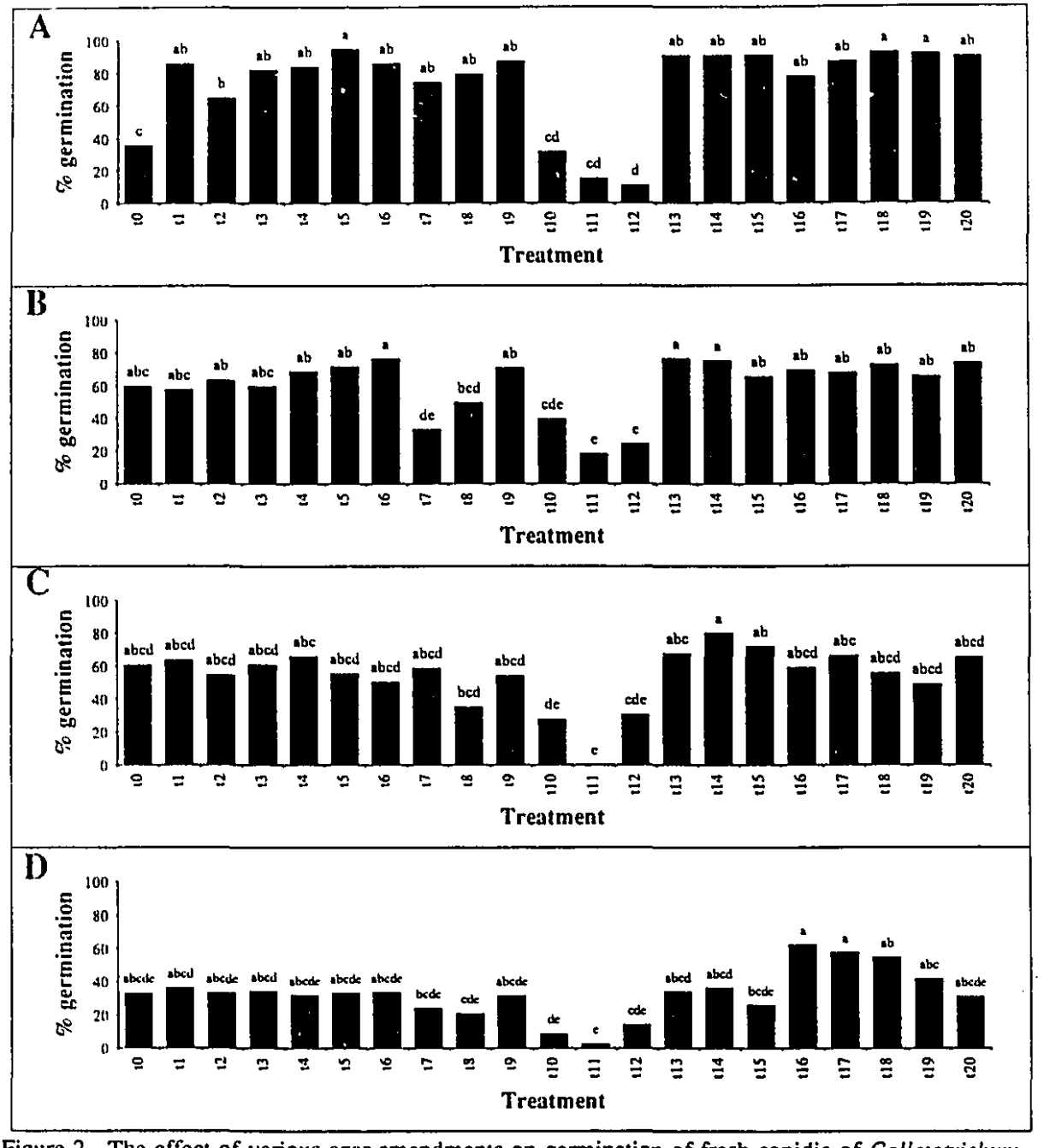


Figure 2. The effect of various agar amendments on germination of fresh conidia of *Colletotrichum coccodes* (A) compared to conidia formulated in kaolin clay (B), talcum powder (C), or soluble starch (D), after 10 hours of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.

Bars with the same letter(s) are not significantly different according to Duncan Multiple Range test.

while conidia in soluble starch required 9-10 hours on WA to which 10^{-3}M rhamnose or 0.1% Sorbo were added, and would have reached 50% germination only after 15 hours on WA to which 10^{-2}M asparagine was added. Conidia formulated in kaolin clay required 6 to 7 hours and conidia in talcum powder 7 to 8 hours to reach the 50% germination level on the same media. The addition of 10^{-2}M asparagine, 10^{-3}M rhamnose, or 0.1% Sorbo to WA did not increase significantly germination of formulated conidia, and the rates at which these conidia germinated on these media were lower than the germination rates of fresh conidia, with conidia in kaolin clay, in all cases, showing rates closest to the fresh ones (Fig. 3A, B, C). This suggests that when conidia are dried in kaolin clay, they do not require a shock to regain their physiological activity. Conidia in talcum powder or soluble starch may have produced an endogenous inhibitor in storage which resulted in decreased germination rates, or they may simply have depleted their food reserves by keeping a relatively high metabolic activity in storage resulting in the death of some, reflecting the decreased germination rates.

Appressoriation of fresh conidia was increased significantly by the addition of 10^{-2}M asparagine to WA. The addition of 10^{-2}M and 10^{-3}M rhamnose - as well as most of the compounds- also increased appressoriation, although not significantly (Fig. 4A). The compounds that decreased germination (high concentrations of anthralinic acid, 0.2% Triton) also resulted in decreased appressoriation, as did $5*10^{-4}$ and $5*10^{-5}$M EDTA, and 10% sucrose. The addition of EDTA to the medium lowers the pH which may not be enough to affect germination (like anthralinic acid) but was sufficient to decrease appressoria formation. The response to 10% sucrose is expected since it was demonstrated by Blakeman and Parbery (1977) that appressoriation of *C. acutatum* Simmonds took place only after excess nutrients

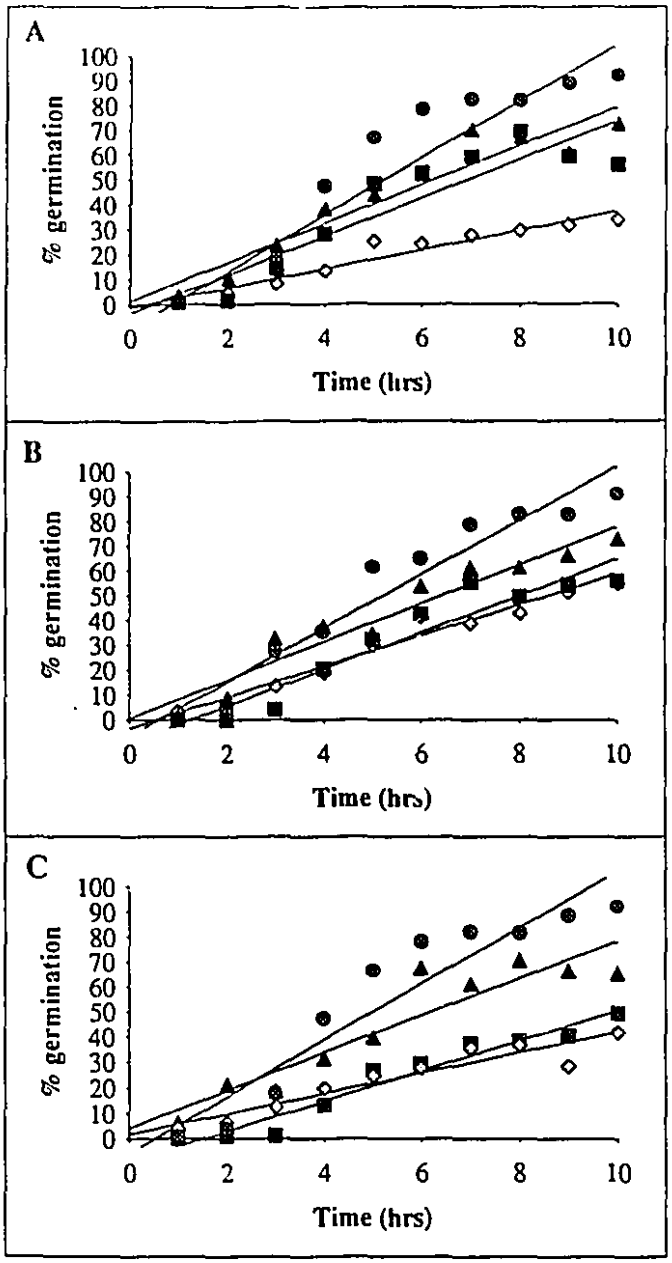


Figure 3. Germination of fresh conidia compared to germination of formulated conidia of *Colletotrichum coccodes* over a 10-hr incubation on WA+10^{-2}M asparagine (A), WA+10^{-3}M rhamnose (B), and WA+0.1% Sorbo (C), where

- Talcum powder, (A) y= -3.71 + 7.67x (R^2=0.83); (B) y= -9.44 + 7.4x (R^2=0.91); (C) y= -9.13 + 5.94x (R^2=0.95)
- Kaolin clay, (A) y= 1.13 + 7.76x (R^2=0.91); (B) y= 0.4 + 7.72x (R^2=0.93); (C) y= 3.98 + 7.43x (R^2=0.86)
- Soluble starch, (A) y= -1.26 + 3.8x (R^2=0.93); (B) y= -3.42 + 6.2x (R^2=0.97); (C) y= 1.62 + 4.01x (R^2=0.89)
- Fresh conidia, (A) y= -10.04 + 11.43x (R^2=0.95); (B) y= -6.56 + 10.83x (R^2=0.95); (C) y= -6.09 + 11.22x (R^2=0.89)

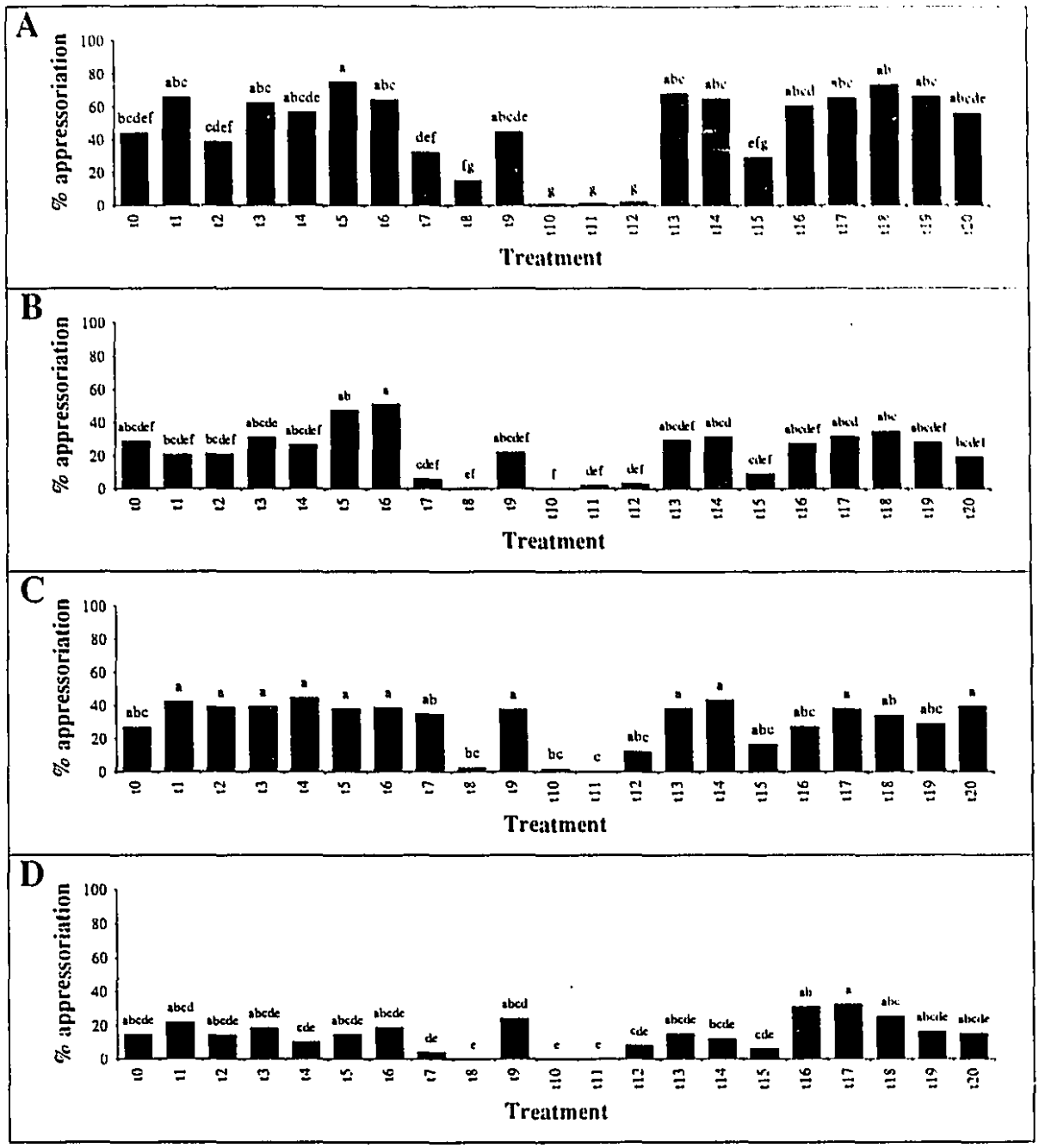


Figure 4. The effect of various agar amendments on appressoriation of fresh conidia of *Colletotrichum coccodes* (A) compared to conidia formulated in kaolin clay (B), talcum powder (C), or soluble starch (D), after 10 hours of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.

