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Effects of carbon concentration and carbon-to-nitrogen ratio on growth, conidiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccodes*

X Yu[1], SG Hallett[1], J Sheppard[2] and AK Watson[1]

[1]*Department of Plant Science;* [2]*Department of Agricultural and Biosystems Engineering, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Québec, Canada H9X 3V9*

The effect of carbon concentration and carbon-to-nitrogen ratio (C:N) as well as their interaction on *Colletotrichum coccodes* growth and sporulation in submerged flask culture were evaluated. When C:N ratios were held constant, both mycelial dry biomass and spore yield increased with increasing carbon concentration. The specific spore yields (spore yield g^{-1} carbon), however, were not significantly different for the same C:N ratio in most cases. The highest spore yields (1.3×10^8 spores per ml) were obtained from media containing 20 g per liter carbon with C:N ratios ranging from 5:1 to 10:1. When the C:N ratio was greater than 15:1, spore yields were significantly decreased with increasing C:N ratios. High carbon concentration (20 g L^{-1}) combined with high C:N ratios (above 15:1) reduced both mycelial growth and sporulation, and increased spore matrix production. Spores produced in medium containing 10 g L^{-1} carbon with C:N ratios from 10:1 to 15:1 had 90% germination on potato dextrose agar after 12 h and caused extensive shoot dry weight reduction on the target weed, velvetleaf. These results suggest that C:N ratios from 10:1 to 15:1 are optimal for *C. coccodes* spore production.

Keywords: carbon concentration; *Colletotrichum coccodes*; conidiation; C:N ratio; mycoherbicide

Introduction

Production of fungal plant pathogens as mycoherbicides to control weeds is a relatively new approach for weed control. Mycoherbicides may provide alternative and complementary weed control tools in integrated weed management systems. Three commercial products have been developed: Collego™ (*Colletotrichum gloeosporioides* (Penz) Penz. & Sacc. fsp *aeschynomene*) for control of northern jointvech (*Aeschynomene virginica* (L.) BSP) in soybean (*Glycine max* (L.) Merril) and rice (*Oryza sativa* L.) in southeastern United States; Devine™ (*Phytophthora palmivora* (Butl.) Butl. MWV pathotype) for stranglervine (*Morrenia odorata* (H. & L.) Lindl.) in Florida citrus groves; and Lubao 2 (*Colletotrichum gloeosporioides* fsp *cuscutae*) for dodder (*Cuscuta* sp) control in soybeans in China [16,17]. BioMal™ (*Colletotrichum gloeosporioides* f sp *malvae*) has been registered in Canada for control of round-leaved mallow (*Malva pusilla* Sm) but has not been marketed [9]. Many other fungal plant pathogens are being evaluated worldwide as potential bioherbicides [1].

Colletotrichum coccodes (Wallr.) Hughes is a fungal pathogen isolated from foliar lesions on velvetleaf (*Abutilon theophrasti* Medic.) [18]. Velvetleaf is a major weed in corn (*Zea mays* L.) and soybeans in Canada and the United States [14]. Application of *C. coccodes* spore suspensions will kill velvetleaf seedlings in the cotyledon stage [2,18]. In addition, *C. coccodes* treatments significantly reduce growth and competitive ability of older velvetleaf [2] and significantly increase soybean yield [3]. Currently, liquid culture fermentation using Modified Richard's Solution has been used to produce *C. coccodes* spores for greenhouse and field testing to evaluate its potential for velvetleaf control [18]. Unfortunately, this medium is not suitable for large-scale production due to its costly V-8 juice component.

A previous study demonstrated that the V-8 juice can be replaced with soy protein and a new medium has been developed for *C. coccodes* spore production [19]. For the commercial production of a bioherbicide, careful selection of carbon and nitrogen sources as well as mineral supplements is necessary for optimum growth and sporulation of fungi. In addition, the carbon to nitrogen (C:N) ratio is also of major importance in balancing the medium nutrient supply [8]. In this paper, the effects of carbon concentration and carbon-to-nitrogen ratio in shake flask culture on *C. coccodes* growth, sporulation, spore germination, and virulence on velvetleaf are reported.

