

## Use of Pregelatinized Starch and Casamino Acids to Improve the Efficacy of *Colletotrichum truncatum* Conidia Produced in Differing Nutritional Environments

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*Colletotrichum truncatum* NRRL 13737 is being studied in a cooperative effort of U.S. Department of Agriculture researchers to investigate the potential of the fungus as a biological control agent against the weed, *Sesbania exaltata*. We have found that nutritional and environmental factors present at the time of inoculum production and host infection influence the ultimate effectiveness of the bioherbicide. In the present study, conidial efficacy was evaluated after providing amendments to spores of *C. truncatum* that had been produced in media with differing carbon to nitrogen (C:N) ratios. Amending aqueous suspensions of conidia with pregelatinized starch reduced appressoria formation on cellophane membranes regardless of the C:N ratio of the medium in which conidia were produced, while Casamino acids increased appressoria formation. In seedling assays, conidia produced in a medium with a C:N ratio of "10:1" incited more severe disease than did spores produced in "30:1" medium, though amending with pregelatinized starch increased the efficacy of "30:1" spores to levels comparable to "10:1" spores without amendment. Casamino acids increased the severity of disease incited by "10:1" spores but generally did not influence "30:1" spore efficacy. The importance of spore production protocols in the development of maximally effective formulations of bioherbicide propagules is discussed.

### Introduction

The feasibility of increasing the effectiveness of biological control agents using specialized delivery systems and/or nutrient amendments has been successfully demonstrated (Walker and Connick 1983, Lewis *et al.* 1991). However, research on delivery systems for aerially applied bioherbicides has been largely restricted to invert emulsions (Connick *et al.* 1991) or agents that apparently enhance the viscosity of aqueous spore suspensions (Daigle and

Connick 1990). Few studies have evaluated the impact of nutrient amendments on bioherbicide efficacy, although amendments have been shown to impact the formation of appressoria in studies of fungal pathogens of agronomic crops (Mercer *et al.* 1970, Harper *et al.* 1980). Additionally, an increase in disease severity has frequently been attributed to an increased availability of nutrients, such as amino acids, to fungal pathogens (Cook and Baker 1983).

Pregelatinized water-dispersible starches have utility as food additives and are effective as

<sup>3</sup> The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

encapsulating agents in sprayable systems for agricultural chemicals (Trimnell and Shasha 1988). McGuire and Shasha (1990) recently demonstrated that an aqueous suspension of sucrose and pregelatinized starch ("Mira-spense", A.E. Staley Company, Decatur, IL, USA) was effective in encapsulating and sticking the microbial insecticide *Bacillus thuringiensis* subsp. *kurstaki* to aerial plant parts. After drying, this formulation strongly resisted washoff during repeated simulated rainfall events. Additionally, this material maintained the effectiveness of the bioinsecticide and was amenable to the addition of UV protectants which could further protect the viability of biological control agents. Whether this material could be effectively employed with propagules of bioherbicidal agents has not been evaluated.

*Colletotrichum truncatum* (Schw.) Andrus & Moore has considerable potential for development as a bioherbicide against the weed *Sesbania exaltata* (Raf.) V.L. Cory (Boyette 1991). Submerged culture studies (Jackson and Bothast 1990) with *C. truncatum* showed that the carbon concentration and the carbon:nitrogen (C:N) ratio of the conidiation medium significantly influenced the number of conidia produced. Media with a C:N ratio of 30:1 produced the highest number of conidia. However, conidia produced in a medium with a C:N ratio of 10:1 are more effective in inciting disease than are conidia produced in media with C:N ratios of 30:1 or 80:1 (Schisler *et al.* 1991a). The purpose of the present study was to determine if sucrose, pregelatinized starch (Mira-spense) and Casamino acids would differentially impact germination and appressoria formation by conidia of *C. truncatum* that had been produced in media with C:N ratios of 30:1 and 10:1. Additionally, we evaluated the effect of these amendments on the ability of 10:1 and 30:1 conidia to induce disease symptoms on *S. exaltata*.