Bars with the same letter(s) are not significantly different according to Duncan Multiple Range test.

were used. Appressoria formation of formulated conidia was decreased by the same compounds and, for conidia in kaolin, was increased, although not significantly, by the addition of 10^{-2} and 10^{-3} M asparagine to the medium (Fig. 4B). In the case of conidia formulated in talc, most compounds increased appressoriation, although not significantly (Fig. 4C). Enhancement of appressoriation in the worst formulation, conidia in soluble starch was not observed (Fig. 4D). When compared to fresh conidia, appressoriation rates of formulated conidia were lower, with conidia in talc being closest to fresh ones (Fig. 5A, B, C). After 6 hours of incubation on WA to which 10^{-2}M asparagine, 10^{-2} or 10^{-3}M rhamnose were added, only Prep. VII had significantly less appressoria than other formulated or fresh conidia. Only Prep. I incubated on WA to which 10^{-2}M asparagine was added, and Prep.IV on WA to which 10^{-2}M asparagine or 10^{-2}M rhamnose produced a number of appressoria that was not significantly different from the controls. Although germination data indicated that the best formulation was conidia in kaolin clay, appressoriation data show a decrease in the performance of this formulation. Many reports state that the minimum a_w at which molds can grow is often affected by the solute which has been used to lower the a_w (Pitt and Hocking, 1977; Troller, 1987). Cells usually react to a lowered a_w by accumulating compatible solutes. The addition of talcum powder to *C. coccodes* conidia may have resulted in an accumulation of solutes that facilitated appressoria formation when the environmental conditions were favourable, which was not the case of conidia in kaolin clay. When 10^{-2}M asparagine was added to WA, no effect on lag period before appressoriation was observed, which was not the case when 10^{-2} or 10^{-3}M rhamnose were added. Even appressoria formation of fresh conidia was delayed in the latter cases, which might be due to the nutrient availability for conidia to

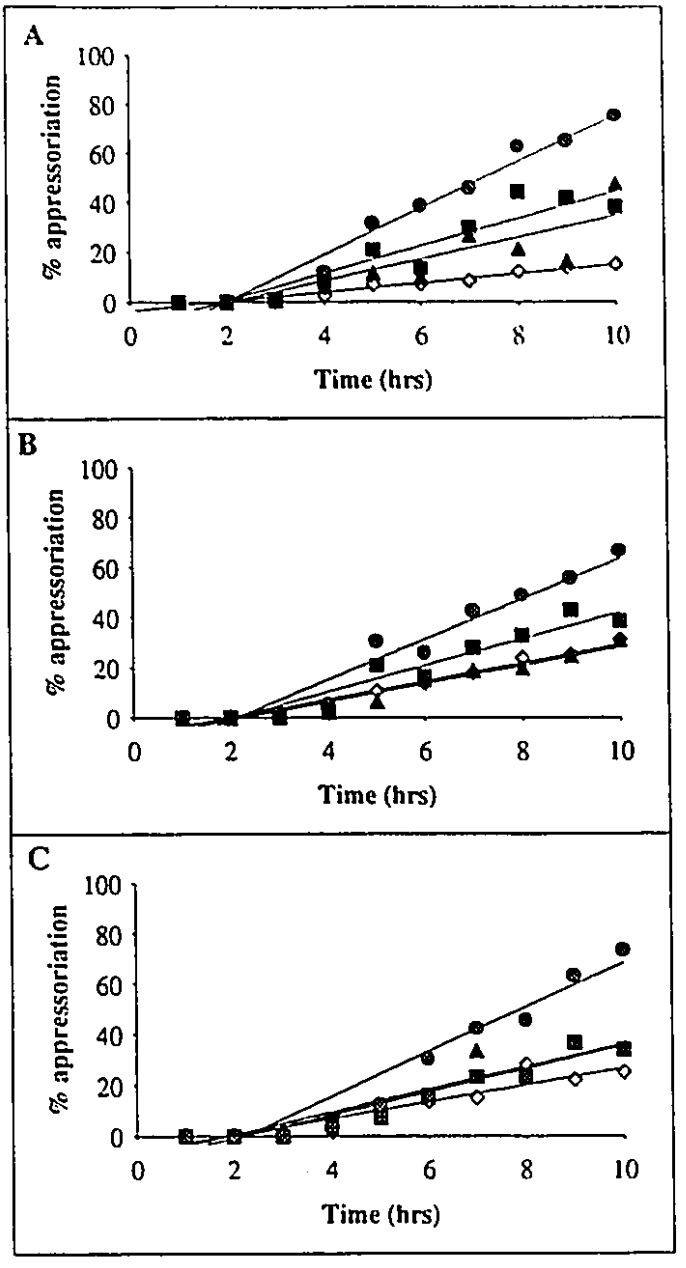


Figure 5. Appressoria formation of fresh and formulated conidia of *Colletotrichum coccodes* over a 10-hr incubation on water agar (WA)+10^{-2}M asparagine (A), WA+10^{-2}M rhamnose (B), and WA+10^{-3}M rhamnose (C), where

- ▣ Talcum powder, (A) y= -10.55 + 5.48x (R^2=0.89); (B) y= -11 + 5.29x (R^2=0.93); (C) y= -10.11 + 4.57x (R^2=0.92)
- △ Kaolin clay, (A) y= -8.98 + 4.34x (R^2=0.77); (B) y= -8.27 + 3.62x (R^2=0.94); (C) y= -8.47 + 4.47x (R^2=0.93)
- ◇ Soluble starch, (A) y= -3.56 + 1.84x (R^2=0.96); (B) y= -7.38 + 3.64x (R^2=0.96); (C) y= -6.38 + 3.31x (R^2=0.95)
- ○ Fresh conidia, (A) y= -18.67 + 9.37x (R^2=0.95); (B) y= -17.07 + 8.04x (R^2=0.94); (C) y= -19.6 + 8.8x (R^2=0.96)

grow, instead of seeking nutrients in a host and thus forming structures for penetration.

Fresh and formulated conidia, to a lesser extent, behaved as expected since the various reported nutrients increased germination and appressoria formation, except for the high concentrations of anthralinic acid which resulted in low pH that might have affected the propagules. Daigle and Cotty (1991) found that the formulation that enhanced germination of *Alternaria cassiae* was one of pH 6.5 and contained 1% Tween 20, 0.02M phosphate buffer and 1% dehydrated potato dextrose broth. Fungal nutrients have been used in bioherbicide formulations including *Fusarium solani* f. sp. *cucurbitae* which controls Texas gourd (*Cucurbita texana*). The fungus was formulated in alginate granules and was found to be more effective when granules were amended with soy flour, a fungus nutrient (Connick *et al.*, 1990). When "stored" in alginate granules, fungi grow and proliferate and therefore are able to respond normally to nutrients, whereas when conidia are stored as a dry powder, they go into a dormant state and are less able to respond to external factors such as nutrients. Of the commercially available bioherbicides (DeVine™, COLLEGO™ and BioMal™), one component of COLLEGO™ is composed of sucrose and other compounds. Unfortunately, specific information of commercial formulations is not available because formulations *per se* are generally "trade secrets".

Effect of cutin on germination and appressoriation of formulated and fresh spores of *C. coccodes*.

Fresh and formulated conidia, independently of the filler, showed decreased germination and appressoriation rates with an increase of cutin in the medium (Fig. 6A, B, C,

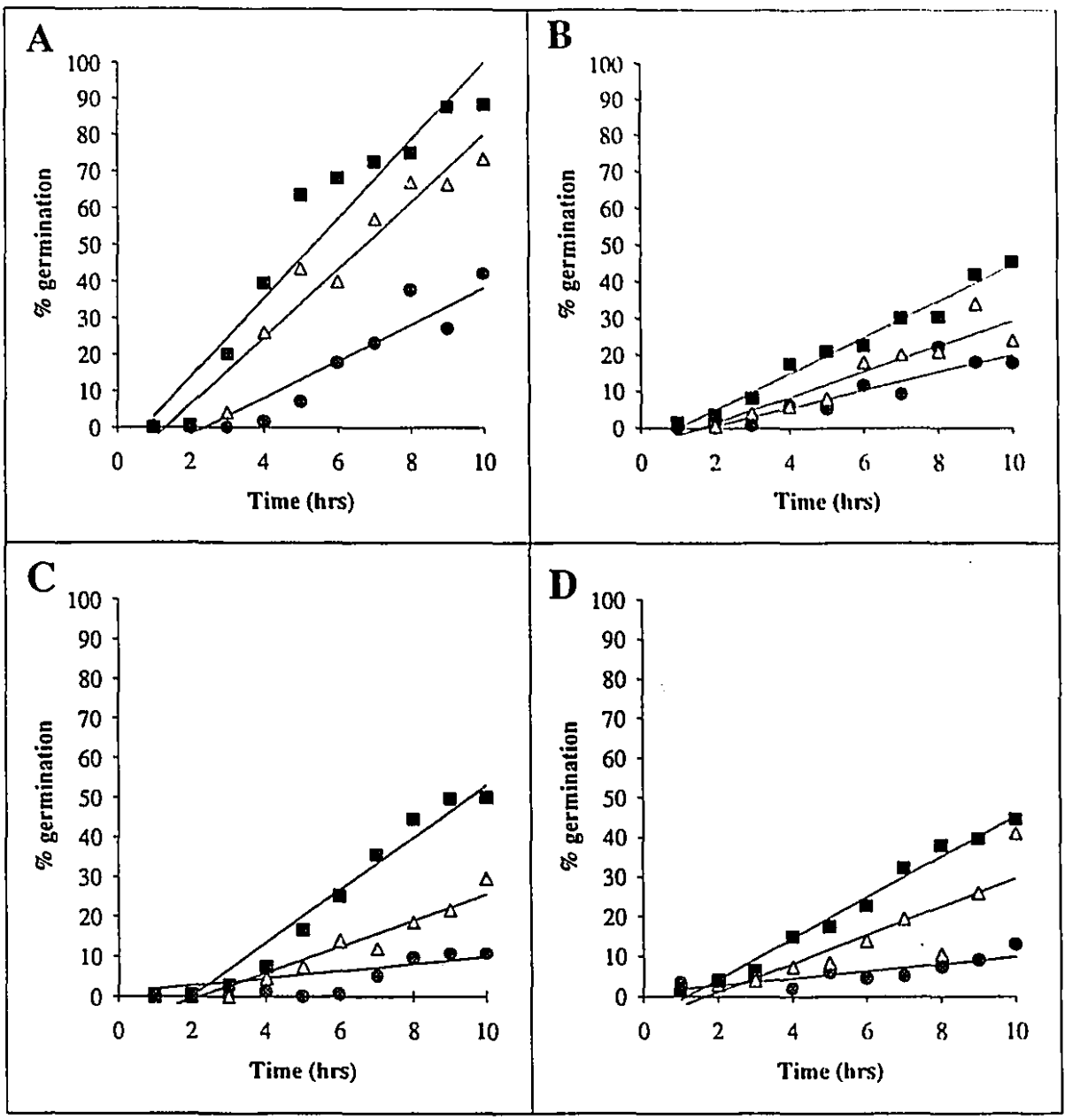


Fig. 6. Germination of fresh conidia of *Colletotrichum coccodes* (A) and conidia formulated in kaolin clay (B), talcum powder (C), or soluble starch (D) over a 10hr period when incubated on potato dextrose agar (PDA) ▣ △ , PDA + 10g/L cutin ○ , or PDA + 40g/L cutin —.

(A) ▣ : y= -7.97 + 10.81x (R^2=0.92); △ : y= -12.93 + 9.23x (R^2=0.95);
○ : y= -12.1 + 5.03x (R^2=0.88).

(B) ▣ : y= -5.03 +4.95x (R^2=0.98); △ : y= -4.27 + 3.49x (R^2=0.85);
○ : y= -4.27 + 2.42x (R^2=0.85).

(C) ▣ : y= -13.03 +6.6x (R^2=0.96); △ : y= -7.17 + 3.26x (R^2=0.93);
○ : y= -3.90 + 1.38x (R^2=0.79).

(D) ▣ : y= -6 + 5.12x (R^2=0.98); △ : y= -5.93 + 3.55x (R^2=0.77);
○ : y= 0.97 + 0.88x (R^2=0.69).

