2006

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Accepted for publication 8 February 2005.

ABSTRACT

Zhang, S., Schisler, D. A., Boehm, M. J., and Slininger, P. J. 2005. Carbon-to-nitrogen ratio and carbon loading of production media influence freeze-drying survival and biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9. Phytopathology 95:626-631.

Fusarium head blight (FHB), caused by *Gibberella zeae*, is a devastating disease of wheat worldwide. *Cryptococcus nodaensis* OH 182.9 is an effective biocontrol agent for this disease. Development of a dried product of OH 182.9 would have potential advantages of ease of handling, favorable economics, and acceptance by end users. Isolate OH 182.9 was grown for 48 and 72 h in semi-defined complete liquid (SDCL) medium with carbon-to-nitrogen (C/N) ratios of 6.5:1, 9:1, 11:1, 15:1, and 30:1, and in SDCL C/N 30:1 media with varied carbon loadings of 7, 14, 21, and 28 g/liter. Total biomass production and cell survival at 15 days after freeze-drying were evaluated. Biomass production of OH 182.9 (CFU per milliliter) was not different for all cultivation time by medium C/N or carbon loading combinations. In general, cells harvested at 48 h survived freeze-drying better than those harvested at 72 h. Survival of freeze-dried cells was greatest for cells grown for 48 h in C/N 30:1 medium. Cells produced in C/N 6.5:1 medium generally exhibited the poorest survival. For the C/N 30:1 media, cells from 7 g/liter carbon loading medium harvested after 48 h had the best survival after freeze-drying. The difference in freeze-dried cell populations between superior and inferior treatments was typically 1 to 2 log units at 15 days after freeze-drying. The biomass of OH 182.9 produced in SDCL with varied C/N ratios and in SDCL C/N 30:1 media with differing carbon loadings was tested for biocontrol efficacy against FHB in greenhouse studies. The biomass harvested from SDCL C/N 9:1, 11:1, and 15:1 media after 48 h significantly reduced symptoms of FHB. None of the treatments with cells harvested at 72 h consistently reduced FHB severity ($P \leq 0.05$). Cells grown in SDCL C/N 30:1 media with 7 and 14 g/liter carbon loading significantly reduced FHB disease severity. Cells harvested from SDCL C/N 9:1, 11:1, and 30:1 with 14 g/liter carbon increased the 100-kernel weight compared with the disease control. The potential of improving OH 182.9 product quality via management of the nutritional environment of the production medium is demonstrated in this study.

Biological control is an important component of integrated pest management. Work on biological control of Fusarium head blight (FHB) has only recently begun but significant success has been achieved (4,5,13,14,19,22,29,30). Using microbial antagonists isolated from wheat anthers, our research group has demonstrated biological control of FHB in greenhouse and field conditions using a variety of wheat cultivars and scales of liquid culture-produced inoculum (3,13,14,22). One of our most efficacious yeast strains, *Cryptococcus nodaensis* OH 182.9, was selected for testing the effectiveness against FHB in fields at different locations in the United States. Frozen biomass of isolate OH 182.9 significantly reduced FHB when results were averaged across 15 field sites in the United States (15). Fresh cell preparations of the wild-type OH 182.9 and a fungicide tolerant variant of OH 182.9 in combination with recommended rates of fungicides Folicur and BAS 505 were tested on two winter wheat cultivars. The biocontrol preparation significantly reduced disease severity of FHB when data from all test sites were pooled and compared favorably with the fungicide Folicur (20).

Though the frozen cell concentrate product and fresh cell preparations reduced FHB in field trials, development of a dried biocontrol product would have potential advantages of ease of handling, convenience in transportation, favorable economics, and consumer acceptance. Freeze-drying is the most convenient and successful method of preserving bacteria, sporulating fungi, and yeast (2). Freeze-drying has advantages of protection from contamination or infestation during storage, long viability, and ease of distribution (27). However, dehydration of microbial biomass can adversely affect its viability and efficacy in the development process (2,27). There are several reports indicating that production medium and/or cultivation time influences the desiccation tolerance and/or efficacy of biocontrol agents (6,8,10,11,16, 17,24). The influence of these production variables on biocontrol efficacy and desiccation tolerance has not been addressed, however, for yeast antagonists. The objective of this research was to investigate the effect of differing carbon-to-nitrogen (C/N) ratio and carbon loading in production media and cultivation duration on the viability of the biocontrol agent *C. nodaensis* OH 182.9 after freeze-drying and on the efficacy of fresh cells of OH 182.9 against FHB.