## Materials and Methods

### *Production and Harvest of Conidia*

*C. truncatum* isolate NRRL 13737 (ARS patent culture collection 18434) was used in all studies. The fungus was stored in 10% glycerol at -80 C

on colonized 2-mm-dia potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) plugs until needed. Conidia of the fungus were produced in liquid culture (Schisler *et al.* 1991a). A defined basal salts medium containing macro- and micronutrients and vitamins was amended with differing levels of Casamino acids and glucose to produce media with a total carbon content of 4 g/l and carbon:nitrogen ratios of 10:1 and 30:1. Casamino acids were added to the basal medium before autoclaving while sterile, autoclaved glucose was added to cooled basal salts medium after autoclaving. The final pH of media was adjusted to 5.0 before autoclaving. Media were dispensed in 500-ml flasks (250 ml/flask) having baffles (Bellco, Inc., Vineland, NJ). Liquid media were inoculated, incubated and conidia harvested as previously described except that conidial solutions were maintained at 25°C and were immediately used after harvest. Cooling aqueous suspensions of *C. truncatum* conidia to near 0°C adversely impacts subsequent conidial performance (Jackson and Schisler 1995).

### *Assay of Amendment Influence on Conidial Germination in vitro*

Solutions of 2% sucrose (w/v) (Difco), pregelatinized starch (PGS) and vitamin free Casamino acids (Difco) were prepared 15 min before use by adding each ingredient separately to sterile 0.08% solutions of Tween 80 (Sigma Chemicals, St. Louis, MO, USA). Immediately prior to use, solutions containing amendments were combined with an equal volume of a conidial suspension composed of spores produced in either 10:1 or 30:1 C:N media ("10:1 spores" or "30:1 spores") to produce 1% solutions of each amendment containing  $5 \times 10^5$  conidia/ml. One-percent amendment solutions were determined to be optimally effective in preliminary *in vitro* assays of conidial germination. Control solutions contained the same concentration of 10:1 or 30:1 conidia suspended in 0.04% solutions of Tween 80. Treatment solutions were then misted onto precut sterile cellophane membrane backing (Bio-Rad Laboratories, Richmond, CA, USA) which had been placed in 9-cm-dia Petri dishes containing 2% water agar (Difco, Bacto-agar).

Dishes were completely randomized and stored in the dark at 24-25°C. The membranes were sprayed with Carnoy's fluid after 6 h to arrest spore germination. Six dishes from each treatment were then examined microscopically with 100 conidia/plate being evaluated for germination and formation of appressoria. The normality of the data permitted analysis without transformation, using a 2 x 4 factorial analysis of variance.

#### *Efficacy of Amended Conidia in Inciting Disease Symptoms on S. exaltata*

Seedlings of *S. exaltata* were grown for 2 wks as previously described (Schisler *et al.* 1991a), except that seedlings were incubated at 25°C in a plant growth chamber with 14 h of fluorescent and incandescent light (Controlled Environment Chambers, G10, Chagrin Falls, Ohio, USA). Seedlings were blocked by size immediately prior to experimental treatment. Conidia and treatment solutions were prepared as described for membrane studies. Four additional control treatments consisted of separate solutions of each individual amendment and no amendment, combined with a sterile solution of Tween 80 containing heat-killed 10:1 and 30:1 spores in equivalent amounts (final concentration of total conidia =  $5 \times 10^5$ /ml; final amendment concentration = 1%). Treatment solutions were sprayed onto 10 seedlings/treatment until runoff. Inoculated seedlings were immediately placed in a dew chamber without lights at 26-27°C. After 6 h, seedlings were removed, arranged using a randomized complete block design, and placed back in the growth chamber for 1 wk. Plants were then harvested and scored for shoot dry weight and number of leaves remaining attached per seedling to determine the relative severity of disease. Use of the described conditions and conidial concentrations resulted in moderate, sublethal levels of seedling disease which permitted the accurate separation of treatment effects. Analysis of variance was performed on the entire dataset and on a 2 x 4 factorial data set which resulted from the removal of data from control treatments. This and all other experiments were repeated at least once.

