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Effects of Carbon and Nitrogen Sources and Carbon-to-Nitrogen Ratio on Production of *Exserohilum longirostratum*

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ABSTRACT

The effects of carbon and nitrogen sources and the carbon-to-nitrogen ratio on the growth and sporulation of *Exserohilum longirostratum* were evaluated. Rice flour and malt extracts as the carbon and nitrogen sources, respectively, produced the greatest amount of mycelium. Sources that produced the most biomass were chosen as carbon and nitrogen sources in a C:N ratio test. However, in further experiments, glucose was chosen as the carbon source for the C:N ratio test because rice flour was found to be easily contaminated. Under the C:N ratio test (fixed carbon test), the highest spore production was obtained with a 5:1 ratio (4.78×10^6 spores/mL) and the highest biomass production was obtained with a 7.5:1 ratio (4.66 g/100 mL). In the fixed nitrogen test, 7.5:1 ratio provided the greatest output (4.08×10^6 spores/mL) whereas a 5:1 ratio produced the most biomass (4.33 g/100 mL). Meanwhile, the control which consisted of V8 agar without additional carbon and nitrogen source produced 1.07×10^6 spores/mL. These results provide information on the influence of carbon and nitrogen source and the C:N ratio that can be used in media for optimum growth and spore yield.

Keywords: Carbon, nitrogen, C:N ratio, *Exserohilum longirostratum*, spore yield

ABSTRAK

Kesan sumber karbon dan nitrogen dan nisbah karbon kepada nitrogen terhadap pertumbuhan dan pensporulan *Exserohilum longirostratum* telah dinilai. Tepung beras dan estrak gandum sebagai sumber karbon dan nitrogen setiap satunya, telah

menghasilkan jumlah miselium yang banyak. Sumber yang menghasilkan biojisim lebih banyak telah dipilih sebagai sumber karbon dan nitrogen dalam ujian nisbah C:N. Walau bagaimanapun, glukosa telah dipilih sebagai sumber karbon oleh kerana tepung beras adalah sangat mudah tercemar. Pada ujian nisbah C:N (ujian pengikatan karbon), penghasilan terbanyak spora adalah pada nisbah 5:1 (4.78×10^6 spora/mL), dan biojisim tertinggi telah dihasilkan pada nisbah 7.5:1 (4.66 g/100 mL). Dalam ujian pengikatan nitrogen, nisbah 7.5:1 memberikan hasil terbaik (4.08×10^6 spora/mL) manakala nisbah 5:1 telah menghasilkan biojisim yang lebih banyak (4.33 g/100 mL). Sementara itu, kawalan yang mengandungi agar V8 tanpa sumber karbon dan nitrogen tambahan telah menghasilkan 1.07×10^6 spora/mL. Keputusan ini memberikan maklumat berkenaan pengaruh sumber karbon dan nitrogen serta nisbah C:N dalam media untuk pertumbuhan dan hasil spora optimum.

Kata kunci: Karbon, nitrogen, nisbah C:N, *Exserohilum longirostratum*, hasil spora

INTRODUCTION

The use of fungal pathogens as bioherbicides is a relatively new approach in weed management. The fungal propagules incorporated as active ingredient would need to be available in large quantities for the efficacious control of target weeds. However, the technology for mass production of fungal inoculates is largely undeveloped, and this has impeded the development of bioherbicides. Suitable low-cost media would first have to be developed to culture the fungi.

Any culture medium needs nutrients – carbon, nitrogen and vitamins – for the fungus to grow. The nutrient intake by the fungus (to a large extent determined by the medium composition) is known to affect the fungal growth, including the quality/quantity of spores and mycelium produced. Thus, the medium would have to be formulated correctly for the most economical culture to lower the cost of the bioherbicide produced. A reliable mass production system is thus the *sine qua non* for the development of commercial bioherbicides.

Azean *et al.* (2002) had reported the potential of *Exserohilum longirostratum* as a bioherbicide for barnyard grass. However, to use the fungus as a bioherbicide on a large scale, the inoculum must first be available in bulk. However, very little is known about its culture although considerable information exists for other fungi. The objective of this study was to evaluate the effect of different carbon and nitrogen sources and the carbon:nitrogen (C:N) ratio in the culture medium for optimum inoculum production of *E. longirostratum*.

MATERIALS AND METHODS

Fungal Culture

Stock culture of *Exserohilum longirostratum* was grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, USA) at room temperature (30 ± 2 °C). The stock cultures were obtained from the Department of Plant Protection, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Basal Medium

The defined basal salts medium for the study consisted of KH_2PO_4 (5.0 g), $MgSO_4.7H_2O$ (0.5 g), $CaCl_2$ (0.5 g), $FeCl_3.6H_2O$ (0.05 g) and $CuSO_4$ (0.05 g) were dissolved in one liter of distilled water.