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DOI: 10.1094/PHYTO-95-0626

MATERIALS AND METHODS

Cultures. A yeast, *C. nodaensis* OH 182.9 (NRRL Y-30216), originally isolated from wheat anthers and proved effective against *Gibberella zeae* (13,14,22) in greenhouse and field trials, was used in this study.

Growth conditions and sample preparation. A stock culture of OH 182.9 was stored in 10% glycerol at –80°C and streaked on 1/5 Tryptic soy broth agar (TSBA/5) (Difco Laboratories, Detroit, MI) to assure purity. A single colony was streaked on TSBA/5 and incubated at 25°C for 48 h before cells were harvested in weak phosphate buffer (PO_4 buffer, 0.004% KH_2PO_4 buffer with 0.019% $MgCl_2$: Aid Pack, Gloucester, MA). Fifty milliliters of semi-defined complete liquid (SDCL) medium (26) in 250-ml Erlenmeyer flasks was inoculated with the cell suspension to an optical density of 0.1 at wavelength 620 nm. Flasks (three replicates per treatment) were incubated at 250 rpm, 2.0-cm eccentricity, and 25°C for 48 and 72 h prior to use. Media of differing C/N ratios were obtained by varying the ratio of glucose and Casamino acid while maintaining constant carbon loading (14 g/liter). SDCL medium with C/N ratios of 6.5:1, 9:1, 15:1, 30:1, and the standard SDCL C/N 11:1 medium were used in this study. For SDCL C/N 30:1 media, changes in carbon loading also were achieved by increasing or decreasing, in proportion, the amount of glucose and Casamino acid. Media with carbon loadings of 7, 21, and 28 g/liter and the standard SDCL medium with 14 g/liter were tested.

Freeze-drying process. For each flask, 2-ml samples were distributed in autoclaved 10-ml serum vials and then placed in a freeze-drier (FTS Systems, Inc., Stone Ridge, NY) with a eutectic temperature of –8°C and operated at –45°C for 48 h.

Survival of cells after freeze-drying. Vials containing freeze-dried samples were sealed and stored at 25°C. After 0, 2, 8, and 15 days, three replicates of each *C. nodaensis* OH 182.9 production treatment were rehydrated to their original volume (2 ml) with PO_4 buffer. Rehydrated samples were shaken by hand for 30 s and placed at room temperature for 15 min before the number of CFU per milliliter was determined by plating aliquots of appropriate dilutions on TSBA/5 and incubating at 25°C for 48 h. Microscopic observations revealed that cell clumps contained five cells or less and accounted for less than 3% of the total cells observed. The experiment was performed twice with combinations of differing C/N ratios in SDCL (6.5:1, 9:1, 11:1, 15:1, and 30:1) and cultivation times (48 and 72 h). The survival of cells produced in SDCL C/N 30:1 media with varied carbon loadings (7, 14, 21, and 28 g/liter) after 48 and 72 h also was evaluated. The CFU per milliliter data were transformed to logarithmic values for statistical analysis.

Production of *C. nodaensis* OH 182.9 inoculum. Inoculum of OH 182.9 was prepared in liquid cultures incubated for 48 or 72 h as described earlier. The SDCL media containing 14 g/liter with C/N ratios of 6.5:1, 9:1, 15:1, 30:1, and the standard media C/N 11:1 were used. For SDCL media with a C/N ratio of 30:1, carbon loadings of 7, 14, 21, and 28 g/liter were used to produce inoculum of OH 182.9.

Preparation of *G. zeae* inoculum. Macroconidia of *G. zeae* isolate Z-3639, originally isolated from wheat in Kansas, were produced on clarified V8 agar (CV8 agar) under a regime of 12 h/day fluorescent light for 7 days at 24°C (23). Conidial suspensions were obtained by flooding the surface of colonized CV8 agar with PO_4 buffer and removing conidia with a sterile inoculation loop.