## Results and Discussion

For the dependent variables of appressoria formation, number of leaves and shoot dry weight, there were significant spore type x amendment interactions which precluded the pooling of data sets to obtain overall spore type and amendment effects. For seedling assays, blocking did not interact with spore type or amendment variables.

#### *Conidiation Medium Influence on Spore Germination and Seedling Disease*

Conidia produced in the 10:1 C:N ratio medium germinated more frequently than those produced in 30:1 C:N medium (94% vs. 81%;  $LSD (.01) = 7$ ). Conidia from 10:1 medium formed more appressoria than 30:1 conidia (Table 1A) and often incited more severe symptoms on the weed host (Tables 1B,C) with the magnitude of the difference being amendment dependent. Schisler *et al.* (1991a) demonstrated that conidia produced in 10:1 medium differed from 30:1 and 80:1 conidia in morphology, nuclear number, germination rate and efficacy in inciting symptoms of disease on *S. exaltata* seedlings. Recently, other studies have reported on the importance of the biomass production medium in producing effective biological control agents of insects (Lane *et al.* 1991) and plant pathogens (Harman *et al.* 1991).

#### *Influence of Amendments on Conidial Germination and on Growth of Control Seedlings*

Conidial germination was not significantly impacted by amendments, with 85-90% of the spores germinated on membranes in all treatments. For 10:1 spores, Casamino acids and sucrose increased, while PGS decreased, appressoria formation (Table 1A). Amendments similarly influenced appressoria formation by 30:1 spores except that sucrose had no effect when compared to the control (Table 1A). None of the amendment solutions containing heat-killed 10:1 and 30:1 conidia influenced the number of leaves/seedling ( $x = 5.5-5.7$ ) while PGS was found to slightly reduce the shoot dry

weight of treated seedlings when compared to the control ( $PGS = 113$  mg/seedling, Control = 136 mg/seedling,  $LSD (.05) = 22$ ). This effect was at least partially attributable to *PGS* solutions causing some leaflets to adhere to adjacent leaflets for a few days after treatment.

#### *Influence of Amendments on Disease Severity*

Casamino acid and *PGS* amendments increased the efficacy of 10:1 spores in inciting disease as determined by a reduced number of leaves (Table 1B) and reduced dry weight/seedling 7 d after infection (Table 1C) while sucrose had no effect compared to controls. Similar results were found for 30:1 spores except *PGS* was the only amendment that increased the efficacy of 30:1 spores compared to control seedlings treated with unamended 30:1 spores. *PGS* may have influenced the level of disease initiated by both 10:1 and 30:1 spores in a number of ways. *PGS* likely induced slight physiological stress within treated seedlings as evidenced by a small loss of shoot dry weight in these seedlings. A wide variety of plant stresses have been reported to increase host susceptibility to pathogens (Schoeneweiss 1975). Additionally, the moisture holding capabilities of *PGS* may have allowed more conidia to germinate and penetrate plant tissues after the removal of plants from the dew chamber. It is noteworthy that *PGS* hindered, rather than stimulated, appressoria formation by both spore types *in vitro*. Furthermore, sucrose and Casamino acids stimulated appressoria formation by 10:1 and 30:1 conidia *in vitro*, respectively, yet in neither case did these combinations increase seedling disease. Successful formation of appressoria does not necessarily infer that the pathogen will successfully penetrate plant host tissues (Suzuki *et al.* 1982, Williamson and Fokkema 1985). Alternatively, differences in chemical, nutrient, topographical and microbial conditions on the phylloplane could radically alter conidial behavior from that exhibited *in vitro*. Preliminary results using epifluorescent microscopy confirm that *PGS* treated spores more readily form appressoria *in situ* than *in vitro* (Schisler, unpublished results). Caution in

using *in vitro* assays to predict the efficacy of spore types and amendments is warranted.