Materials and methods

Cultures

Stock cultures of *Colletotrichum coccodes* (DAOM 183088, deposited in the Biosystematics Research Institute, Ottawa, Canada) were stored on potato dextrose agar (PDA) slants under mineral oil, or in soil at 4°C. Small pieces of stock culture were transferred to PDA plates and incubated for 7–14 days at 24°C. Small plugs were removed from the plates and placed into 50 ml of Modified Richard's liquid medium, composed of sucrose, 10 g L^{-1}; KNO_3, 10 g L^{-1}; KH_2PO_4, 5.0 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 2.5 g

Correspondence: AK Watson, Department of Plant Science, Macdonald Campus of McGill University, 21 111 Lakeshore Road, Ste-Anne-de-Bellevue, Québec, Canada H9X 3V9
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L^{-1}; $FeCl_3 \cdot 6H_2O$, 0.02 g L^{-1}; V-8 juice 150 ml L^{-1} and distilled water to make the total volume 1 liter, in 250-ml Erlenmeyer flasks. Cultures were incubated for 5 days on a rotary shaker at 250 rpm at room temperature. Spores obtained from this liquid culture were used as initial inoculum.

Media

The basal salts medium for carbon and carbon-to-nitrogen ratio studies was composed of KH_2PO_4, 5.0 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}; $CaCl_2$, 0.5 g L^{-1}, $FeCl_3 \cdot 6H_2O$, 0.05 g L^{-1}; $CuSO_4$, 0.05 g L^{-1}. Stock solutions of sucrose (10%, w/v), KNO_3 (10%, w/v) and soy protein (ICN Biomedicals, Amsterdam) (5%, w/v) were added to the basal salts medium as required to obtain desired carbon and carbon-to-nitrogen ratios.

In each treatment, the supplemented nitrogen was composed of 50% nitrogen from KNO_3 and 50% from soy protein. Molecular carbon concentration and carbon-to-nitrogen ratio were calculated based on the presence of carbon and nitrogen in sucrose (42% carbon, KNO_3 (14% nitrogen) and soy protein. According to the supplier, soy protein contains 92% protein, which is approximately 52% elemental carbon and 16% nitrogen. Carbon concentrations of 5 g L^{-1}, 10 and 20 g L^{-1} were tested. Carbon-to-nitrogen ratios of 5:1, 7.5:1, 10:1, 15:1, 20:1, 30:1 and 40:1 were tested at each carbon concentration. The exact amounts of sucrose, soy protein and KNO_3 for a carbon concentration of 5 g L^{-1} along with various carbon to nitrogen ratios, are listed in Table 1. For carbon concentrations of 10 g L^{-1} and 20 g L^{-1}, the amount of sucrose, KNO_3 and soy protein were two-fold and four-fold of that in the concentration of 5 g L^{-1}, respectively. During the studies, the initial pH was adjusted to 5.5 by addition of 0.5 N NaOH or 0.5 N HCl.

Growth and sporulation

Experiments were conducted in 250-ml Erlenmeyer flasks with 50 ml of medium inoculated with 2.5 ml of a spore suspension (2×10^7 spores per ml) in each flask. The cultures were incubated at 24°C on a rotary shaker at 250 rpm for 5 days. Spores were separated from mycelium by filtering the culture through four layers of cheese cloth in a funnel. Spore suspensions were centrifuged at $65\,000 \times g$ for 10 min. The supernatant medium was discarded, the pellets were washed with distilled water, centrifuged a second time under the same conditions, and then the pellets were resuspended in distilled water to the original volume. Spore concentrations were determined microscopically with the aid of a haemocytometer. Mycelium biomass was determined by removing the mycelium from cheese cloth and drying at 60 ± 1°C for 24 h. Germination tests were conducted by spraying conidia onto 1-cm diameter PDA plugs and incubating them in petri dishes with moist filter paper for 12 h at room temperature. After incubation, spores were stained with a fast green solution (0.2% w/v), and germinating spores were counted microscopically. Specific spore yield and specific mycelial biomass yield were calculated as follows:

Specific spore yield (Y_{sp}):
Y_{sp} (spores g^{-1}) = Spore concentration (spores L^{-1})/initial carbon concentration (g L^{-1});
Specific mycelial biomass yields (Y_{ms}):
Y_{ms} (g g^{-1}) = mycelial dry weight (g L^{-1})/initial carbon concentration (g L^{-1}).