D). The decrease in germination and appressoriation of conidia on dialysis membranes was obvious as early as after three and six hours of incubation respectively (Appendix A, Figures 5, 6), and was maintained for 10 hours (Fig. 6). After 10 hours of incubation, cutin levels as low as 2.5 and 0.25 g/L resulted in a decrease in germination and appressoriation of fresh conidia (Fig. 7A, B). Conidia formulated in kaolin clay, talcum powder, or soluble starch needed higher levels to be significantly affected: 10 and 30 g cutin /L decreased germination and appressoriation of conidia formulated in kaolin clay and even higher levels were needed to affect conidia formulated in talcum powder or soluble starch (Fig. 7C, D, E, F, G, H). Parallel to the response to germination stimulants, the formulated conidia, less physiologically active than fresh conidia, responded to the presence of cutin, which seemed to act like a germination and appressoriation inhibitor at higher concentrations, in a similar way but at a lower magnitude. Several reports on the role of cutinase in penetration (Kolattukudy and Köller, 1983, Kolattukudy *et al.*, 1983, 1987a&b), the purification and characterization of cutinase (Kolattukudy *et al.*, 1989), and the clarification of the mechanism by which the cutinase gene is activated (Woloshuk and Kolattukudy, 1986), but there is no report on the effect of cutin on germination or appressoriation of fungal conidia and results cannot be compared. The inhibitory activity of cutin at high concentrations might be the result of an incomplete purification of cutin from apple peels (stopping the procedure at a stage that showed the highest cutinase induction, A-M. L'Heureux, personal communication) , leaving an inhibitor in the end product. This experiment was run to determine the best concentration of cutin (the one that did not lower germination and/or appressoriation), which was found to be 0.25g cutin/L. This concentration was tested in subsequent bioassays, alone and in

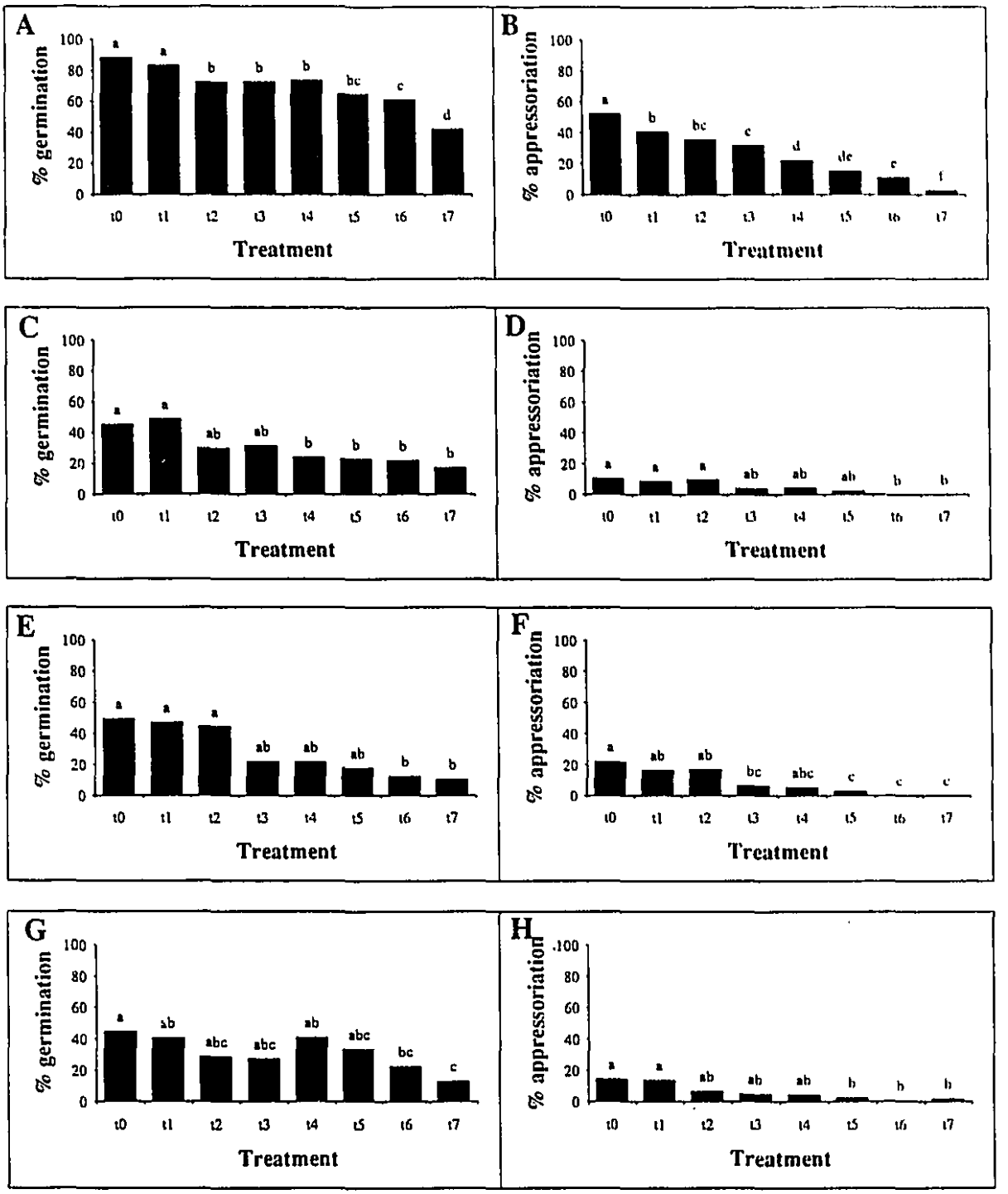


Figure 7. The effect of cutin on germination and appressoria formation of fresh conidia of *Colletotrichum coccodes* (A, B), compared to conidia formulated in kaolin clay (C, D), talcum powder (E, F), or soluble starch (G, H), after a 10hr incubation on agar, where t_0= potato dextrose agar (PDA), t_1= PDA + 0.25g/L cutin, t_2= PDA + 2.5g/L cutin, t_3= PDA + 5g/L cutin, t_4= PDA + 10g/L cutin, t_5= PDA + 20g/L cutin, t_6= PDA + 30g/L cutin, t_7= PDA + 40g/L cutin. Bars with the same letter(s) are not significantly different.

combination with other treatments, to determine the effect of cutin on disease severity.

Effect of the "best" germination stimulants/"best" cutin concentration/three reported pectinase inducers alone or in combination on disease incidence and development

Treatments did not significantly enhance disease severity caused by formulated or fresh conidia except Prep. VII, which resulted in a poor disease development when sprayed without additives (Table 1). Treatments consisting of Prep. VII to which cutin + rhamnose + calcium chloride, cutin + Sorbo + PGA, cutin + Sorbo + potassium phosphate, calcium chloride, sucrose + potassium phosphate or sucrose + calcium chloride were added, resulted in higher disease severities than did Prep. VII alone when sprayed on velvetleaf plants (Table 1). When applied alone, Prep. I, IV and fresh conidia were not significantly different from any other treatment (Table 1). However, the addition of sucrose to fresh conidia and to conidia formulated in kaolin clay or talcum powder resulted in increased disease severities. When sucrose was added to Prep. I, the resulting treatment was significantly different from Prep. I to which cutin was added (Table 1). Prep. IV + sucrose was significantly different from Prep. IV + cutin + calcium chloride, Prep. IV + rhamnose + potassium phosphate, Prep. IV + Sorbo + calcium chloride, Prep. IV + cutin + rhamnose + calcium chloride, Prep. IV + cutin + Sorbo + PGA, and Prep. IV + cutin + Sorbo + calcium chloride (Table 1). The treatment consisting of fresh conidia to which sucrose was added was significantly different from fresh conidia to which cutin + PGA, Sorbo + PGA, cutin + rhamnose + PGA, cutin + Sorbo + calcium chloride, or cutin + Sorbo + PGA were added (Table 1).

Different species of *Colletotrichum* have been reported to have different germination

Table 1. Disease ratings on 14 day old velvetleaf plants 9 days after inoculation with fresh conidia of *Colletotrichum coccodes* or conidia formulated in kaolin clay (KC), talcum powder (TP), or soluble starch (SS) to which germination stimulants, cutin, and/or pectinase inducers were added.

	Disease rating[1]			
Treatment (additives)	Fresh conidia	Prepared in KC	Prepared in TP	Prepared in SS
None	8.2 abc[2]	6.7 ab	6.2 abcde	2.3 d
1% sucrose	8.6 a	8.3 a	7.9 a	4.0 abcd
10^{-2}M rhamnose	8.2 abc	8.0 ab	7.9 abc	3.1 bcd
10^{-3}M asparagine	7.7 abc	8.3 ab	7.8 ab	3.8 abcd
0.1% Sorbo	7.9 abc	6.4 ab	7.8 ab	4.7 abcd
0.25g/L cutin	7.8 abc	5.6 b	7.0 abcde	4.7 abcd
0.25 g/L cutin + 1% sucrose	8.0 abc	6.4 ab	6.1 abcde	5.1 abcd
0.25 g/L cutin + 10^{-2}M rhamnose	8.0 abc	7.4 ab	5.3 abcde	4.2 abcd
0.25 g/L cutin + 10^{-3}M asparagine	8.4 ab	7.7 ab	5.0 abcde	5.3 abcd
0.25 g/L cutin + 0.1% Sorbo	8.0 abc	7.4 ab	6.1 abcde	3.7 abcd
1 g/L polygalacturonic acid (PGA)	7.2 abc	6.4 ab	6.9 abcde	3.3 abcd
0.1M potassium phosphate (KH_2PO_4)	8.4 ab	7.0 ab	6.9 abcde	4.2 abcd
0.001M calcium chloride ($CaCl_2$)	7.3 abc	6.6 ab	6.4 abcde	7.0 ab
0.25g/L cutin + 1 g/L PGA	6.7 c	5.8 ab	6.4 abcde	5.7 abcd
0.25 g/L cutin + 0.1M KH_2PO_4	7.4 abc	6.4 ab	6.6 abcde	4.0 abcd
0.25 g/L cutin + 0.001 M $CaCl_2$	7.9 abc	6.4 ab	4.4 de	5.3 abcd
0.1% sucrose + 1g/L PGA	7.9 abc	6.3 ab	6.0 abcde	4.8 abcd
1% sucrose + 0.1M KH_2PO_4	7.9 abc	7.4 ab	7.0 abcde	6.2 abc
1% sucrose + 0.001M $CaCl_2$	7.8 abc	6.3 ab	7.0 abcde	6.3 abc
10^{-2}M rhamnose + 1g/L PGA	7.4 abc	7.2 ab	4.9 abcde	6.1 abcd
10^{-2}M rhamnose + 0.1M KH_2PO_4	7.9 abc	5.9 ab	3.6 de	3.4 abcd
10^{-2}M rhamnose + 0.001M $CaCl_2$	8.2 abc	7.6 ab	7.9 abcd	3.6 abcd
10^{-3}M asparagine + 1g/L PGA	7.8 abc	6.6 ab	7.0 abcde	3.7 abcd
10^{-3}M asparagine + 0.1M KH_2PO_4	8.0 abc	7.2 ab	6.1 abcde	5.1 abcd
10^{-3}M asparagine + 0.001M $CaCl_2$	8.0 abc	7.0 ab	6.2 abcde	2.4 cd
0.1% Sorbo + 1g/L PGA	6.7 bc	7.1 ab	6.7 abcde	5.4 abcd
0.1% Sorbo + 0.1M KH_2PO_4	7.0 abc	6.9 ab	5.1 abcde	4.7 abcd

0.1% Sorbo + 0.001M $CaCl_2$	8.2 abc	6.3 ab	3.1 e	4.2 abcd
0.25 g/L cutin + 10^{-2}M rhamnose + 1g/L PGA	6.7 c	6.2 ab	6.6 abcde	4.3 abcd
0.25 g/L cutin + 10^{-2}M rhamnose + 0.1M KH_2PO_4	7.7 abc	5.6 ab	5.7 abcde	6.2 abcd
0.25 g/L cutin + 10^{-2}M rhamnose + 0.001M $CaCl_2$	7.8 abc	6.8 ab	4.7 cde	7.9 a
0.25 g/L cutin + 0.1% Sorbo + 1g/L PGA	6.8 bc	6.3 ab	4.3 de	6.4 ab
0.25 g/L cutin + 0.1% Sorbo + 0.1M KH_2PO_4	7.7 abc	5.2 ab	5.9 abcde	6.9 ab
0.25 g/L cutin + 0.1% Sorbo + 0.001M $CaCl_2$	6.7 bc	6.2 ab	3.9 bcde	4.3 abcd

[1] Mean disease rating of 3 pots where 0 = no lesions and 10>90% of the sprayed area.
[2] Values followed by the same letter(s) are not significantly different according to Friedmann multiple comparison test.

and/or appressoriation stimulants. Conidial germination of *C. papayae* was found to increase with increasing concentrations of sucrose (Gupta, 1979), whereas L-rhamnose was reported to be the best carbon source for germination and appressorium formation of *C. capsici* (Solanki *et al.*, 1974). *C. coccodes* conidia seem to be more favourably affected by sucrose than by rhamnose. These results concur with the previous study regarding the effect of various agar amendments on germination of fresh and formulated conidia. The addition of 10^{-3}M rhamnose to water agar significantly increased germination of fresh conidia, but the increase was similar to the one caused by the addition of 0.1% sucrose. However, sucrose performance was slightly better than rhamnose when formulated conidia were concerned. In this experiment, although the germination stimulants used resulted in increased disease severities, these were not detectable with nonparametric analyses. Cutin, added to formulated and fresh conidia at 0.25g/L, did not result in a treatment significantly different from the controls (formulated/fresh conidia without additives) (Table 1). It might be that the cutinase gene is not turned on before spores land on a surface, in which case the cutin fragments added to conidial suspensions before being placed on a shaker were of little use. Although the addition of potassium phosphate to conidia formulated in kaolin clay or talcum powder and fresh conidia seem to result in increased velvetleaf disease severities when compared to the controls, they were not significantly different from the controls. The addition of calcium chloride increased significantly the "infectivity" of Prep. VII (Table 1). Prep. VII, the worst formulation, was the only one to be significantly improved by the incorporation of additives in the conidial suspension. The best treatment proved to be the addition of the combination of cutin, rhamnose and calcium chloride to resuspended conidia. When cutin + Sorbo +

potassium phosphate or cutin + Sorbo + PGA were added to Prep. VII, velvetleaf disease severities were significantly increased from the control (Prep. VII alone).