Screening of Carbon Sources by Fungal Growth

Four carbon (C) sources (glucose, fructose, sucrose and rice flour) of 10 g each were tested for their effect on the production of mycelium. As carbohydrates, they all had 40% C (Jackson and Jaronski, 2008). They were each placed in a 250 mL Erlenmeyer flask with 100 mL basal medium. The mixtures were autoclaved at 121 °C for 15-20 minutes. Five mycelium plugs from a 7 day-old culture were transferred to the Erlenmeyer flask and the flask shaken for one week at room temperature (30 ± 2 °C) on a rotary shaker at 100 rpm. At harvest, the fresh mycelium was weighed on a balance (AND Model GF300) then dried in an oven at 70 °C for one week before weighing again for its dry weight. The C source producing the highest mycelium yield was chosen as the carbon source in the following C:N ratio test.

Screening of Nitrogen Sources by Fungal Growth

Four nitrogen (N) sources (10 g each) [ammonium nitrate (34%N), potassium nitrate (14% N), yeast extract (10% N) and malt extract (1.5% N)] were tested for the production of mycelium. The method used was the same as that used in the screening of carbon sources. The N source producing the highest mycelium yield was chosen as the sole nitrogen source in the following C:N ratio test.

Effect of C:N Ratio on Fungal Growth

Based on the results from the two previous experiments, glucose and malt extracts were chosen as the carbon and nitrogen sources for the C:N ratio studies. Glucose and malt extracts with various amounts were added in the C:N ratios of 5:1, 7.5:1, 10:1, 15:1, 20:1 and 40:1 to 100 mL basal medium in 250 mL Erlenmeyer flasks

with four replicates for each ratio. The method used was the same as that used in the screening of carbon and nitrogen sources for fungal growth.

Effect of C:N Ratio on Sporulation

Five mycelium plugs were transferred to 100 mL V8 broth (20 mL V8 juice in 80 mL distilled water) in a 250 mL flask. The flask was then shaken (100 rpm) for 2 days at 28 °C, after which the broth was added (to inoculate) to another 400 mL V8 broth in a one-liter flask and shaken for another 2 days. The content of the flask was drained and blended in a Warring blender for 30-60 sec, and 25 mL of the suspension was poured onto a layer of modified agar medium (glucose, malt extracts, 12 g water agar-granulated agar; Difco) (250 mL) in a tray (35 × 26 × 2.5 cm). Glucose and malt extract were mixed at C:N ratios of 5:1, 7.5:1, 10:1, 15:1, 20:1 and 40:1 with four replications. 250 mL of the mixture was poured into a tray and incubated in a chamber at room temperature (30 ± 2 °C) for 5 days. The trays were exposed to fluorescent light (12 h light: 12 h dark) for 1-2 days. The spores were first harvested 24 h after incubation by scraping them off gently and the residual spores rinsed off the medium surface with 100 mL of sterile water. The conidia suspensions were sieved through two layers of cheese cloth. The trays were kept in the chamber and harvested at 5 days after inoculation. A control of V8 agar (200 mL of V8 juice [Cambell Soup Company, Camden, NJ, USA] in 800 mL distilled water, 12 g agar) devoid of any external carbon and nitrogen source was used. The concentration of the spores harvested from each ratio was determined by using a Hemacytometer (Reichert Scientific Instruments, Buffalo, N.Y., USA). The morphology of spores harvested was reported based on their length, width and number of septa. The measurements were taken on a random sample of 200 conidia by using a calibrated Ocular Micrometer with four replications for each ratio.

Data Analysis

All the experiments were conducted in a completely randomized design (CRD) with four replications. The data were log-transformed before analysis by the standard SAS procedure (SAS Institute, 2001). Means separation were done if the treatments showed significant differences by Tukey's HSD test.

RESULTS

Effect of Carbon Source on Growth

Of the four carbon sources tested for production of mycelial biomass of *Exserohilum longirostratum*, rice flour (0.813 g), glucose (0.733 g) and sucrose (0.732 g) supported significantly higher mycelia dry weight compared to fructose (0.262 g)

(Figure 1A). Glucose was chosen as the carbon source for subsequent tests because rice flour was easily contaminated.