A completely randomized design with three replicates per treatment was used in all experiments. All treatments were performed three times and data were pooled based on Bartlett's test of the homogeneity of variances [4]. Differences between treatments were established with Duncan's multiple range test ($\alpha = 0.05$) [15]. The interaction of carbon concentration and carbon-to-nitrogen ratio on *C. coccodes* spore yield, germination rate and mycelial biomass were analyzed by SAS [11] and fitted to the general linear model. Results were presented using surface response methodology.

Virulence test

Velvetleaf seeds were placed on moist filter paper in petri dishes at 4°C for 24 h then incubated at 30°C for 48 h. Four germinated seeds were planted in 10-cm diameter plastic pots filled with Pro-Mix 'BX' general purpose growth medium (Les Tourbieres Premier Ltee, Riviere-du-loup, Quebec, Canada). Plants were grown in controlled environment chambers (Conviron®, Model E-15, Controlled Environments, Winnipeg, Manitoba, Canada) for 14 days at 24/18°C day/night temperature and a 14-h photoperiod with 400 μE m^{-2} s^{-1}. *C. coccodes* spores were suspended in 20 ml of distilled water and sprayed on the 14-day-old plants in a spray chamber at the rate of 10^9 spores per m^2. Three pots (four plants per pot) were sprayed per treatment. The virulence of spores produced in media with 10 g L^{-1} carbon and C:N ratios of 5:1, 7.5:1, 10:1, 15:1, and 20:1 was assessed by evaluating their ability to reduce plant biomass on velvetleaf. Spores produced in Modified Richard's solution were used as the standard or positive control and plants sprayed with distilled water were the negative control. After spraying them, velvetleaf plants were immediately incubated in a dark dew chamber at 24°C for 18 h and then transferred to a growth chamber in a random design at the conditions mentioned above. Plants were incubated for 14 days after inoculation, harvested at soil level, dried at 60°C for 1 week, and their dry weight was recorded. The experiments were conducted three times. Data for velvetleaf shoot dry weight were analyzed using

Table 1 Concentrations of sucrose, soy protein and KNO_3 in media at 5 g L^{-1} total carbon. For total carbon concentration at 10 and 20 g L^{-1}, the amount of sucrose, soy protein and KNO_3 are two- and four-fold, respectively, of the data below

C:N ratio	Sucrose (g L^{-1})	Soy protein (g L^{-1})	KNO_3 (g L^{-1})
5:1	7.95	3.40	3.61
7.5:1	9.26	2.26	2.41
10:1	9.92	1.70	1.81
15:1	10.57	1.13	1.20
20:1	10.9	0.85	0.90
30:1	11.23	0.57	0.60
40:1	11.39	0.43	0.45

the ANOVA procedure in SAS [11]. Treatment means were separated using Duncan's multiple range test.

Results

Sporulation

The carbon-to-nitrogen ratio significantly affected *C. coccodes* sporulation at the different carbon concentrations tested. When the carbon concentration was 5 g L^{-1}, spore yields decreased with increasing C:N ratio. Spore yields were significantly higher within the C:N ratio range of 5:1 to 10:1 than in the range of 15:1 to 40:1. In media with 10 g L^{-1} carbon, *C. coccodes* produced similar amounts of spores at C:N ratios of 5:1, 7.5:1 and 10:1. Spore yields were significantly reduced when the C:N ratio was greater than 15:1. At 20 g L^{-1} carbon, highest spore yields were obtained at C:N ratios of 7.5:1 and 10:1. Spore yields were significantly lower at a C:N ratio of 5:1 and above 15:1 than those obtained at 7.5:1 and 10:1 (Table 2). As carbon concentration increased, spore yields decreased with the increase of the C:N ratio when carbon concentration was low and medium. However, spore yield decreased at a C:N ratio of 5:1 when the carbon concentration was high (Table 2). Maximum spore yields were obtained at C:N ratios between 7.5:1 and 10:1 with 20 g L^{-1} carbon (Figure 1). Although spore yield increased with the increase of carbon concentration with a constant C:N ratio, the specific spore yields were not significantly different amongst C:N ratios of 5:1, 7.5:1 and 20:1 to 40:1 (Figure 2a). With C:N ratios of 10:1 and 15:1, the specific spore yields with 20 g L^{-1} carbon concentration were significantly higher than those with 5 and 10 g L^{-1} of carbon concentration.