Greenhouse assays of *C. nodaensis* OH 182.9 against FHB. Studies were conducted in a climate-controlled greenhouse where temperatures ranged from 15 to 20°C at night and 23 to 28°C during the day. Natural sunlight was supplemented by high-pressure sodium lights for 14 h/day during the winter season. Two wheat (cv. Norm) seedlings were grown in a 19-cm-diameter plastic pot containing air-steam pasteurized (60°C for 30 min) potting mix (Terra-lite Redearth Mix, W.R. Grace, Cambridge, MA) in a growth chamber (25°C, 14-h light per day, 600 μmol/[m^2/s]) for 7 to 8 weeks prior to use. Pots were fertilized after 1 week and weekly thereafter with 50 ml of a solution containing Peters 20-20-20 (1.25 g/liter) (Grace-Sierra Horticultural Products, Milpitas, CA) and iron chelate (0.079 g/liter) (Sprint 330, Becker Underwood, Inc., Ames, IA). Wheat heads were inoculated at anthesis (Feeke's growth stage 10.5.1) by spraying 5 ml of 25% suspensions of 48- or 72-h cultures of OH 182.9 for each plant. The microbial inoculum was obtained by diluting OH 182.9 cultures with weak PO_4 buffer and adding 0.036% Tween 80 (Sigma Chemical, St. Louis, MO). For each treatment, 20 ml of inoculum was used to inoculate four plants representing a total of 12 to 16 heads. Heads then were challenged by spraying 12 ml of a conidial suspension of *G. zeae* (1 to 2×10^4 conidia per ml) in weak PO_4 buffer with 0.036% Tween 80. Treatments were arranged in a completely randomized design ($n = 4$). Each experiment was performed two to three times. Wheat heads inoculated only with a suspension of *G. zeae* or with PO_4 buffer and Tween 80 alone served as controls. Inoculated plants were misted slightly with water and incubated in a plastic humidity chamber at 22 to 25°C for 3 days before being transferred to greenhouse benches. The severity of FHB was visually estimated using a 0 to 100% scale (28) at 14 days after inoculation. Wheat heads were then allowed to mature and dry, hand-threshed, and 100-kernel weights were determined for each replicate.

Statistical analysis. Data of cell survival were analyzed separately for each repeated experiment at each time point individually and were submitted to analysis of variance (ANOVA) (SAS PC Windows version 8.2, SAS Institute, Cary, NC). Without the control treatments, factorial analyses of ANOVA were performed with C/N ratio or carbon loading and cultivation time as categorical variables for disease severity and cell survival after freeze-drying using PROC GLM. One-way ANOVAs, including the controls, were performed for each C/N ratio or carbon loading by cultivation time combination. Disease severity data were normalized when needed using the arcsine transformation before ANOVA. Data of disease were pooled after statistical analysis demonstrated that experiment by treatment interactions were not significant ($P \leq 0.05$). The significance of treatment effects was determined by the magnitude of the F value ($P \leq 0.05$). Separation of means was accomplished using Fisher's protected least significant difference test ($P \leq 0.05$).

RESULTS

Effects of C/N ratio of production media and cultivation time on cell survival of *C. nodaensis* OH 182.9 after freeze-drying. With 14 g/liter carbon, cells grown in SDCL C/N 30:1 media for 48 h survived to a greater extent than cells from other media by incubation time combinations (Fig. 1A). The population size ($\log_{10}$ CFU/ml) of the viable cells was as high as 8 at 2 days after freeze-drying. By 15 days, the population size was 7.7. For the experiments with higher C/N ratios, i.e., 11:1, 15:1, and 30:1, with two-factor ANOVA, there was a significant C/N ratio by cultivation time interaction effect with OH 182.9 cells at 0, 2, 8, and 15 days after freeze-drying ($P < 0.0001$, $P = 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively), which precluded obtaining overall C/N or cultivation time effects. In contrast to results for cells harvested after 48 h from C/N 30:1 medium, cells grown in SDCL C/N 6.5:1 had the least survival after freeze-drying (Fig. 1B). The logarithmic value of viable cells dramatically dropped from 8 to 5.6 after freeze-drying for cells harvested from SDCL C/N 6.5:1 at 48 h, and dropped further to 4.3 at the end of the experiment. Based on factorial analysis of the experiments with lower C/N ratios (11:9, 9:1, and 6.5:1), there was a significant C/N ratio by cultivation time interaction effect at 0, 8, and

15 days after freeze-drying ($P = 0.0054$, $P < 0.0001$, and $P < 0.0001$, respectively), which precluded obtaining overall C/N ratio or cultivation time effects. Cells harvested at 48 h survived better than those from 72 h (average logarithmic values were 8.21 and 8.07 for 48 and 72 h, respectively) at 2 days after freeze-drying ($P < 0.0001$). Survival of OH 182.9 cells grown in the standard SDCL C/N 11:1 medium was in the midrange of the treatments tested (Fig. 1). In general, cells produced in higher C/N ratio SDCL media survived better than those from lower C/N ratio media. Cells harvested at 48 h generally survived better than those at 72 h, except for few treatments such as C/N 15:1 (Fig. 1A) and C/N 6.5:1 at some time points (Fig. 1B). Total biomass produced from all combinations of C/N ratio by cultivation time was similar, with the logarithmic values ranging from 8.0 to 8.5 (Fig. 1).