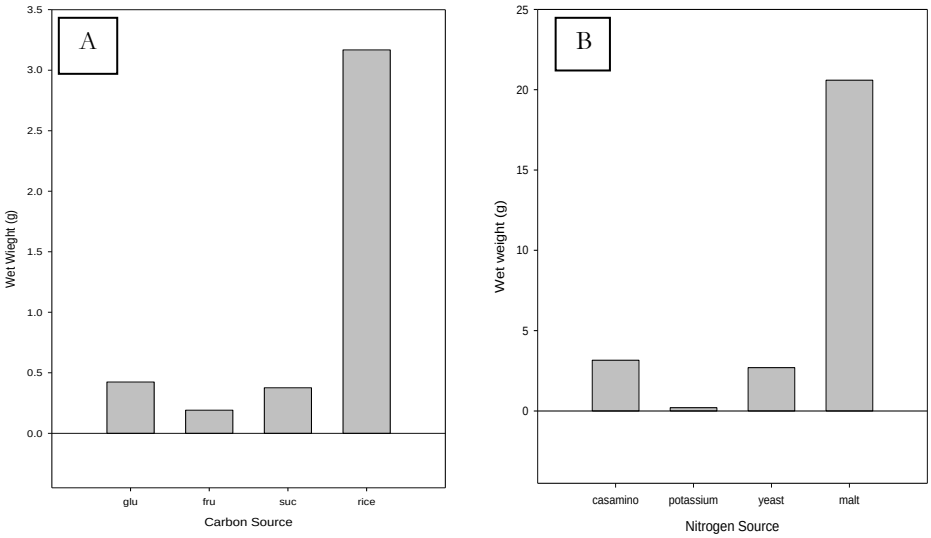

Fig. 1. Effect of different sources of carbon (A) and nitrogen (B) on mycelium biomass of *Exserohilum longirostratum*.

Effect of Nitrogen Source on Growth

For mycelial growth, among the nitrogen sources, malt extract produced the highest yield of mycelium (8.881 g). This was followed by yeast extract (1.598 g), ammonium nitrate (0.266 g) and potassium nitrate (0.113 g) (Fig. 1B). Therefore, malt extract was chosen as the nitrogen source for the C:N ratio test.

Effect of C:N Ratio on Growth

The ratio of C and N did not significantly affect the yield of mycelial dry weight. The C:N ratio of 10:1 resulted in greater production of biomass (Fig. 2). The mycelial production was not influenced by the amount of C added to the medium, whereby the yields were similar for the ratios of 5:1, 7.5:1 and 40:1. The production of mycelial biomass at low C:N ratio was not significantly different compared with the high C:N ratio despite using up the most sugar.

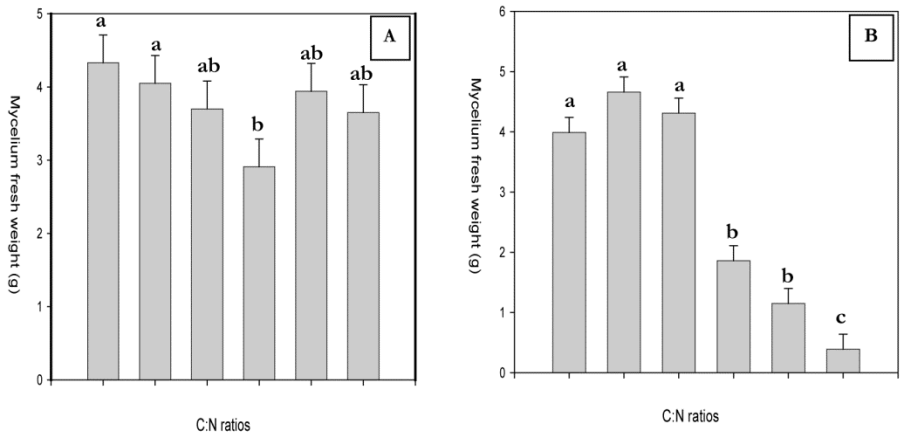

Fig. 2. Effect of different C:N ratios on mycelium biomass of *Exserohilum longirostratum* in fixed nitrogen test (A) and in fixed carbon test (B). Means with similar letter are not significantly different according to Tukey's HSD test ($P < 0.05$).

Effect of C:N Ratio on Sporulation

Sporulation of *Exserohilum longirostratum* was significantly affected by the C:N ratios (Fig. 3). Both the 7.5:1 and 10:1 ratios yielded more spores compared to other ratios but they were not significantly different from each other (6.4×10^5 spores/mL and 6.6×10^5 spores/mL, respectively). The control V8 agar without any added carbon or nitrogen yielded only 1.4×10^5 spores/mL, and thus was about a quarter of the production by the other ratios (except 5:1 and 15:1) (Fig. 3). Without any added nutrients, it was expected that the control production would be low. The spore production was generally the highest on day 2 of the test. The production capacity was high on day 2 because the stress on the mycelium induced more spore production after cutting off the spores during the first harvest. The ratio of 5:1 produced the most spores although the production capacity of all the ratios tested was high on day 2 (Figure 4).