Spore germination rate

Media carbon concentration and C:N ratio also had an effect on the spore germination rate. When media carbon concentration was 5 g L^{-1} and 10 g L^{-1}, spore germination rate decreased with an increase of the C:N ratio (Table 2). Spore germination rates were significantly higher with C:N ratios of 5:1 to 10:1 than in the range of 15:1 to 40:1 at 5 g L^{-1} carbon. When media had 10 g L^{-1} carbon, spore germination rates were not different in media with C:N ratios from 5:1 to 20:1. At C:N ratios of 30:1 and 40:1,

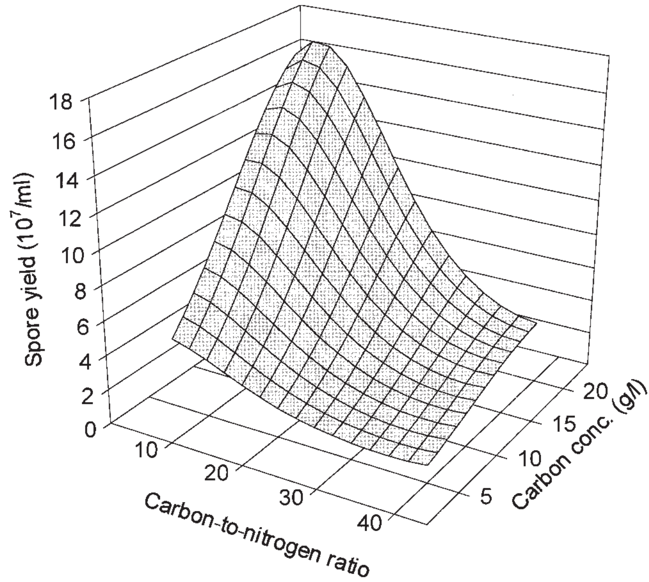

Figure 1 Effect of carbon concentration and C:N ratio on spore yield. Data were fitted to the following model: $z = 7.2427 + 0.0815x + 0.002225xy - 0.00204x^2 - 0.00204y^2 + 0.000041y^3 - 0.000052xy^2$; $r^2 = 0.95$ where x is the carbon concentration, y is the C:N ratio and z is the spore yield.

germination was significantly less than for C:N ratios between 5:1 to 20:1. With 20 g L^{-1} carbon concentration, highest spore germination rates were obtained with C:N ratios of 7.5:1 and 10:1. Spore germination rates were significantly lower with C:N ratios of 5:1 and above 15:1 than those obtained at 7.5:1 and 10:1 (Table 2). Although carbon concentration and C:N ratio both affected spore germination rate, the C:N ratio was the most important factor. Spore germination rates decreased with increasing C:N ratios.

Mycelial biomass

Mycelial biomass increased with increasing C:N ratio when the media contained 5 g L^{-1} carbon (Table 2). In media with 10 g L^{-1} of carbon, similar mycelial dry biomass production occurred with C:N ratios from 5:1 to 20:1. Mycelial dry biomass was significantly decreased when C:N ratios

Table 2 Spore yield, mycelium production, and spore germination rate in medium with different carbon concentrations and carbon-to-nitrogen ratios. Means were separated by Duncan's multiple range test. Means in the same column with the same letter are not significantly different ($P < 0.05$)