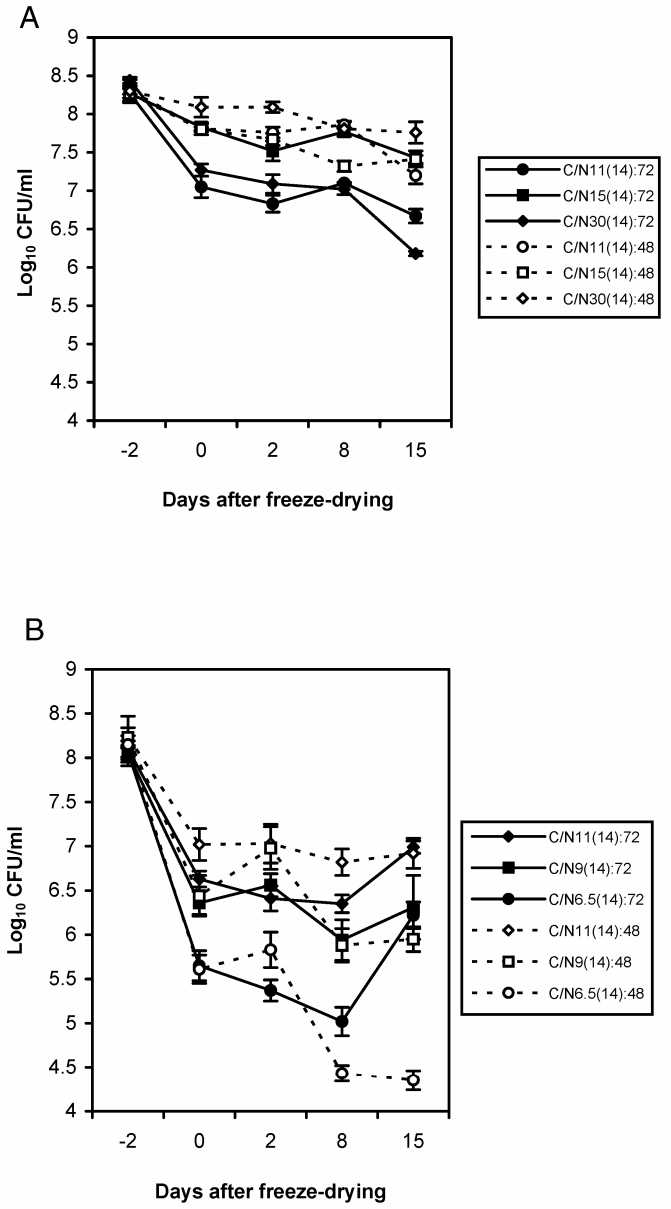

Fig. 1. Effect of C/N ratio of production media and cell age (48 and 72 h) on the survival of *Cryptococcus nodaensis* OH 182.9 cells after freeze-drying. The semi-defined complete liquid (SDCL) media contained 14 g of carbon per liter. Day –2 on the *x* axis indicates the date at which the cells were harvested, i.e., before being placed for 2 days in a freeze-drier. Each data point represents the mean (± standard error of the mean) from a representative of two replicate experiments. **A,** Effect of higher C/N ratio: C/N = 11(14):1, 15(14):1, and 30(14):1. Cells of OH 182.9 grown in SDCL C/N 30:1 media for 48 h (◇) survived to a greater extent than those from other media by incubation time combinations. A significant interaction between C/N ratio and cultivation time exists with OH 182.9 cells at 0, 2, 8, and 15 days after freeze-drying. **B,** Effect of lower C/N ratio: C/N = 6.5(14):1, 9(14):1, and 11(14):1. Cells grown in SDCL C/N 6.5:1 (○, ●) had the least survival after freeze-drying. A significant C/N ratio by cultivation time interaction was found at 0, 8, and 15 days after freeze-drying. Cells harvested at 48 h survived better than those from 72 h at 2 days after freeze-drying.

Effect of C/N ratio of production media and cultivation time on the efficacy of *C. nodaensis* OH 182.9. Treatments with cells harvested from standard SDCL C/N 11:1 media at 48 h reduced FHB severity ($P \leq 0.05$) by 29 to 57% (Table 1) compared with the disease controls. Treatment with cells harvested from SDCL C/N 15:1 media and C/N 9:1 media at 48 h also reduced FHB severity ($P \leq 0.05$). Cells grown in SDCL media for 72 h did not have biocontrol activity against FHB except for those grown in the standard SDCL C/N 11:1 in one of the experiments. Treatment with cells harvested after 48 h from SDCL C/N 30:1 reduced FHB severity, albeit not significantly, relative to the disease control in this experiment. Factorial analysis showed no significant differences in C/N ratios and cultivation time effects and no significant interactions.