Effect of fatty acids/surfactants on velvetleaf infection

The addition of surfactants or fatty acids did not affect disease incidence and severity. After 7 days of inoculation, velvetleaf was mostly affected by fresh spores although in a manner comparable to many of the other treatments (Table 2; Figure 8A, B). After 10 days, the addition of oleic and stearic acids to spores formulated in talcum powder (Figure 8C) or kaolin clay (Figure 8D) respectively resulted in increased lesion occurrence on velvetleaf leaves, but this increase was not significant when all the treatments were compared. Nonparametric analyses require large differences for significance, especially when the number of treatments is large. When compared among themselves, formulations in kaolin clay and talcum powder showed better performances (significantly different from the control formulation, to which nothing was added, in case of talcum powder) when stearic and oleic acids respectively were added. Linoleic and oleic acids decreased the efficacy of spores formulated in soluble starch, and Brij 35 affected the efficacy of spores formulated in either soluble starch or talcum powder, when all treatments were compared (Table 2). When compared among themselves, formulations in soluble starch were not affected by the addition of stearic acid, whereas the addition of any other fatty acid/surfactant decreased disease severity. Fresh conidia were not affected by the addition of oleic or linoleic acids, and the addition of stearic acid, Brij 35 and Tween resulted in decreased disease severities (Table 2).

Table 2. Disease ratings of velvetleaf plants 7 and 10 days after inoculation with formulated or fresh conidia of *Colletotrichum coccodes* to which fatty acids were added.

	Disease ratings[1] after		
Treatments[2]	7 days	10 days	
Prep.I	5.0 ab[3,4]	7.3 ab	a[5]
Prep.I + Brij 35	3.4 ab	5.1 abc	b
Prep.I + linoleic acid	4.9 ab	5.8 abc	b
Prep.I + oleic acid	6.0 ab	7.2 ab	a
Prep.I + stearic acid	6.0 ab	8.1 a	a
Prep.I + Tween 20	3.6 ab	5.0 abc	b
Prep.IV	3.7 ab	3.7 abc	d
Prep.IV + Brij 35	2.3 b	2.3 c	de
Prep.IV + linoleic acid	5.0 ab	6.2 abc	b
Prep.I V + oleic acid	5.8 ab	7.4 a	a
Prep.IV + stearic acid	4.7 ab	4.4 abc	c
Prep.IV + Tween 20	3.6 ab	3.4 abc	d
Prep.VII	4.1 ab	4.4 abc	a
Prep.VII + Brij 35	2.1 ab	3.1 bc	bc
Prep.VII + linoleic acid	1.8 b	1.1 c	cd
Prep.VII + oleic acid	3.4 ab	3.2 bc	bc
Prep.VII + stearic acid	3.7 ab	4.9 abc	a
Prep.VII + Tween 20	2.7 b	3.7 abc	b
Fresh conidia	7.2 a	7.1 ab	a
Fresh conidia + Brij 35	3.8 ab	4.2 abc	d
Fresh conidia + linoleic ac	6.8 ab	5.3 abc	bc
Fresh conidia + oleic acid	7.3 ab	7.0 ab	a
Fresh conidia + stearic aci	6.9 ab	6.4 abc	a
Fresh conidia + Tween 20	4.2 ab	5.0 abc	c

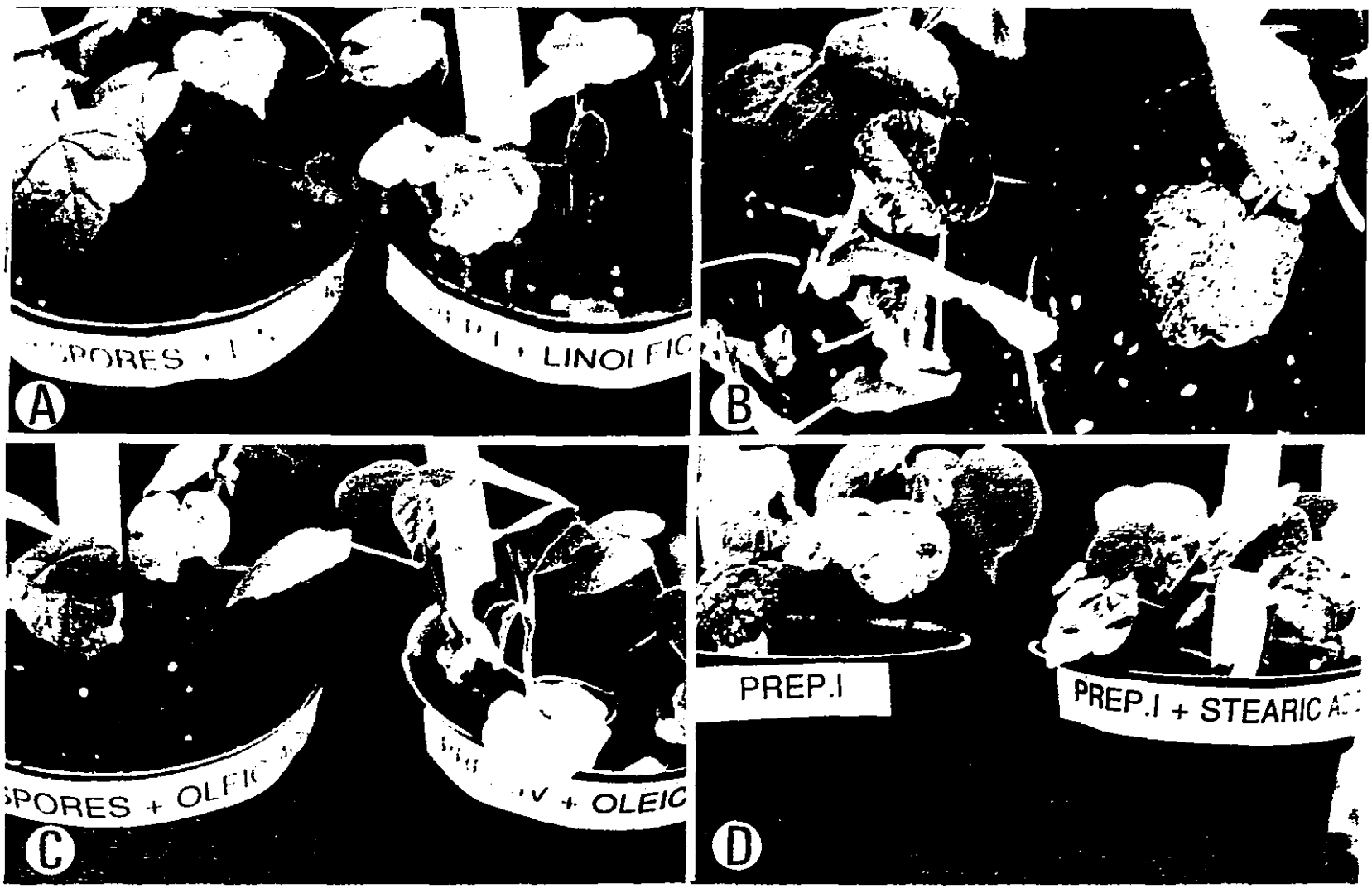
[1] Values are the mean of 3 replicates.

[2] Prep. I=1 ml *Colletotrichum coccodes* (Cc):2.78g kaolin clay (KC), Prep. II=1ml Cc:3.34g KC, Prep. III=1 ml Cc:3.07g KC, Prep. IV=1 ml Cc:2.79g talcum powder (TP), Prep. V=1 ml Cc:3.98g TP, Prep. VI=1 ml Cc:4.98g TP, Prep. VII=1 ml Cc:2.54g soluble starch (SS), Prep. VIII=1 ml Cc:3.78g SS, and Prep. IX=1 ml Cc:5.04g SS.

[3] Disease ratings based on a visual scale where 0 = no lesions and 10>90% of the sprayed area.

[4] Means followed by the same letter(s) are not significantly different according to Friedmann multiple comparison test.ea is covered with lesions, and values are means of three replicates.

Figure 8. Disease severity of fourteen day-old velvetleaf seedlings 7 days after inoculation (A, B) with fresh conidia or conidia formulated in kaolin clay (A), or talcum powder (B), to which linoleic acid was added, and 10 days after inoculation (C, D) with fresh conidia or conidia formulated in talcum powder to which oleic acid was added (C), or conidia formulated in kaolin clay with and without stearic acid (D).


A
B
C
D
PREP.I

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IV. GENERAL DISCUSSION AND CONCLUSION

Colletotrichum coccodes possesses the essential characteristics to be an effective bioherbicide to control velvetleaf. In addition to host specificity, easy mass production and rapid disease symptom production (Poirier, 1984; Wymore *et al.*, 1988), effective formulation is feasible.

The success of formulating conidia by mixing with fillers represents a great asset for future industrial production. The use of water activity concept in bioherbicide studies is essential for a better understanding of conidial physiology, therefore better formulation abilities. The integration of a second component in the formulation which role is not only to enhance germination, but also to trigger conidia to produce the necessary enzymes for a faster host penetration differs from all other known bioherbicide formulations. This aspect is important for bioherbicide development.

Colletotrichum coccodes conidia formulated in kaolin clay or talcum powder were viable and infective, in a manner similar to fresh conidia, for at least six months when stored at room temperature. Formulated conidia stored at 4, 30C or at room temperature in bags permeable to oxygen lost infectivity very slowly, and the rate of decline in infectivity was greater for conidia at 40C. It would be interesting to determine if the fungus would survive at these temperatures had it been under vacuum, without the influence of the surrounding relative humidity.

The addition of germination stimulants, fatty acids, cutinase and / or pectinase inducers to conidia formulated in kaolin clay or talcum powder did not accelerate disease initiation or significantly increase disease severity of velvetleaf seedlings. Monitoring disease expression

of velvetleaf plants after spraying with formulated or fresh conidia with additives and providing dew periods shorter than 18 hours might prove to be interesting. Since sub-optimal moisture conditions represent a major disease constraint, further research in formulation studies is needed. The development of genetically engineered organisms that require less moisture to germinate and infect plant tissue (Charudattan, 1985) would overcome the absolute requirement for free water on the plant surface.

Colletotrichum coccodes is certainly a good candidate to be used as a bioherbicide to suppress velvetleaf populations and its effective formulation makes its marketing possible. The use of plant pathogens on weeds represent an alternative control measure that could exert an additional stress on this aggressive, troublesome weed. Integration of this promising bioherbicide with other chemical agricultural products are ongoing in an attempt to plan an adequate weed control program for velvetleaf (Armour, personal communication, Wymore *et al.*, 1987).

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APPENDIX A
Results not presented in the text

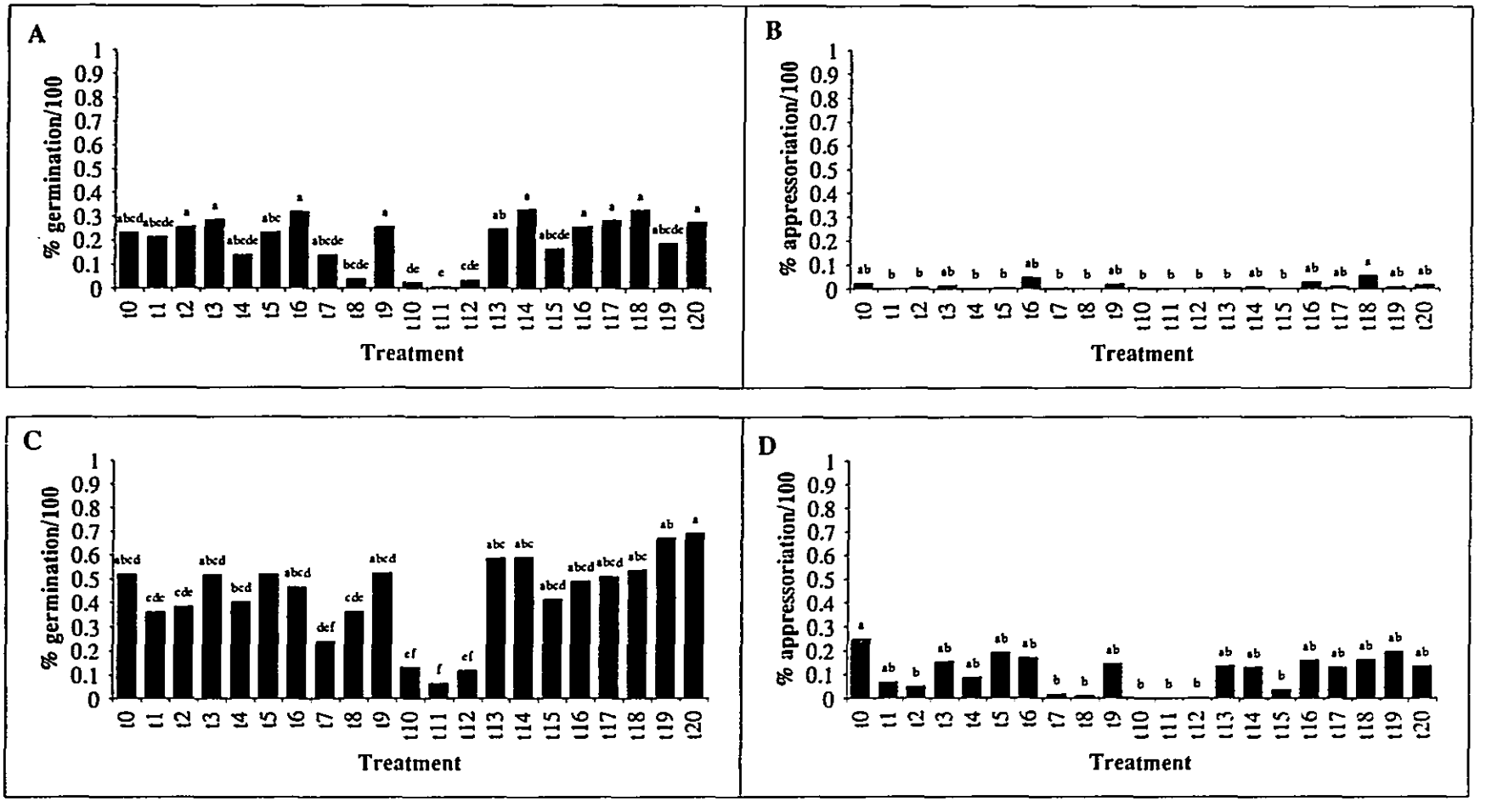

Figure 1. The effect of various agar amendments on germination and appressoriation of conidia of *Colletotrichum coccodes* formulated in kaolin clay after 3 (A, B) and 6 hours (C, D) of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.
Bars in the same graph with the same letter(s) are not significantly different according to Duncan Multiple Range test.