C:N ratio	Carbon concentration (g L^{-1}) 5			10			20		
	Spores (10^7 ml^{-1})	Mycelial dry weight (g L^{-1})	Germination (%)	Spores (10^7 ml^{-1})	Mycelial dry weight (g L^{-1})	Germination (%)	Spores (10^7 ml^{-1})	Mycelial dry weight (g L^{-1})	Germination (%)
5:1	3.8 a	2.60 e	98.9 a	7.4 a	6.3 ab	93.0 a	13.2 b	13.5 a	88.8 b
7.5:1	3.9 a	3.16 d	98.1 a	8.5 a	6.4 a	95.5 a	18.5 a	13.4 a	97.0 a
10:1	3.4 a	3.41 c	95.8 a	8.2 a	6.2 ab	94.4 a	21.3 a	13.4 a	98.2 a
15:1	2.3 b	3.68 b	91.5 b	5.1 b	6.2 ab	92.8 a	14.6 b	12.1 b	91.4 b
20:1	1.8 b	3.84 ab	89.9 b	3.6 c	6.0 b	89.6 a	8.9 c	9.3 c	81.0 c
30:1	0.96 c	3.97 a	86.4 c	2.5 d	4.7 c	83.3 b	5.7 d	8.4 d	82.8 c
40:1	0.99 c	3.74 ab	86.4 c	1.9 d	4.8 c	81.4 b	3.5 e	7.3 e	75.7 d

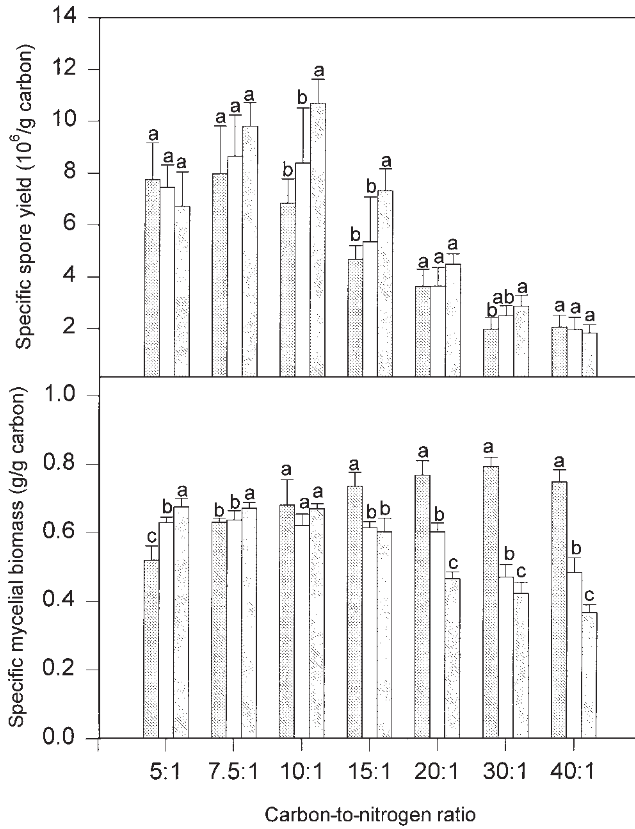

Figure 2 Effect of C:N ratio on specific spore yield (top) and specific mycelial biomass yield (bottom). Data were separated by Duncan's multiple range test. Means with the same letter in the same C:N ratio are not significantly different ($P < 0.05$). ▨ 5 g L^{-1} carbon sources; □ 10 g L^{-1} carbon sources; ▧ 20 g L^{-1} carbon sources.