The 100-kernel weight from the treatment with *C. nodaensis* OH 182.9 cells produced in SDCL C/N 9:1 at 48 h was higher than that from the disease control (Table 1). None of the other treatments resulted in increased 100-kernel weight compared with the disease control, except for the treatment with cells harvested at 48 h from the standard SDCL C/N 11:1 medium in one of the two repeated experiments.

Influence of carbon loading of production media (C/N 30:1) on the survival of *C. nodaensis* OH 182.9 after freeze-drying. Because cells grown in SDCL C/N 30:1 containing 14 g/liter were superior in surviving freeze-drying in earlier studies, cells were produced in C/N 30:1 media with carbon loadings of 7, 21, and 28 g/liter and the standard 14 g/liter and assessed for survival after freeze-drying. Cells harvested from 7 g/liter carbon SDCL

TABLE 1. Effect of C/N ratio of production media with carbon loading of 14 g/liter on biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9 against Fusarium head blight (FHB)[w]

Treatment[x]	Disease severity (%)[y]	100-Kernel weight (g)[y]
C/N 11(14):72[z]	37.8 b	3.09 ab
C/N 11(14):48	30.5 bc	3.08 ab
C/N 15(14):72	45.2 ab	2.84 b
C/N 15(14):48	37.5 b	2.92 ab
C/N 30(14):72	45.1 ab	2.83 b
C/N 30(14):48	39.2 ab	2.88 ab
Disease control	70.7 a	2.39 b
Nontreated control	0.0 c	3.62 a
$LSD_{0.05}$	32.4	0.77
C/N 11(14):72[z]	85.0 a	2.34 cd
C/N 11(14):48	62.8 bc	3.36 ab
C/N 9(14):72	82.5 ab	2.61 bcd
C/N 9(14):48	55.5 c	3.16 abc
C/N 6.5(14):72	82.1 ab	2.52 bcd
C/N 6.5(14):48	82.8 ab	2.42 bcd
Disease control	88.8 a	2.14 d
Nontreated control	0.0 d	4.09 a
$LSD_{0.05}$	20.9	1.00

[w] Data are from two separate experiments in which the effect of higher and lower C/N ratios was tested, respectively, due to the space limitation of the humidity chamber. Each experiment was conducted three times.

[x] Wheat heads were inoculated at flowering by spraying 25% suspensions of fully colonized cultures of OH 182.9, and were immediately challenged by spraying conidial suspensions of *Gibberella zeae* (1 to 2 × 10^4 conidia per ml). FHB disease was visually rated at 14 days after pathogen inoculation.

[y] Data from three trials for each experiment were pooled and arcsine-transformed before statistical analysis. Means followed by a common letter within a column are not significantly different at $P \leq 0.05$.

[z] C/N 11(14):72 indicates that the C/N ratio of the semi-defined complete liquid medium was 11:1 with 14 g/liter carbon, and the cultivation time of OH 182.9 was 72 h.

media after 48 h maintained the highest viable cell counts after freeze-drying (Fig. 2), with the highest $\log_{10}$ CFU per milliliter (8.1) at 5 days after freeze-drying. However, the number of viable cells after freeze-drying dramatically decreased for cells grown in SDCL with carbon loading at 14 g/liter for 72 h, where the $\log_{10}$ CFU per milliliter was 5 at the end of the experiment. Cells produced from other treatments with carbon loadings of 21 and 28 g/liter also maintained log values in the range of 7 to 8. Cells from SDCL harvested at 48 h generally survived freeze-drying better than those harvested at 72 h. Based on two-factor analysis, there was a significant carbon loading and cultivation time interaction effect at 0, 2, 8, and 15 days after freeze-drying ($P < 0.0001$, $P = 0.004$, $P = 0.028$, $P < 0.0001$, and $P < 0.0001$, respectively), which precluded obtaining overall carbon loading and cultivation effects.