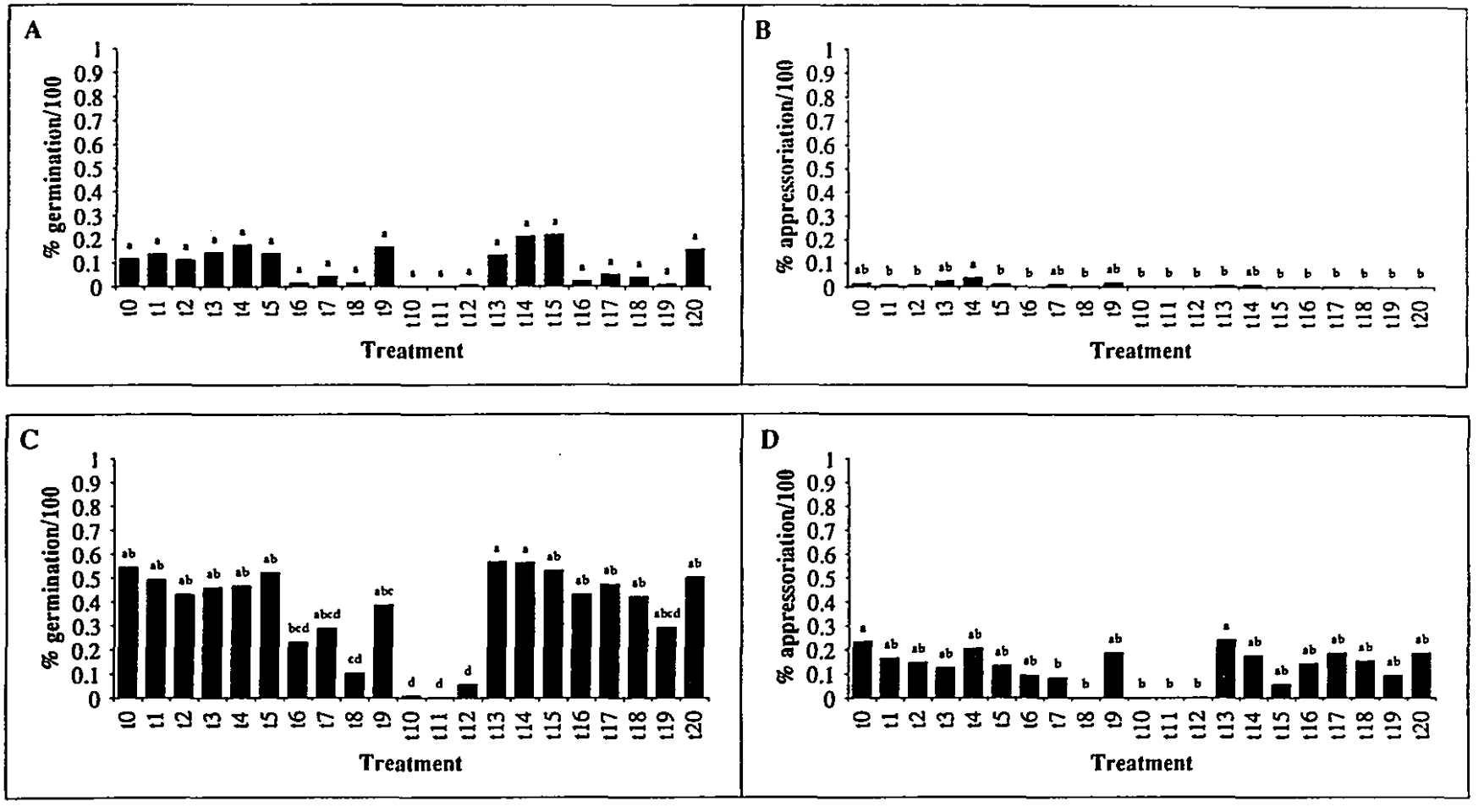

Figure 2. The effect of various agar amendments on germination and appressoriation of conidia of *Colletotrichum coccodes* formulated in talcum powder after 3 (A, B) and 6 hours (C, D) of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.
Bars in the same graph with the same letter(s) are not significantly different according to Duncan Multiple Range test.

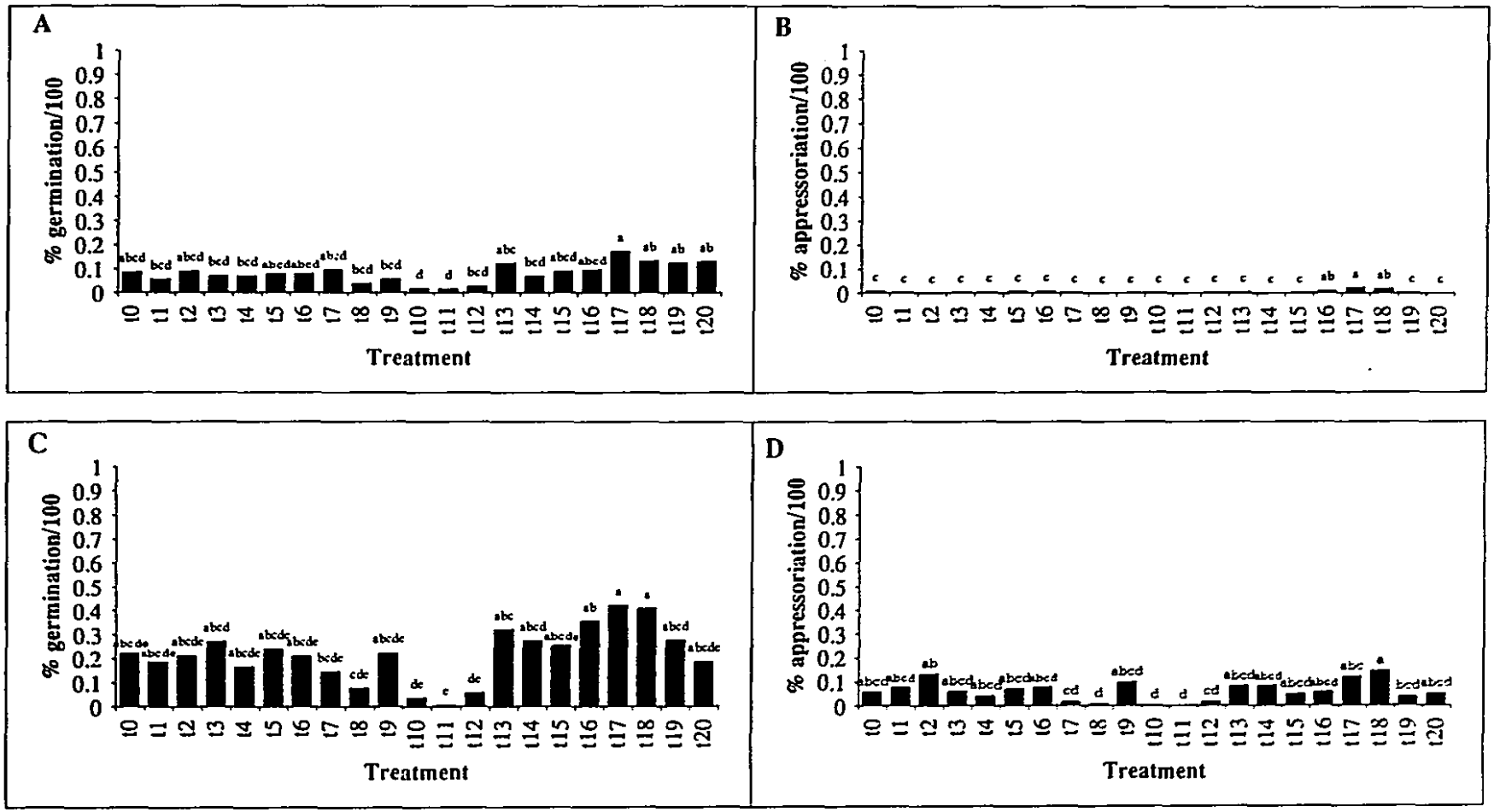

Figure 3. The effect of various agar amendments on germination and appressoriation of conidia of *Colletotrichum coccodes* formulated in soluble starch after 3 (A, B) and 6 hours (C, D) of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.
Bars in the same graph with the same letter(s) are not significantly different according to Duncan Multiple Range test.

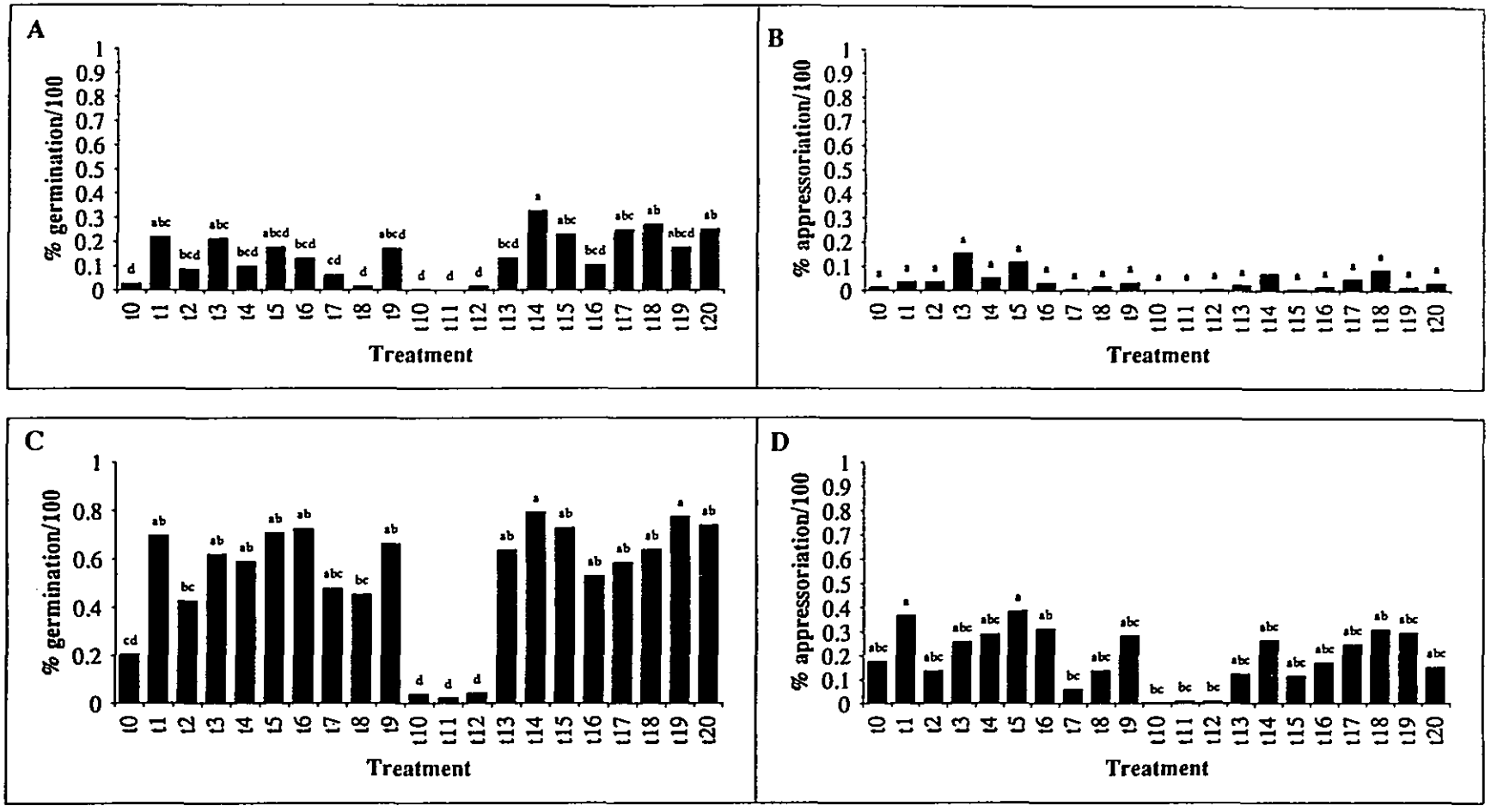

Figure 4. The effect of various agar amendments on germination and appressoriation of fresh conidia of *Colletotrichum coccodes* after 3 (A, B) and 6 hours (C, D) of incubation, where t_0= water agar (WA) only, t_1= WA + $2*10^{-2}$M glycine, t_2= WA + 10^{-2}M glycine, t_3= WA + 10^{-3}M glycine, t_4= WA + $2*10^{-2}$M asparagine, t_5= WA + 10^{-2}M asparagine, t_6= WA + 10^{-3}M asparagine, t_7= WA + $5*10^{-5}$M EDTA, t_8= WA + $5*10^{-4}$ M EDTA, t_9= WA + 10^{-4}M anthralinic acid (AA), t_{10}= WA + 10^{-3}M AA, t_{11}= WA + 10^{-2}M AA, t_{12}= WA + 0.2% Triton, t_{13}= WA + 0.1% sucrose, t_{14}= WA + 1% sucrose, t_{15}= WA + 10% sucrose, t_{16}= WA + 0.1% Sorbo, t_{17}= WA + 1% Sorbo, t_{18}= WA + $2*10^{-2}$M rhamnose, t_{19}= WA + 10^{-2}M rhamnose and t_{20}= WA + 10^{-3}M rhamnose.
Bars in the same graph with the same letter(s) are not significantly different according to Duncan Multiple Range test.