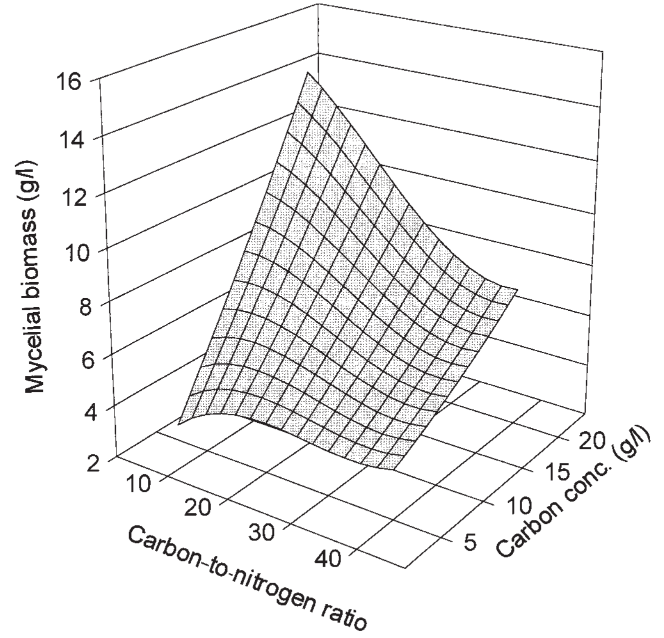

Figure 3 Effect of carbon concentration and C:N ratio on mycelial biomass. Data were fitted to the following model: $z = -2.8446 + 0.79x + 0.499y - 0.031xy + 0.0043x^2 - 0.016y^2 + 0.00018y^3 + 0.00035xy^2$; $r^2 = 0.98$. where x is the carbon concentration, y is the C:N ratio and z is the mycelial dry biomass.

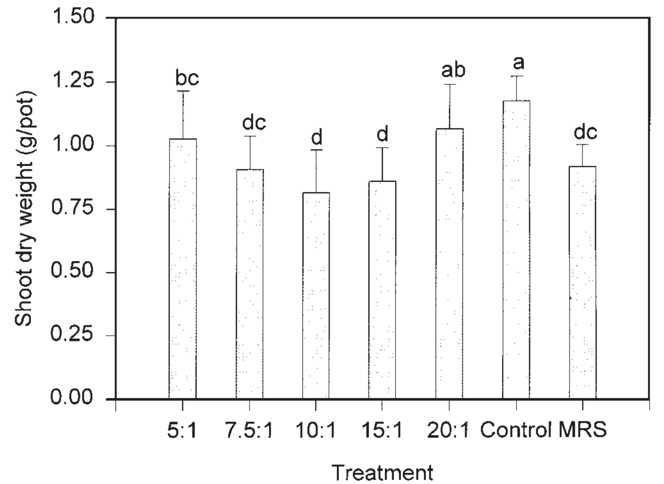

Figure 4 Effect of spores produced from various C:N ratios on velvetleaf shoot dry weight reduction. Data were separated by Duncan's multiple range test. Means with the same letter are not significantly different ($P<0.05$). MRS: Modified Richard's Solution.

were above 30:1. With 20 g L^{-1} carbon, mycelial biomass was not significantly different with C:N ratios of 5:1, 7.5:1, and 10:1. However, with C:N ratios of 15:1 and above, biomass significantly decreased with increasing C:N ratio (Table 2). Media became viscous after 5 days of incubation at C:N ratios above 15:1 in 20 g L^{-1} carbon. Interaction of carbon concentration and carbon-to-nitrogen ratio is shown in Figure 3. At low C:N ratios, *C. coccodes* mycelial biomass increased with the increasing carbon concentration. Mycelial biomass decreased with increasing C:N ratios when carbon concentration was high. The maximum mycelial biomass was obtained in 20 g L^{-1} carbon with a 5:1 C:N ratio (Figure 3). The specific yield of mycelial biomass for a constant C:N ratio varied depending upon the carbon concentration. At C:N ratios of 5:1 and 7.5:1, specific mycelial biomass yields for 20 g L^{-1} carbon concentration were significantly greater than those for 5 and 10 g L^{-1} carbon. With a C:N ratio of 10:1, there was no effect of carbon concentration. However, when the C:N ratio was greater than 15:1, the specific mycelial biomass yields increased significantly with decreasing carbon concentration (Figure 2b).

Virulence test

Spores produced from all C:N ratios, except 20:1, significantly reduced velvetleaf shoot dry weight (Figure 4). The virulence of spores produced in media with C:N ratios from 5:1 to 15:1 were not significantly different from the spores produced in Modified Richard's solution. However, spores produced in media with C:N ratios of 10:1 and 15:1 resulted in a greater reduction in velvetleaf biomass as compared to spores produced in medium with C:N ratios of 5:1 and 20:1.