Influence of carbon loadings in production SDCL media (C/N 30:1) on FHB biocontrol efficacy by *C. nodaensis* OH 182.9. Treatments with cells harvested after 48 h from SDCL C/N 30:1 media with the standard carbon loading of 14 g/liter consistently protected wheat from FHB ($P \leq 0.05$) (Table 2) with disease reduction of more than 60% relative to the disease controls. Treatments with cells produced in the standard SDCL C/N 11:1 media for 48 h reduced disease severity of FHB by up to 83%. In addition, cells from SDCL media with 7 g/liter carbon harvested at 48 h significantly protected wheat from FHB. None of the wheat plants treated with cells harvested at 72 h had significantly lower disease severity compared with the disease control ($P \leq 0.05$). Based on factorial analysis, there was a significant cultivation time effect with $P = 0.0399$ and 0.0006 on FHB disease severity for the experiment with higher and lower carbon loadings, respectively. Disease severity averaged 47 and 65% for 48- and 72-h harvested cells, respectively, for the higher carbon loading experiment. For the lower carbon loading experiment, disease severity averaged 25 and 47% for the cells harvested after 48 and 72 h, respectively.

Treatments with cells harvested from SDCL C/N 30:1 media with 14 and 21 g/liter carbon at 48 h increased the 100-kernel weight compared with the disease control (Table 2). The treatment with cells harvested from the standard SDCL C/N 11:1 media at 48 h showed an increase of 100-kernel weight in one of the two experiments.

DISCUSSION

Based on this study, C/N ratio, carbon loading of the production media, and cultivation time influence the survival of *C. nodaensis* OH 182.9 after freeze-drying and the biocontrol efficacy of fresh cells against FHB. The data presented here provide valuable information for selecting production media and optimizing cultivation time in developing freeze-dried OH 182.9 products for FHB control. Cells of OH 182.9 harvested after 48 h from SDCL C/N 30:1 media with 7 and 14 g/liter carbon survived better after freeze-drying than others and demonstrated levels of biocontrol efficacy comparable to cells harvested after 48 h from the standard SDCL C/N 11:1 media. The use of these improved media formulations for producing more effective biocontrol products of OH 182.9, therefore, is advisable.

There are some studies on the effect of nutritional factors during production of biomass of biocontrol agents on agent efficacy. Bacterial strains vary in their efficacy in reducing Fusarium dry