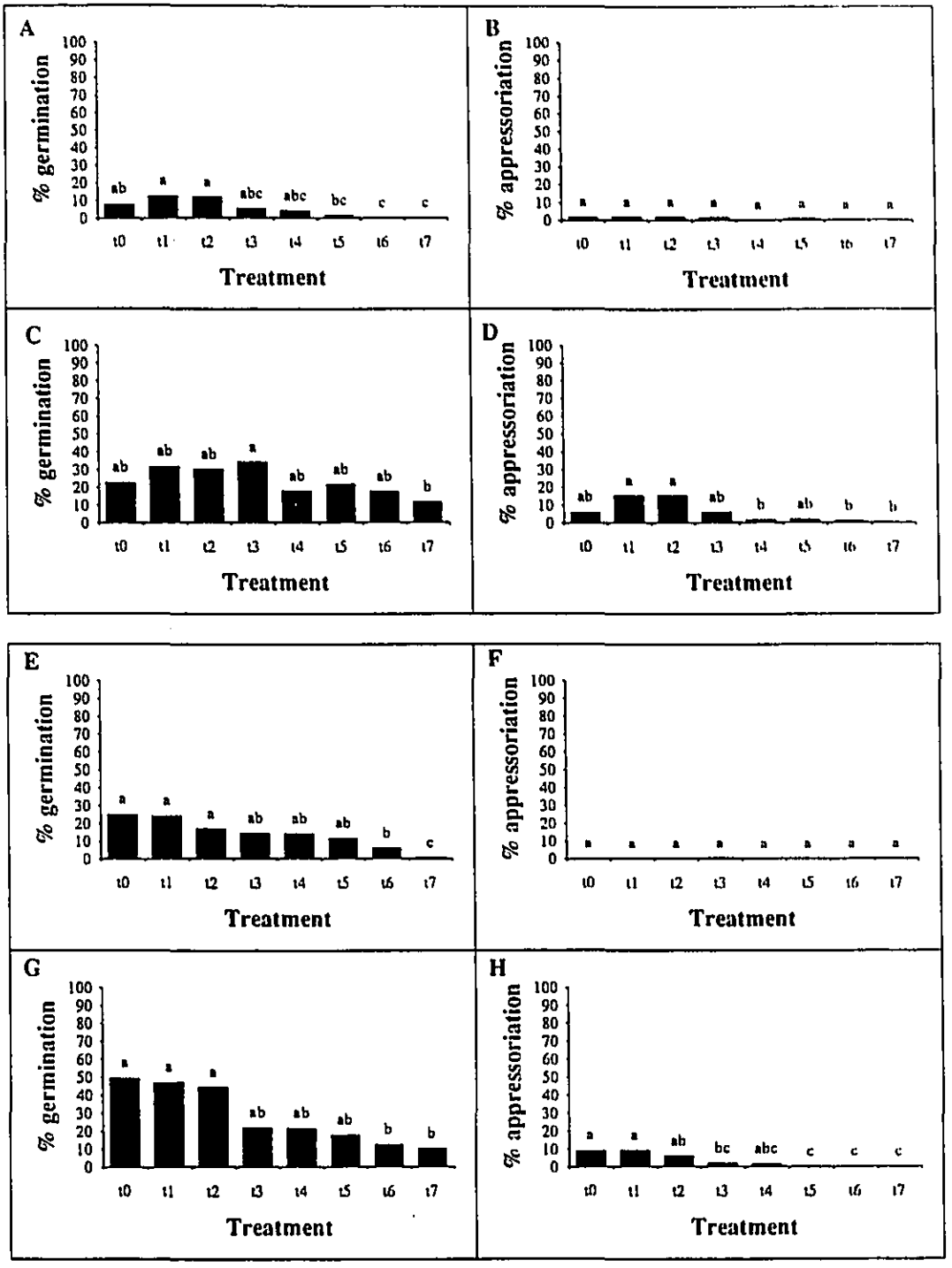

Figure 5. The effect of cutin on germination and appressoria formation of conidia of *Colletotrichum coccodes* formulated in kaolin clay (A, B, C, D) compared to conidia formulated in talcum powder (E, F, G, H) after a 3 (A, B / E, F) or 6 hours (C, D / G, H) of incubation on agar, where t_0= potato dextrose agar (PDA), t_1= PDA + 0.25g/L cutin, t_2= PDA + 2.5g/L cutin, t_3= PDA + 5g/L cutin, t_4= PDA + 10g/L cutin, t_5= PDA + 20g/L cutin, t_6= PDA + 30g/L cutin, t_7= PDA + 40g/L cutin. Bars in the same graph with the same letter(s) are not significantly different.

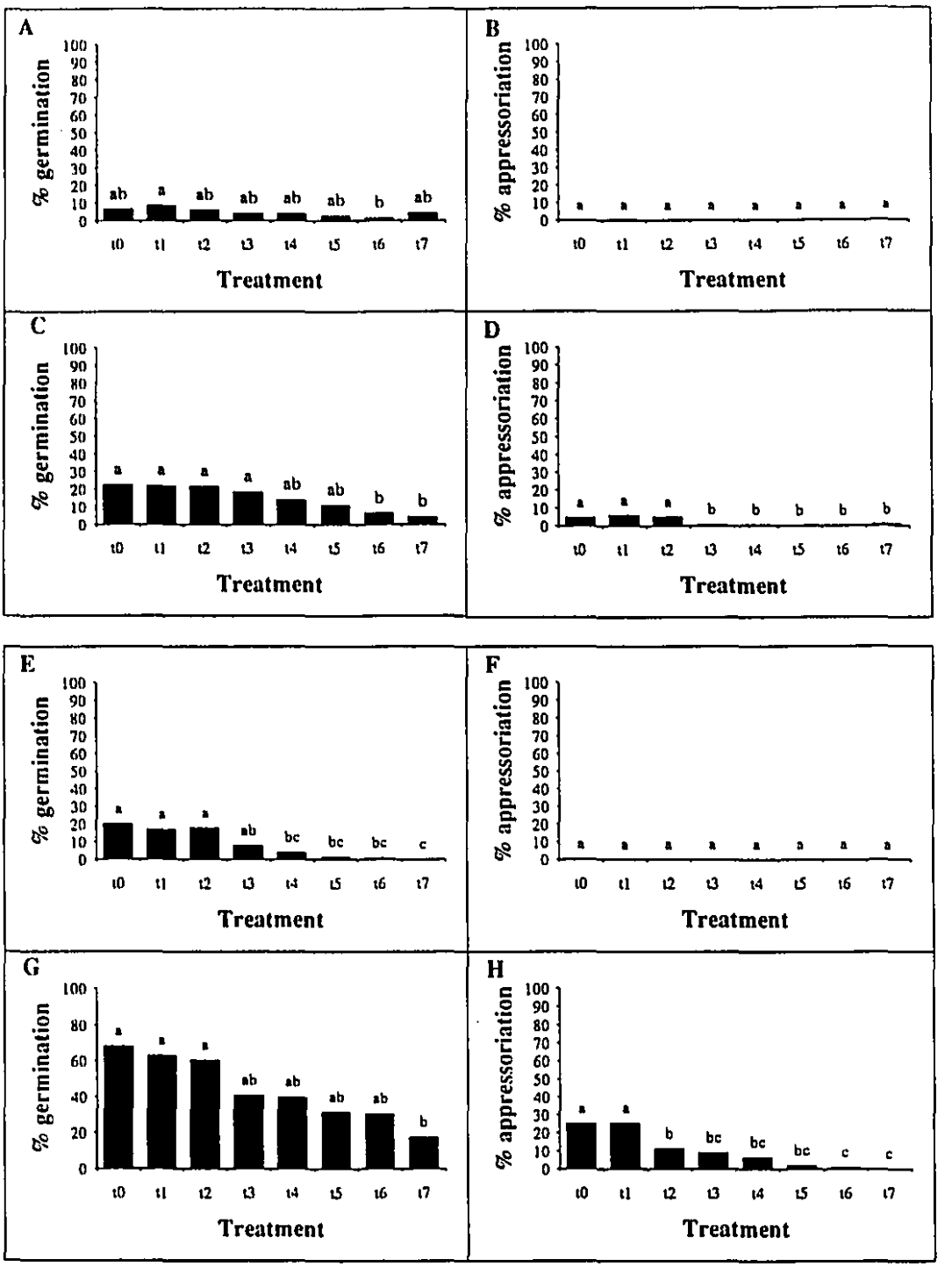

Figure 6. The effect of cutin on germination and appressoria formation of conidia of *Colletotrichum coccodes* formulated in soluble starch (A, B, C, D) compared to fresh conidia (E, F, G, H) after a 3 (A, B / E, F) or 6 hours (C, D / G, H) of incubation on agar, where t_0= potato dextrose agar (PDA), t_1= PDA + 0.25g/L cutin, t_2= PDA + 2.5g/L cutin, t_3= PDA + 5g/L cutin, t_4= PDA + 10g/L cutin, t_5= PDA + 20g/L cutin, t_6= PDA + 30g/L cutin, t_7= PDA + 40g/L cutin. Bars with the same letter(s) are not significantly different.

APPENDIX B
Analysis of parametric data

Analysis 1. Effect of time on water activity in the bags.

A) Non-Linear Least Squares Summary Statistics Dependent Variable a_w

Prep.	Source	DF	Sum of Squares	Mean Square
I	Regression	2	1.8558989643	0.9279494822
	Residual	5	0.1196110357	0.0239222071
	Uncorrected Total	7	1.9755100000	
	(Corrected Total)	6	0.3182477143	
II	Regression	2	1.8465204679	0.9232602340
	Residual	5	0.1176865321	0.0235373064
	Uncorrected Total	7	1.9642070000	
	(Corrected Total)	6	0.3273177143	
III	Regression	2	1.8954697370	0.9477348685
	Residual	5	0.0888572630	0.0177714526
	Uncorrected Total	7	1.9843270000	
	(Corrected Total)	6	0.3319268571	
IV	Regression	2	1.8255538658	0.9127769329
	Residual	5	0.1384021342	0.0276804268
	Uncorrected Total	7	1.9639560000	
	(Corrected Total)	6	0.3511560000	
V	Regression	2	1.8031657793	0.9015828896
	Residual	5	0.1443102207	0.0288620441
	Uncorrected Total	7	1.9474760000	
	(Corrected Total)	6	0.3557268571	
VI	Regression	2	1.7965353828	0.8982676914
	Residual	5	0.1429326172	0.0285865234
	Uncorrected Total	7	1.9394680000	
	(Corrected Total)	6	0.3553394286	
VII	Regression	2	1.9841590876	0.9920795438
	Residual	5	0.0553889124	0.0110777825
	Uncorrected Total	7	2.0395480000	
	(Corrected Total)	6	0.2835428571	
VIII	Regression	2	1.9707998716	0.9853999358
	Residual	5	0.0410501284	0.0082100257
	Uncorrected Total	7	2.0118500000	
	(Corrected Total)	6	0.2357534286	
IX	Regression	2	1.8382065482	0.9191032741
	Residual	5	0.0403964518	0.0080792904
	Uncorrected Total	7	1.8786030000	
	(Corrected Total)	6	0.2008417143	

(B) Asymptotic 95 %.

Prep.		Parameter Estimate	Std. Error	Confidence Interval	
				Lower	Upper
I	B_0	0.4000000000	0.06362830438	0.2364406072	0.5635593928
	B_1	0.9000000000	0.94013241010	-1.5166522693	3.3166522693
II	B_0	0.4000000000	0.06311434904	0.2377617523	0.5622382477
	B_1	0.9000000000	0.93253852441	-1.4971318476	3.2971318476
III	B_0	0.411241642	0.0453387182	0.2946964461	0.527786839
	B_1	2.570391012	4.5423588361	-9.1059448649	14.246726889
IV	B_0	0.4000000000	0.0684441159	0.2240613490	0.5759386510
	B_1	0.9000000000	1.0112878580	-1.6995605201	3.4995605201
V	B_0	0.4000000000	0.0698897151	0.2203453718	0.5796546282
	B_1	0.9000000000	1.0326471361	-1.7544654965	3.5544654965
VI	B_0	0.4000000000	0.0695553271	0.2212049309	0.5787950691
	B_1	0.9000000000	1.0277064270	-1.7417651836	3.5417651836
VII	B_0	0.424458856	0.0361411186	0.3315564992	0.5173612122
	B_1	2.243689650	2.5125944890	-4.2150464896	8.7024257905
VIII	B_0	0.422867221	0.0309954035	0.3431921548	0.5025422876
	B_1	2.356343878	2.4283676545	-3.8858834297	8.5985711851
IX	B_0	0.417413812	0.0302697779	0.339603998	0.495223625
	B_1	3.170563436	5.4676660836	-10.884315969	17.225442841

Analysis 2. Effect of water activity on weight loss of the preparations in the bags.

Prep. I. Dependent Variable: wtls.