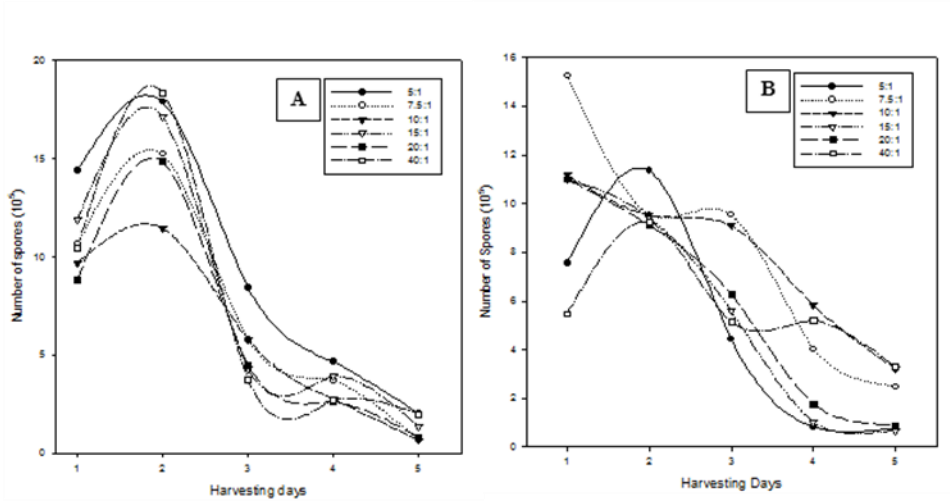

Fig. 3. Effect of different C:N ratios on spore production of *Exserohilum longirostratum* in fixed carbon test (A) and in fixed nitrogen test (B).

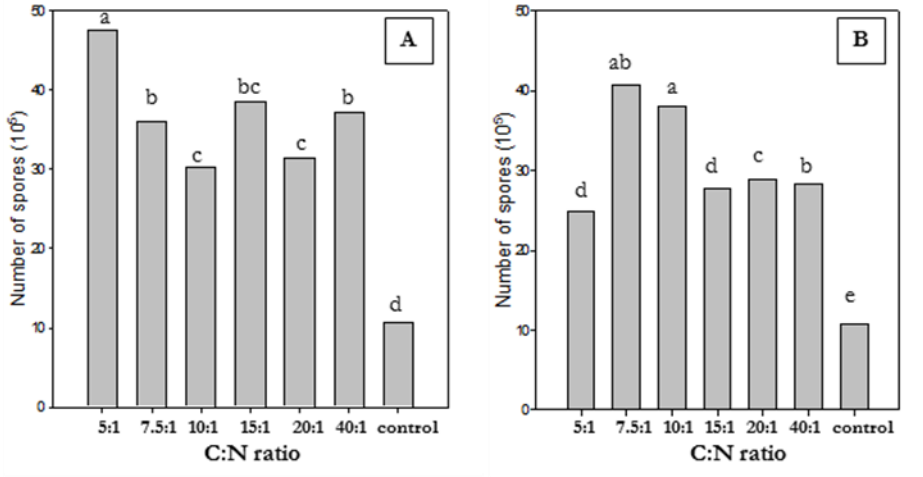

Fig. 4. Effect of C:N ratios on spores production of *Exserohilum longirostratum* in fixed carbon test (A) and fixed nitrogen test (B) on day 2. Means with the same letter are not significantly different ($P < 0.05$)

Effect of C:N Ratio on Conidia Morphology

The effect of C:N ratios on conidia morphology was determined based on the length, width, shape and color of the conidia. The conidia were olive brown, broadest around the basal area and narrowing towards the apex into a long beak, and had rounded ends with the end cells often cut off by a dark, thick septum *i.e.* the hilum. The dimensions of the spores fit well the description for *E. longirostratum* (Subram) sivan spores (Sivanesan, 1987; Alcon, 1988). The addition of external carbon and nitrogen did not alter the morphology of the conidia (Table 1). Manipulating the C:N ratio also did not affect the morphology of the conidia produced (Table 2).

Table 1: Comparison of 200 conidial dimensions produced in different C:N ratio tests with those described in the literature.