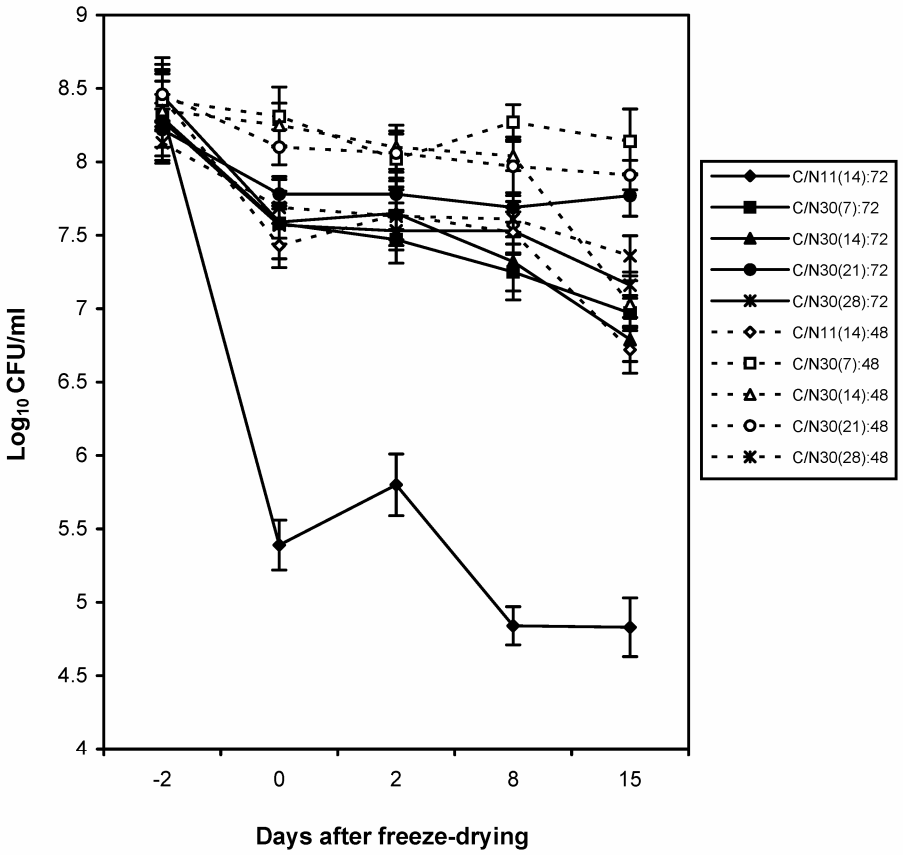

Fig. 2. Influence of carbon loading in semi-defined complete liquid (SDCL) media (C/N 30:1) on the survival of *Cryptococcus nodaensis* OH 182.9 cells after freeze-drying. Day –2 on the *x* axis indicates the date at which the cells were harvested, i.e., before being placed for 2 days in a freeze-drier. Each data point represents the mean (± standard error of the mean) from a representative of two replicate experiments. Cells of OH 182.9 that were harvested after 48 h (□) in SDCL media that contained 7 g of carbon per liter maintained the highest viable cell counts after freeze-drying. The number of viable cells after freeze-drying dramatically decreased for cells grown for 72 h (◆) in SDCL with 14 g of carbon loading per liter. Cells from SDCL harvested at 48 h generally survived freeze-drying better than those harvested at 72 h. There was a significant interaction between carbon loading and cultivation time at all time points.

rot on stored potato tubers depending on the composition of the liquid production media (26). Schisler et al. (21) found that the C/N ratio of the production media influenced the number and morphology of conidia of *Colletotrichum truncatum*, a mycoherbicide for weed control, as well as the biocontrol efficacy. A medium with C/N 30:1 produced the greatest number of conidia, yet conidia grown at C/N 10:1 were more successful in controlling the weed hemp sesbania (*Sesbania exaltata*) than those produced in 30:1 or 80:1. In a subsequent study, conidia produced in C/N 10:1 medium contained significantly more protein and less lipid than those produced in 30:1 or 80:1 (11). Engelkes et al. (8) reported that the number of ascospores of *Talaromyces flavus* increased as the C/N ratio increased from 5:1 to 30:1. In addition, C and N sources that slightly increased ascospore production of *T. flavus* reduced the efficacy of biological control of Verticillium wilt compared with ascospores produced on potato dextrose agar, indicating that optimum nutrient conditions for production of biocontrol agents with maximum efficacy may not necessarily be the conditions that support the greatest populations of propagules. The discrepancy in FHB biocontrol efficacy by OH 182.9 produced in media with different C/N or carbon loadings may result from variations in production of some specific compounds such as proteins and lipids in OH 182.9 cells during liquid production. In order to answer this, it would be worth testing the content of protein and lipid in OH 182.9 cells produced from different C/N media or production media with different carbon loadings.

With the FHB biocontrol agent OH 182.9, no effect of nutritional factors on total biomass accumulation was obtained. However, the biocontrol efficacy of fresh biomass of *C. nodaensis* OH 182.9 was consistently superior for cells harvested after 48 h compared with cells harvested after 72 h. Biocontrol efficacy of FHB was most pronounced when cells were produced in C/N 9:1, 11:1, and 15:1 media containing 14 g/liter carbon and harvested at 48 h. For cells harvested after 48 h from C/N 30:1 media, efficacy was superior for cells produced in media with the lowest carbon loading. Variations in biocontrol efficacy may result from specific changes in cell physiology and survival on the plant surface, which is complex and is likely due to many interacting factors. One of these factors is nutrient starvation imposed on microbial cells. Nitrogen or carbon starvation can lead to freeze-thaw tolerance, and likely general stress tolerance in cells of *Saccharomyces cerevisiae* but the tolerance is not maintained if starvation persists (18). Temporary glucose starvation results in drastic reduction in mRNA degradation, which preserves resources while maintaining cell ability to quickly recover if nutrients become available without a prolonged delay (12). In our study, cells grown in C/N 6.5:1 medium for 72 h versus 48 h survived in an environment depleted of carbon for an extended period and were therefore likely to have been less tolerant to drying stress when applied to wheat plant surfaces. Addition of glucose to *S. cerevisiae* in stationary growth in the absence of additional nutrients results in rapid viability loss after initial cell physiological changes that are characteristic of mitotic growth (9). Similarly, the availability of bioavailable carbon on the wheat head or during anthesis may not be beneficial to isolate OH 182.9 cells in late stationary stage growth. Insuring that cells have reached, but have not been maintained for long periods at stationary growth appears critical to the efficacy of *C. nodaensis* OH 182.9. This may be the reason that the efficacy of fresh biomass of *C. nodaensis* OH 182.9 against FHB was superior for cells harvested after 48 h compared with cells harvested after 72 h.