Source	DF	SS	MS	F Value	Pr > F	R-Square	C.V.	Root MSE	wtlsMean
Model	2	0.05952312	0.02976156	147.38	0.0002	0.986612	6.291750	0.014210	0.22585714
Error	4	0.00080774	0.00020193						
Corr. Total	6	0.06033086							

Source	DF	Type I SS	Mean Square	F Value	Pr > F
A_w	1	0.05072948	0.05072948	251.22	0.0001
A_w*A_w	1	0.00879364	0.00879364	43.55	0.0027

Source	DF	Type III SS	Mean Square	F Value	Pr > F
A_w	1	0.00342913	0.00342913	16.98	0.0146
A_w*A_w	1	0.00879364	0.00879364	43.55	0.0027

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.1352395538	3.00	0.0399	0.04507965
A_w	0.6832155023	4.12	0.0146	0.16579491
A_w*A_w	-.8564411957	-6.60	0.0027	0.12978322

Prep. II. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.06276470	0.03138235	117.46	0.0003	0.983257	7.067333	0.016346	0.23128571
Error	4	0.00106873	0.00026718						
Corr. Total	6	0.06383343							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
AW	1	0.05309738	0.05309738	198.73	0.0001
AW*AW	1	0.00966731	0.00966731	36.18	0.0038

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00381880	0.00381880	14.29	0.0194
A_w*A_w	1	0.00966731	0.00966731	36.18	0.0038

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.1363120250	2.70	0.0541	0.05048969
A_w	0.7038585367	3.78	0.0194	0.18617712
A_w*A_w	-.8745235719	-6.02	0.0038	0.14538613

Prep. III. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.05901197	0.02950598	232.45	0.0001	0.991469	5.039393	0.011267	0.22357143
Error	4	0.00050775	0.00012694						
Corr. Total	6	0.05951971							

Source	DF	Type I SS	Mean Square	F Value	Pr > F
A_w	1	0.05193144	0.05193144	409.11	0.0001
A_w*A_w	1	0.00708053	0.00708053	55.78	0.0017

Source	DF	Type III SS	Mean Square	F Value	Pr > F
A_w	1	0.00214816	0.00214816	16.92	0.0147
A_w*A_w	1	0.00708053	0.00708053	55.78	0.0017

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.1834455993	5.59	0.0050	0.03280048
AW	0.4995385473	4.11	0.0147	0.12143126
AW*AW	-.7146250566	7.47	0.0017	0.09568415

Prep. IV. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.06842355	0.03421178	104.68	0.0004	0.981252	7.413495	0.018078	0.24385714
Error	4	0.00130731	0.00032683						
Corr. Total	6	0.06973086							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
A_w	1	0.05564833	0.05564833	170.27	0.0002
A_w*A_w	1	0.01277522	0.01277522	39.09	0.0033

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00540631	0.00540631	16.54	0.0153
A_w*A_w	1	0.01277522	0.01277522	39.09	0.0033

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.1348979971	2.66	0.0562	0.05063788
AW	0.7698230737	4.07	0.0153	0.18927739
AW*AW	-.9286824693	-6.25	0.0033	0.14853933

Prep. V. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.07920095	0.03960047	67.93	0.0008	0.971399	9.363683	0.024145	0.25785714
Error	4	0.00233191	0.00058298						
Corr. Total	6	0.08153286							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
A_w	1	0.07234918	0.07234918	124.10	0.0004
A_w*A_w	1	0.00685176	0.00685176	11.75	0.0266

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
AW	1	0.00144500	0.00144500	2.48	0.1905
AW*AW	1	0.00685176	0.00685176	11.75	0.0266

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.2588292434	3.92	0.0172	0.06599569
A_w	0.3926137958	1.57	0.1905	0.24937779
A_w*A_w	-.6764394294	-3.43	0.0266	0.19731203

Prep. VI. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.08759416	0.04379708	128.29	0.0002	0.984650	6.807199	0.018477	0.27142857
Error	4	0.00136555	0.00034139						
Corr. Total	6	0.08895971							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
A_w	1	0.07690332	0.07690332	225.27	0.0001
A_w*A_w	1	0.01069084	0.01069084	31.32	0.0050

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00329944	0.00329944	9.66	0.0359
A_w*A_w	1	0.01069084	0.01069084	31.32	0.0050

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.2222253689	4.36	0.0121	0.05101869
AW	0.5990271008	3.11	0.0359	0.19268600
AW*AW	-.8509229480	-5.60	0.0050	0.15205764

Prep. VII. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.03354611	0.01677306	169.72	0.0001	0.988353	5.892387	0.009941	0.16871429
Error	4	0.00039532	0.00009883						
Corr. Total	6	0.03394143							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
A_w	1	0.02887218	0.02887218	292.14	0.0001
A_w*A_w	1	0.00467393	0.00467393	47.29	0.0023

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00200315	0.00200315	20.27	0.0108
A_w*A_w	1	0.00467393	0.00467393	47.29	0.0023

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.0685698777	1.75	0.1545	0.03912299
A_w	0.6214580299	4.50	0.0108	0.13803775
A_w*A_w	-.7245826020	-6.88	0.0023	0.10536346

Prep. VIII. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.04543808	0.02271904	257.61	0.0001	0.992296	4.913142	0.009391	0.19114286
Error	4	0.00035277	0.00008819						
Corr. Total	6	0.04579086							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
A_w	1	0.04455215	0.04455215	505.17	0.0001
A_w*A_w	1	0.00088594	0.00088594	10.05	0.0339

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00002798	0.00002798	0.32	0.6033
A_w*A_w	1	0.00088594	0.00088594	10.05	0.0339

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.2615908888	5.45	0.0055	0.04799474
A_w	0.0947199293	0.56	0.6033	0.16815937
A_w*A_w	-.4111234396	-3.17	0.0339	0.12971443

Prep. IX. Dependent Variable: wtls.

Source	DF	SS	MS	F-Value	Pr > F	R-Square	C.V.	Root MSE	wtls Mean
Model	2	0.05003166	0.02501583	26.61	0.0049	0.930095	14.60039	0.030661	0.21000000
Error	4	0.00376034	0.00094009						
Corr. Total	6	0.05379200							

Source	DF	Type I SS	Mean Square	F-Value	Pr > F
AW	1	0.03710215	0.03710215	39.47	0.0033
AW*AW	1	0.01292950	0.01292950	13.75	0.0207

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
A_w	1	0.00755280	0.00755280	8.03	0.0471
A_w*A_w	1	0.01292950	0.01292950	13.75	0.0207

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	-0.079236133	-0.57	0.5999	0.13931401
A_w	1.449017820	2.83	0.0471	0.51121485
A_w*A_w	-1.565594828	-3.71	0.0207	0.42215535

Analysis 3. The effect of 0, 10, or 20% glycerol on growth of *Colletotrichum coccodes* (measured by colony diameter) when incubated at 24C in the dark.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} level of B.
E_{ij} is the random error associated with the ij^{th} experimental unit.

Dependent variable: CD3

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	TRT	2	11.84222222	5.92111111	761.29	0.0001
	error	6	0.04666667	0.00777778		
2	TRT	2	12.83555556	6.41777778	5776.00	0.0001
	error	6	0.00666667	0.00111111		
pooled	TRT	2	24.66777778	12.33388889	2775.13	0.0001
error		8	0.03555556	0.00444444		

Dependent variable: CD11

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
	TRT	2	66.54888889	33.27444444	49.83	0.0002
	error	6	4.00666667	0.66777778		
	TRT	2	61.04000000	30.52000000	78.26	0.0001
	error	6	2.34000000	0.39000000		
	TRT	2	127.5211111	63.7605556	90.80	0.0001
	Error	8	5.6177778	0.7022222		

Dependent variable: CD19

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
	TRT	2	110.7488889	55.3744444	68.84	0.0001
	error	6	4.8266667	0.8044444		
	TRT	2	113.0955556	56.5477778	107.60	0.0001
	error	6	3.1533333	0.5255556		
	TRT	2	223.8211111	111.9105556	167.80	0.0001
	Error	8	5.3355556	0.6669444		

Dependent variable: CD25

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
	TRT	2	111.3355556	55.6677778	360.44	0.0001
	error	6	0.9266667	0.1544444		
	TRT	2	113.8200000	56.9100000	437.77	0.0001
	error	6	0.7800000	0.1300000		
	TRT	2	224.9677778	112.4838889	790.90	0.0001
	Error	8	1.1377778	0.1422222		

Analysis 4. The effect of 0, 0.1, 0.2, or 0.4% glycerol on *Colletotrichum coccodes* (measured by colony diameter) when incubated at 24C in the dark.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} level of B.
E_{ij} is the random error associated with the ij^{th} experimental unit.

Dependent variable: CD3.

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	TRT	3	0.89395833	0.29798611	18.58	0.0006
	error	8	0.12833333	0.01604167		
2	TRT	3	1.12229167	0.37409722	149.64	0.0001
	error	8	0.02000000	0.00250000		
pooled		3	1.96750000	0.65583333	60.54	0.0001
	error	12	0.13000000	0.01083333		

Dependent variable: CD7.

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	TRT	3	0.18000000	0.06000000	1.20	0.3700
	error	8	0.40000000	0.05000000		
2	TRT	3	0.24666667	0.08222222	6.58	0.0149
	error	8	0.10000000	0.01250000		
pooled		3	0.40333333	0.13444444	5.69	0.0116
	error	12	0.28333333	0.02361111		

Dependent variable: CD15.

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	TRT	3	1.08666667	0.36222222	4.39	0.0419
	error	8	0.66000000	0.08250000		
2	TRT	3	0.55000000	0.18333333	7.86	0.0091
	error	8	0.18666667	0.02333333		
pooled		3	1.54833333	0.51611111	8.39	0.0028
	error	12	0.73833333	0.06152778		

Dependent variable: CD21.

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	TRT	3	0	0	99999.99	0.0
	error	8	0	0		
2	TRT	3	0	0	99999.99	0.0
	error	8	0	0		
pooled		3	0	0	99999.99	0.0
	error	12	0	0		

Analysis 5. Effect of polyethylene glycol (PEG) on growth of *Colletotrichum coccodes* (measured by colony diameter) after 6, 9, 12 and 14 days of incubation at 24C in the dark.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} level of B.
E_{ij} is the random error associated with the ij^{th} experimental unit.

Dependent Variable: colony diameter after 6 days (CD6).

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	PEG	3	0.18916667	0.06305556	1.48	0.2908
error		8	0.34000000	0.04250000		
2	PEG	3	0.19000000	0.06333333	6.33	0.0166
error		8	0.08000000	0.01000000		
pooled	TRT	3	0.27125000	0.09041667	5.76	0.0112
error		12	0.18833333	0.01569444		

Dependent Variable: CD9

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	PEG	3	0.38333333	0.12777778	3.07	0.0911
error		8	0.33333333	0.04166667		
2	PEG	3	0.21666667	0.07222222	1.73	0.2373
error		8	0.33333333	0.04166667		
pooled	TRT	3	0.47666667	0.15888889	5.53	0.0128
error		12	0.34500000	0.02875000		

Dependent Variable: CD12

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	PEG	3	1.16250000	0.38750000	3.14	0.0868
error		8	0.98666667	0.12333333		
2	PEG	3	0.90916667	0.30305556	4.91	0.0319
error		8	0.49333333	0.06166667		
pooled	TRT	3	1.94333333	0.64777778	6.62	0.0069
error		12	1.17333333	0.09777778		

Dependent Variable: CD14.

Experiment	Source	DF	Anova SS	Mean Square	F Value	Pr > F
1	PEG	3	1.81666667	0.60555556	3.80	0.0580
error		8	1.27333333	0.15916667		
2	PEG	3	1.23666667	0.41222222	3.30	0.0787
error		8	1.00000000	0.12500000		
pooled	TRT	3	3.01000000	1.00333333	5.56	0.0126
error		12	2.16666667	0.18055556		

Analysis 6. Effect of germination stimulants on germination and appressoria formation of fresh and formulated conidia after 3, 6 and 10 hours of incubation.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} level of B.
E_{ij} is the random error associated with the ij^{th} experimental unit.

Prep. 1.
Dependent variable: germination after 3 hours (G3).

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.67835238	0.33917619	29.06	0.0001
TRT	20	0.63674286	0.03183714	2.73	0.0034
Error	40	0.46684762	0.01167119		

Dependent variable: appressoriation after 3 hours (A3).

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.00854603	0.00427302	7.29	0.0020
TRT	20	0.01490794	0.00074540	1.27	0.2530
Error	40	0.02345397	0.00058635		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	27368.66667	13684.33333	64.78	0.0001
TRT	20	18145.33333	907.26667	4.29	0.0001
Error	40	8450.0000	211.25000		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.57842222	0.28921111	29.37	0.0001
TRT	20	0.33628889	0.01681444	1.71	0.0740
Error	40	0.39384444	0.00984611		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	26027.65079	13013.82540	78.76	0.0001
TRT	20	18145.33333	907.26667	5.44	0.0001
Error	40	6609.68254	165.24206		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	1.88669524	0.94334762	39.75	0.0001
TRT	20	1.26575238	0.06328762	2.67	0.0041
Error	40	0.94923810	0.02373095		

Prep. IV.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.54862222	0.27431111	18.28	0.0001
TRT	20	0.33470794	0.01673540	1.12	0.3731
Error	40	0.60031111	0.01500778		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.00402222	0.00201111	8.83	0.0007
TRT	20	0.00543175	0.00027159	1.19	0.3094
Error	40	0.00911111	0.00022778		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.50402222	0.25201111	9.84	0.0003
TRT	20	2.07950794	0.10397540	4.06	0.0001
Error	40	1.02484444	0.02562111		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.10932381	0.05466190	5.08	0.0108
TRT	20	0.35661905	0.01783095	1.66	0.0856
Error	40	0.43014286	0.01075357		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.38303810	0.19151905	5.34	0.0088
TRT	20	1.91146667	0.09557333	2.66	0.0041
Error	40	1.43529524	0.03588238		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.17946667	0.08973333	2.89	0.0674
TRT	20	1.24754286	0.06237714	2.01	0.0301
Error	40	1.24313333	0.03107833		

Prep. VII.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.29640317	0.14820159	58.50	0.0001
TRT	20	0.09666032	0.00483302	1.91	0.0406
Error	40	0.10133016	0.00253325		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.00028889	0.00014444	2.83	0.0711
TRT	20	0.00259365	0.00012968	2.54	0.0060
Error	40	0.00204444	0.00005111		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	1.57966032	0.78983016	44.98	0.0001
TRT	20	0.75886032	0.03794302	2.16	0.0188
Error	40	0.70240635	0.01756016		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.17258413	0.08629206	30.47	0.0001
TRT	20	0.10172698	0.00508635	1.80	0.0569
Error	40	0.11328254	0.00283206		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	2.54762857	1.27381429	50.85	0.0001
TRT	20	1.25228571	0.06261429	2.50	0.0067
Error	40	1.00197143	0.02504929		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.60091746	0.30045873	28.67	0.0001
TRT	20	0.53128889	0.02656444	2.54	0.0060
Error	40	0.41914921	0.01047873		

Fresh conidia.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.38564127	0.19282063	21.17	0.0001
TRT	20	0.58737460	0.02936873	3.22	0.0008
Error	40	0.36429206	0.00910730		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.00046984	0.00023492	1.96	0.1543
TRT	20	0.00224127	0.00011206	0.93	0.5515
Error	40	0.00479683	0.00011992		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	1.24206667	0.62103333	23.33	0.0001
TRT	20	3.68614286	0.18430714	6.92	0.0001
Error	40	1.06473333	0.02661833		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.52018095	0.26009048	11.20	0.0001
TRT	20	0.83017143	0.04150857	1.79	0.0583
Error	40	0.92881905	0.02322048		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.18604127	0.09302063	5.45	0.0081
TRT	20	4.12990794	0.20649540	12.09	0.0001
Error	40	0.68322540	0.01708063		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	2	0.54388571	0.27194286	11.04	0.0002
TRT	20	3.62653333	0.18132667	7.36	0.0001
Error	40	0.98518095	0.02462952		

Analysis 7. Effect of different cutin concentrations on germination and appressoriation of fresh and formulated conidia after 3, 6 and 10 hours of incubation.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} level of B.
E_{ij} is the random error associated with the ij^{th} experimental unit.