Ratio (C:N)	Fixed Carbon Test			Fixed Nitrogen Test		
	Length (µm)	Width (µm)	No. of septum	Length (µm)	Width (µm)	No. of septum
5:1	123.925	12.813	10	148.48	12.147	11
7.5:1	127.725	13.091	10	145.462	12.659	11
10:1	149.664	12.470	11	137.808	12.682	11
15:1	127.962	13.251	10	136.41	12.384	11
20:1	126.957	13.526	10	131.716	12.271	11

According to Sivanesan (1987): conidia dimension: 100 - 434 µm × 12-20.5 µm, number of septa 6-26.

Table 2: The effect of different C:N ratios on mycelial biomass and spore production of *Exserohilum longirostratum* in a basal salt medium for both fixed carbon and fixed nitrogen test.

C:N ratio	Mycelial Biomass (g/100 mL)		Spore Yield (10^5)	
	Fixed Carbon	Fixed Nitrogen	Fixed Carbon	Fixed Nitrogen
5:1	3.993 a	4.330 a	47.460 a	24.890 d
7.5:1	4.661 a	4.048 a	36.015 bc	40.800 ab
10:1	4.313 a	3.702 ab	30.305 c	37.970 a
15:1	1.862 b	2.910 b	38.545 b	27.650 d
20:1	1.149 b	3.939 ab	31.365 c	28.960 c
40:1	0.392 c	3.646 ab	37.215 b	28.340 b

Means followed by the same letter within a column are not significantly different according to Tukey's HSD test ($P < 0.05$).

DISCUSSION

Carbon concentration and C:N ratios are known to affect spore yield and quality, including germinability, pathogenicity and virulence (Jackson and Bothast*,* 1990). There are significant compositional differences in conidia produced in different nutritional environments, such as variation in protein and lipid contents (Jackson and Bothast*,* 1990). Any attempt to optimize spore production must take into account not only the number of spores but also spore efficacy. Glucose is the most widely used carbon source in tissue culture (Griffin, 1981), and the transport and enzyme systems for assimilating it are inherent in fungi so that they need not adapt to the substance before they start to grow. Fungi break down oligosaccharides to monosaccharides for absorption, for example sucrose to glucose and fructose outside the cell membrane before both are absorbed, although glucose is preferentially absorbed. Fungi also require nitrogen for synthesis of amino acids. The nitrogen sources containing protein or protein hydrolysates can support high levels of mycelium production while the inorganic nitrogen sources produce much less. This was probably the main reason for the significantly lower mycelium production with potassium nitrate than malt extract.

The production of mycelia biomass was low with higher C:N ratio. In other words, the biomass production decreased with lower supply of N, and complete absence of nitrogen stopped growth altogether. Thus, if more carbon were to be applied resulting in a high C:N ratio, nitrogen may become a growth-limiting factor. In a nitrogen-scarce situation, *E. longirostratum* may change from growth (producing more mycelium) to reproduction (sporulation) (Yu *et al.,* 1998). Therefore, growth can be increased by higher supply of carbon source only within limits, contingent on nitrogen being adequate. However, the results showed that the fungi remain in the vegetative stage and induced more mycelium production when provided carbon source was in excess of that required.

The highest spore production occurred in the cultures supplied with malt extract and glucose with C:N ratio of 7.5:1 and 10:1. Nevertheless, all the ratios produced higher spore yields than the control (V8). This indicates that some form of optimal balance of carbon and nitrogen are required for high conidia production. However, it is noteworthy that when the carbon applied was increased, it did not support higher spore production yields. This suggests that some inhibitory compound(s) could be produced together with sporulation (Bodo *et al.,* 1985). The spores are on the hyphal tips, so more branching of the mycelium (producing more tips) should increase the spore production. However, this relationship varied under different nutritional conditions, and should be further clarified in future experimentation. Both the mycelium biomass and spore yield were greatly affected by the C:N ratio. Some of the carbon was respired off, while some incorporated into the biomass. Hence, the ideal growth medium should have plentiful carbon so that even with some of it respired off, there would still be an adequate supply for ensuing growth.

The intricacy on the preferential consumption of one nutrient over others remains a frontier issue in culture control. The shortage or surplus of a nutrient(s) may cause the fungus to produce more of some metabolites and less of others, making the production capacity rather unpredictable. One way to do this is to have the medium purposefully unbalanced to veer the fungus to produce the specific metabolite(s) wanted, which can be ascertained and quantified easily.

Manipulating the C:N ratio did not change the morphology of *Exserohilum longirostratum*. The fungus probably retained its potential as a bio-control agent. An understanding of how nutrients influence the growth and sporulation of the fungus is very important for its mass production. Finding the optimal C:N ratio and developing a low-cost medium are currently the most important steps forward. Maintaining or, if possible, enhancing the efficacy of the pathogen by manipulating the culture medium will be the next step to consider.

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