TABLE 2. Influence of carbon loading of production media (semi-defined complete liquid [SDCL] C/N 30:1) on biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9 against Fusarium head blight (FHB)[w]

Treatment[x]	Disease severity (%)[y]	100-Kernel weight (g)[y]
C/N 30(14):72[z]	70.6 ab	1.90 cd
C/N 30(14):48	28.3 bc	2.99 ab
C/N 30(21):72	66.2 ab	2.00 bcd
C/N 30(21):48	45.3 abc	2.45 abc
C/N 30(28):72	58.3 abc	2.21 bcd
C/N 30(28):48	67.3 ab	2.15 bcd
C/N 11(14):72	56.1 abc	2.30 abcd
C/N 11(14):48	15.0 c	3.30 a
Disease control	86.4 a	1.37 d
$LSD_{0.05}$	46.3	1.02
C/N 30(7):72[z]	42.8 abc	2.70 bc
C/N 30(7):48	30.6 bcd	3.16 bc
C/N 30(14):72	51.6 ab	2.62 bc
C/N 30(14):48	20.3 cde	3.23 ab
C/N 11(14):72	36.1 abcd	2.93 bc
C/N 11(14):48	16.4 de	3.14 bc
Disease control	58.6 a	2.50 c
Nontreated control	0.0 e	3.94 a
$LSD_{0.05}$	23.4	0.73

[w] Data are from two separate experiments in which the effect of higher and lower carbon loading was tested, respectively, due to the space limitation of the humidity chamber. Each experiment was conducted at least twice.

[x] Wheat heads were inoculated at flowering by spraying 25% suspensions of fully colonized cultures of OH 182.9, and were immediately challenged by spraying conidial suspensions of *Gibberella zeae* (1 to 2×10^4 conidia per ml). FHB disease was visually rated at 14 days after pathogen inoculation.

[y] Data from repeated trials for each experiment were pooled and arcsine-transformed before statistical analysis. Means followed by a common letter within a column are not significantly different at $P \leq 0.05$.

[z] C/N 30(14):72 indicates the C/N ratio of the SDCL medium was 30:1 with carbon loading 14 g/liter, and the cultivation time of OH 182.9 was 72 h.

To be of practical use, microbial agents must be formulated as products capable of being stored, distributed and applied under a wide range of field conditions. Further information is needed regarding the effects of nutrient factors on stress tolerance. There are some reports that addition of cryoprotectants increases the viability of microbial antagonists after freeze-drying (1,7,25). However, some carbon source based protectants may also enhance pathogen activity (25). Alteration of nutrients in production media may be an alternative for increasing the resistance to stress imposed by the drying and formulation process. In general, we found that production media with higher C/N ratios produced cells that survived better than those produced in media with lower C/N ratios. For the SDCL C/N 30:1 media, most of the carbon concentrations tested produced biomass that survived better than biomass from the standard C/N 11:1 media. Interestingly, the highest survival was observed with cells harvested after 48 h from medium containing 7 g/liter carbon. Cells produced in media with an excess of carbon compared to nitrogen will often store excess carbon internally (10,11,21). Trehalose accumulation is associated with drying stress tolerance (7). It is also possible that leftover carbon in the spent culture broth of 48-h cultures increased the freeze-drying tolerance of cells. Carbohydrates can be effective as cryoprotectants (1,2) which may partially explain why carbon-depleted cells from C/N 11:1 media did not survive freeze-drying well. To address this, further work needs to be accomplished to test the amount of proteins (especially those involved in catalytic activities), carbohydrates, and lipids in cells of isolate OH 182.9 harvested from production media at different C/N ratios.

In our study, cells from young cultures (48 h) survived better than cells from old cultures (72 h). This result coincides with Montazeri and Greaves (16) who found that tolerance of desiccation in conidia of *Colletotrichum truncatum* declined as the age of the culture from which they were harvested increased. The authors suggested that the increase of desiccation tolerance in conidia from 1-week-old cultures compared with that of the 2- or 3-week-old cultures resulted from changes in cell wall structure or in conidial physiology. For *C. nodaensis* OH 182.9, this may be another possible reason for the difference in freeze-drying tolerance in 48- and 72-h cultures.

Taken together, results from this research suggest the potential of improving the product quality of OH 182.9 by managing the

nutritional environment of production media without compromising biocontrol efficacy. To achieve this goal, further research needs to be conducted to confirm the biocontrol efficacy of the freeze-dried products under field conditions.

ACKNOWLEDGMENTS

This material is based upon work supported by the U.S. Department of Agriculture and is, in part, a cooperative project with the U.S. Wheat & Barley Scab Initiative. We thank J. Sloan for providing excellent technical assistance and wheat plants used in the greenhouse experiments; M. Jackson and A. Payne for helping with the freeze-drying work; and D. Palmquist for providing statistical analysis.

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