Prep. I.
Dependent variable: germination after 3 hours (G3).

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	52.5625000	52.5625000	3.23	0.1154
TRT	7	331.4375000	47.3482143	2.91	0.0911
Error	7	113.9375000	16.2767857		

Dependent variable: appressoriation (A3).

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	16.00000000	16.00000000	8.62	0.0219
TRT	7	13.00000000	1.85714286	1.00	0.5000
Error	7	13.00000000	1.85714286		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	18.0625000	18.0625000	0.23	0.6452
TRT	7	862.9375000	123.2767857	1.58	0.2807
Error	7	546.4375000	78.0625000		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	18.0625000	18.0625000	0.59	0.4687
TRT	7	555.4375000	79.3482143	2.58	0.1173
Error	7	215.4375000	30.7767857		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	612.562500	612.562500	11.88	0.0107
TRT	7	1815.937500	259.419643	5.03	0.0245
Error	7	360.937500	51.562500		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	162.5625000	162.5625000	6.74	0.0357
TRT	7	257.4375000	36.7767857	1.52	0.2960
Error	7	168.9375000	24.1339286		

Prep. IV.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	1.00000000	1.00000000	0.70	0.4304
TRT	7	30.00000000	4.28571429	3.00	0.0852
Error	7	10.00000000	1.42857143		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	0.06250000	0.06250000	1.00	0.3506
TRT	7	0.43750000	0.06250000	1.00	0.5000
Error	7	0.43750000	0.06250000		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	5.0625000	5.0625000	0.26	0.6260
TRT	7	965.4375000	137.9196429	7.08	0.0097
Error	7	136.4375000	19.4910714		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	7.5625000	7.5625000	0.69	0.4342
TRT	7	219.4375000	31.3482143	2.85	0.0951
Error	7	76.9375000	10.9910714		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	0.062500	0.062500	0.00	0.9830
TRT	7	3416.937500	488.133929	3.82	0.0488
Error	7	893.437500	127.633929		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	60.062500	60.062500	2.89	0.1329
TRT	7	1010.937500	144.419643	6.95	0.0102
Error	7	145.437500	20.776786		

Prep.VII.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	64.00000000	64.00000000	12.44	0.0096
TRT	7	67.00000000	9.57142857	1.86	0.2156
Error	7	36.0000000	5.1428571		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	0.25000000	0.25000000	2.33	0.1705
TRT	7	0.75000000	0.10714286	1.00	0.5000
Error	7	0.75000000	0.10714286		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	715.5625000	715.5625000	34.56	0.0006
TRT	7	718.4375000	102.6339286	4.96	0.0255
Error	7	144.937500	20.705357		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	18.06250000	18.06250000	9.41	0.0181
TRT	7	88.93750000	12.70535714	6.62	0.0117
Error	7	13.4375000	1.9196429		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	1425.062500	1425.062500	20.72	0.0026
TRT	7	1592.937500	227.562500	3.31	0.0685
Error	7	481.437500	68.776786		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	22.5625000	22.5625000	1.34	0.2851
TRT	7	369.9375000	52.?482143	3.14	0.0773
Error	7	117.9375000	16.8482143		

Fresh conidia.
Dependent variable: G3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	451.5625000	451.5625000	9.97	0.0160
TRT	7	984.9375000	140.7053571	3.11	0.0789
Error	7	316.937500	45.276786		

Dependent variable: A3.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	0.06250000	0.06250000	1.00	0.3506
TRT	7	0.43750000	0.06250000	1.00	0.5000
Error	7	0.43750000	0.06250000		

Dependent variable: G6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	4.000000	4.000000	0.02	0.8906
TRT	7	4550.000000	650.000000	3.31	0.0686
Error	7	1376.000000	196.571429		

Dependent variable: A6.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	42.250000	42.250000	3.06	0.1239
TRT	7	1449.000000	207.000000	14.98	0.0010
Error	7	96.750000	13.821429		

Dependent variable: G10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	33.062500	33.062500	2.33	0.1709
TRT	7	2877.937500	411.133929	28.94	0.0001
Error	7	99.437500	14.205357		

Dependent variable: A10.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLK	1	25.000000	25.000000	2.46	0.1604
TRT	7	3841.000000	548.714286	54.10	0.0001
Error	7	71.000000	10.142857		

Analysis 8. Effect of some germination stimulants (treatments) on germination/appressoriation of *Colletotrichum coccodes* over time.

Statistical model : $Y_{ij} = \mu + \tau_j + E_{ij}$

Definitions: Y_{ij} is the observation associated with the j^{th} treatment.
μ is the overall mean.
τ_j is the effect due to the j^{th} treatment.
E_{ij} is the random error associated with the ij^{th} experimental unit.

(A) Treatment : water agar (WA, t_6).

a) germination

		Prep. I				Prep. IV				Prep. VII				Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	3525.73423	98.112	0.0001	1	5173.90637	67.804	0.0001	1	1149.90773	95.862	0.0001	1	2179.31375	0.0001	
error	8	35.93582			8	76.30666			8	11.99549			8	25.88778		

Dependent variable	Regression equation[a]	R^2[b]	Pr[c]
germination of conidia in Prep. I	y = 3.777200 + 6.537291 X	0.9246	0.0001
germination of conidia in Prep. IV	y = -5.355000+ 7.919218 X	0.8945	0.0001
germination of conidia in Prep. VII	y = 0.067000 + 3.733400 X	0.9230	0.0001
germination of fresh conidia	y = -10.534333 + 5.139642 X	0.9132	0.0001

[a] y is the dependent variable and X is time.
[b] Coefficient of determination
[c] Level of significance

b) appressoriation

		Prep. I				Prep. IV				Prep. VII				Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	1081.66814	48.366	0.0001	1	1214.56718	62.844	0.0001	1	350.92629	83.648	0.0001	1	2659.03585	78.909	0.0001
error	8	22.36433			8	19.32682			8	4.19526			8	33.69750		

Dependent variable	Regression equation	R^2	Pr
appressoriation of conidia in Prep. I	y = -6.271778 + 3.620929 X	0.8581	0.0001
appressoriation of conidia in Prep. IV	y = -5.059778 + 3.836929 X	0.8871	0.0001
appressoriation of conidia in Prep. VII	y = -4.263111 + 2.062438 X	0.9127	0.0001
appressoriation of fresh conidia	y = -13.991333 + 5.677212 X	0.9079	0.0001

(B) Treatment : WA + 10^{-3}M asparagine (t_3).

a) germination.

	Prep. I			Prep. IV			Prep. VII			Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	4973.09821	76.412	0.0001	1	4859.53754	40.014	0.0002	1	3169.14896	83.648	0.0001	1	10782.89106	141.636	0.0001
error	8	65.08277			8	121.44520			8	12.56533			8	76.13112		

Dependent variable	Regression equation	R^2	Pr
germination of conidia in Prep. I	y = 1.131200 + 7.764018 X	0.9052	0.0001
germination of conidia in Prep. IV	y = -3.711133 + 7.674861 X	0.8334	0.0002
germination of conidia in Prep. VII	y = -1.265467 + 3.805594 X	0.9304	0.0001
germination of fresh conidia	y = -10.044667 + 11.432485 X	0.9465	0.0001

b) appressoriation

	Prep. I			Prep. IV			Prep. VII			Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	1551.48587	27.386	0.0008	1	2480.62710	62.681	0.0001	1	278.50299	221.359	0.0001	1	7245.21208	208.035	0.0001
error	8	56.65235			8	39.57569			8	1.25815			8	34.82694		

Dependent variable	Regression equation	R^2	Pr
appressoriation of conidia in Prep. I	y = -8.979867 + 4.336576 X	0.7739	0.0008
appressoriation of conidia in Prep. IV	y = -10.555267 + 5.483448 X	0.8868	0.0001
appressoriation of conidia in Prep. VII	y = -3.559333 + 1.837333 X	0.9651	0.0001
appressoriation of fresh conidia	y = -18.666000+ 9.371273 X	0.9630	0.0001

(C) Treatment : WA + $2*10^{-3}$M rhamnose (t_{16}).

a) germination.

Source	DF	Prep. I MS	Prep. I F Value	Prep. I Prob>F	DF	Prep. IV MS	Prep. IV F Value	Prep. IV Prob>F	DF	Prep. VII MS	Prep. VII F Value	Prep. VII Prob>F	DF	Fresh conidia MS	Fresh conidia F Value	Fresh conidia Prob>F
model	1	4926.32182	48.366	0.0001	1	4527.54736	62.844	0.0001	1	3169.14896	83.648	0.0001	1	9684.30677	78.909	0.0001
error	8	45.98481			8	53.83289			8	12.56533			8	64.54127		

Dependent variable	Regression equation	R^2	Pr
germination of conidia in Prep. I	y = 0.400200 + 7.727418 X	0.9305	0.0001
germination of conidia in Prep. IV	y = -9.445333 + 7.408061 X	0.9131	0.0001
germination of conidia in Prep. VII	y = -3.422133 + 6.197897 X	0.9693	0.0001
germination of fresh conidia	y = -6.557533 + 10.834461 X	0.9494	0.0001

b) appressoriation.

Source	DF	Prep. I MS	Prep. I F Value	Prep. I Prob>F	DF	Prep. IV MS	Prep. IV F Value	Prep. IV Prob>F	DF	Prep. VII MS	Prep. VII F Value	Prep. VII Prob>F	DF	Fresh conidia MS	Fresh conidia F Value	Fresh conidia Prob>F
model	1	1650.36927	105.118	0.0001	1	1724.10580	93.759	0.0001	1	904.47415	153.393	0.0001	1	6388.52720	192.388	0.0001
error	8	15.70014			8	18.38867			8	5.89643			8	33.20642		

Dependent variable	Regression equation	R^2	Pr
appressoriationof conidia in Prep. I	y = -8.466800 + 4.472636 X	0.9293	0.0001
appressoriation of conidia in Prep. IV	y = -10.110733 + 4.571461 X	0.9214	0.0001
appressoriation of conidia in Prep. VII	y = -6.378000 + 3.311091 7 X	0.9504	0.0001
appressoriation of fresh conidia	y = -19.599267 + 8.799812 X	0.9601	0.0001

(D) Treatment: WA + 1% Sorbo (t_{11}).
- appressoriation

		Prep. I				Prep. IV				Prep. VII				Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	1079.89702	123.745	0.0001	1	2309.40246	116.543	0.0001	1	1092.01483	189.140	0.0001	1	5335.73833	120.891	0.0001
error	8	8.72682			8	19.81582			8	5.77359			8	44.13681		

Dependent variable	Regression equation	R^2	Pr
appressoriationof conidia in Prep. I	y = -8.265800 + 3.617964 X	0.9393	0.0001
appressoriation of conidia in Prep. IV	y = -10.999200 + 5.290818X	0.9358	0.0001
appressoriation of conidia in Prep. VII	y = -7.377533 + 3.638206 X	0.9594	0.0001
appressoriation of fresh conidia	y = -17.065933 + 8.042115 X	0.9379	0.0001

E) Treatment: WA + 10^{-3}M rhamnose (t_{11}).
- germination.

		Prep. I				Prep. IV				Prep. VII				Fresh conidia		
Source	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F	DF	MS	F Value	Prob>F
model	1	4552.56922	51.521	0.0001	1	2915.89468	143.206	0.0001	1	1325.16103	66.984	0.0001	1	10390.21415	67.591	0.0001
error	8	88.36262			8	20.36157			8	19.78327			8	153.72218		

Dependent variable	Regression equation	R^2	Pr
germination of conidia in Prep. I	y = 3.976533 + 7.428503 X	0.8656	0.0001
germination of conidia in Prep. IV	y = -9.132333 + 5.945097 X	0.9471	0.0001
germination of conidia in Prep. VII	y = 1.624333 + 4.007812 7 X	0.8933	0.0001
germination of fresh conidia	y = -6.089133 + 11.222388X	0.8942	0.0001