Discussion

C. coccodes grows and sporulates well in a medium containing soy protein, but due to the poor solubility of soy protein in water, it was not suitable as the sole nitrogen source [19]. In this study, soy protein and KNO_3 were used

as nitrogen sources to supply equal amounts of molecular nitrogen in the medium. KNO_3 is used as a nitrogen source in Modified Richard's solution, however, without an organic component (V-8 juice or soy protein), KNO_3 supported limited *C. coccodes* mycelial growth and poor spore yield (data not shown).

The effect of carbon concentration on *C. coccodes* sporulation varied for different C:N ratios. Although spore yield increased with increasing carbon concentration with all C:N ratios tested, the specific spore yields with a constant C:N ratio were usually not significantly different. This indicates that carbon concentration had little effect on the conversion of raw materials to spores by *C. coccodes*. However, the specific spore yields were different with different C:N ratios, demonstrating that the C:N ratio is a key factor influencing *C. coccodes* sporulation. Highest spore yields were obtained with C:N ratios between 7.5:1 and 10:1.

Jackson *et al* [6] and Schisler *et al* [12] reported that the C:N ratio of the sporulation medium significantly influences spore yield and fitness as well as ultimate effectiveness of the potential mycoherbicide, *Colletotrichum truncatum*. *C. truncatum* spore yields were maximum in media with a C:N ratio of 30:1. However, media with a C:N ratio of 10:1 produced spores that germinated more rapidly, formed appressoria more frequently and incited more disease in its host weed, *Sesbania exaltata*. In this study, high germination rates of *C. coccodes* spores corresponded to high spore yields within the same carbon concentration. Spores produced with optimal C:N ratios for sporulation also had high germination rates, incited more severe disease and caused more shoot dry weight reduction in velvetleaf.

When carbon concentration was higher than 10 g L^{-1}, *C. coccodes* spore yields and mycelial biomass had similar responses to C:N ratios. High spore yield and mycelial biomass were obtained at C:N ratios of 5:1 to 10:1. When C:N ratios were greater than 10:1, both spore yield and biomass production decreased with increasing C:N ratios. Since *C. coccodes* spores are produced from hyphal tips, more mycelium branching should lead to more spore production. However, at 5 g L^{-1} carbon, spore yields and mycelial biomass had opposite responses to C:N ratios. Spore yields decreased and mycelial biomass increased with increasing C:N ratios. Low C:N ratios favored growth and sporulation, since nutrients were exhausted faster in low C:N ratios. It is possible that mycelium lysed before harvesting in the 5 g L^{-1} carbon media. Since mycelium of *C. coccodes* is not invasive, it cannot be used as the infection propagule. Therefore, methodologies to reduce mycelial biomass by inducing branching and enhancing sporulation need to be developed.

C. coccodes cultures become very viscous and spore yield and mycelial biomass decreased significantly in media containing 20 g L^{-1} carbon with high C:N ratios (above 15:1). This indicated that in high carbon concentration and high C:N ratios, nitrogen became a growth-limiting factor. In this nitrogen-limited condition, *C. coccodes* apparently changed its metabolic pathway and the extra carbon sources were converted to spore matrix. Regulation of differentiation by carbon concentration and C:N ratio was also found in other *Colletotrichum* species [5].

The presence of a spore matrix can significantly inhibit spore germination and generally enhance the survival of spores [7,10,13]. In this study, increased spore matrix corresponded to reduced germination of *C. coccodes* spores, but the effect of a spore matrix on survival was not tested.

Understanding how nutrients influence growth and sporulation is important for developing a cost-effective medium for bioherbicide mass production. This study showed that carbon concentration and carbon-to-nitrogen ratio are important factors for *C. coccodes* sporulation in submerged culture. These results are very different from those obtained for another bioherbicide fungus, *C. truncatum*. A well-balanced C:N ratio is essential, not only for the optimum production of *C. coccodes* spores, but also for pathogen virulence. A thorough understanding of the nutritional requirement is required to obtain optimum spore yield and efficacy. Through this study, a medium to produce a high number of viable and virulent *C. coccodes* spores was developed. This medium is being used in continuing fermentation optimization experiments addressing design, feeding, pH, temperature, and oxygen transfer effects on sporulation of *C. coccodes.*

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