The sucrose amendment did not affect the level of disease induced by either spore type in plant growth cabinet assays (Table 1B,C). However, biological control agents adhere more readily to plant surfaces and resist washoff when delivered in a solution of *PGS* and sucrose (McGuire and Shasha 1991). Sucrose may positively impact the effectiveness of a *PGS* formulation of *C. truncatum* in field tests or in growth cabinet assays which include simulated rainfall. Casamino acids, which aided 10:1, but not 30:1, spores in inducing plant disease, contain a variety of amino acids. Amino acids have been demonstrated to promote vegetative growth subsequent to conidial germination for several *Colletotrichum* spp. (Emmett and Parbery 1975). Spores produced in 10:1 medium contain a higher percentage of protein while 30:1 spores have a higher percentage of lipid (Jackson and Schisler 1991). The reason for the differential impact of Casamino acids on these 2 spore types remains open to speculation.

#### **Conclusion**

These results, as well as those of other studies (Harman *et al.* 1991, Lane *et al.* 1991, Schisler *et al.* 1991a) show that developing media solely to maximize biomass production of biological control agents is not justified in light of the impact of medium environment on the effectiveness, durability during formulation and shelf-life of the biomass produced. Additionally, we have found that both nutrient amendments and pregelatinized starch have considerable potential for incorporation into sprayable delivery systems for *C. truncatum* and possibly for other bioherbicide agents. Pregelatinized starch sequesters water, sticks to plant surfaces when combined with sucrose, and can be amended with UV protectants, all features which would increase its utility as a formulating agent for aerially applied bioherbicide agents. We continue studies directed towards optimizing bioherbicide shelf-life and efficacy by adjusting the liquid conidiation environment and conidial formulations. We concur with Van Dyke (1989) that formulation research must integrate, among

**Table 1. Effect of amendments of 10:1 and 30:1 conidia of *C. truncatum* on: A. the frequency of appressoria formation after 6 h on cellophane membranes; B. the number of leaves remaining attached 7 d after inoculation of *S. exaltata* seedlings; and C. shoot dry weights (mg) of *S. exaltata* seedlings 7 d after inoculation.**

Amendments <sup>a</sup>	CN = 10:1 <sup>b</sup>	CN = 30:1 <sup>b</sup>	LSD <sup>c</sup>
<b>A. % Appressoria formation</b>			
SUC	66	7	8
PGS	12	0	12
CAS	67	24	9
Con	42	11	12
LSD <sup>c</sup>	11	7	
<b>B. Number of leaves/seedling remaining attached 7d after inoculation</b>			
SUC	4.2	4.6	0.7
PGS	2.2	2.8	1.3
CAS	2.2	4.6	0.4
Con	3.5	4.4	1.0
LSD	0.9	1.0	
<b>C. Shoot dry weight</b>			
SUC	93	104	26
PGS	40	52	14
CAS	52	100	29
Con	70	90	22
LSD	23	20	

<sup>a</sup>Aqueous 1% amendment suspensions (wt/vol) in 0.04% Tween 80 and containing  $5 \times 10^8$  conidia per ml. SUC = sucrose, PGS = pregelatinized starch, CAS = Casamino acids.

<sup>b</sup>Conidia produced in liquid medium with listed carbon:nitrogen ratio (10:1 or 30:1).

<sup>c</sup>LSD(0.05) values for comparing row or column entries are listed at the end of rows and columns, respectively.

other fields, knowledge of fungal biology and phylloplane microbial ecology (Schisler *et al.* 1991b) if researchers wish to succeed in producing reliable and effective bioherbicide products.

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