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Effects of carbon sources, nitrogen sources and minerals on mycelial growth of *Cryphonectria parasitica*

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The study was carried out to assess whether nutrients could potentially affect chestnut trees caused by chestnut blight disease. These strains (JS-6, TC-4 and LT-1) were isolated and identified as *Cryphonectria* parasitica by morphologic and molecular method. Cultures in the medium replaced carbon by one of six carbon sources, or nitrogen replaced by one of six nitrogen sources, or element added by one of four different minerals. The results showed the strains used a monosaccharide more efficiently than a disaccharide. The mycelial growth of strains cultured in the medium containing soluble amylum was not significantly different from the one in medium lacking a carbon source. Mycelial growth was more rapid when cultured in the medium containing $Ca(NO_3)_2$ rather than the other nitrogen sources investigated. However, there was no significant difference in mycelial growth in the medium where $CO(NH_2)_2$ was nitrogen and the medium that lacked nitrogen. The medium with 600 mg/L of $MnCl_2$•$4H_2O$ inhibited the mycelial growth of strain LT-1, whereas 600 mg/L of $Na_2B_4O_7$•$10H_2O$ increased mycelial growth. Strains JS-6 and TC-4 showed increased mycelial growth when $CaCl_2$•$4H_2O$ was present in the medium. The results show the medium containing glucose, or $CaCl_2$•$4H_2O$, or $Ca(NO_3)_2$ could be used to increase the rate of mycelial growth in the laboratory and suggest avoiding soils with high calcium content for chestnut production and avoiding use of calcium fertilizers could potentially reduce the severity of the disease in chestnut trees.

Key words: Chestnut blight, nutrient components, growth of mycelia.

INTRODUCTION

Chestnut blight, which is caused by the fungus *Cryphonectria parasitica* (Murr.) Barr, is a serious disease of chestnut trees. *C. parasitica* was first identified in the USA in 1904, and had infected and killed almost all the American chestnut [*Castanea dentata* (Marsh) Borkh] trees in the USA (Anagnostakis, 1982, 1988). When Fairchild (1913) first identified *C. parasitica* in the Chinese chestnut (*Castanea mollissima* Blume) in China, chestnut blight disease was already present in the main chestnut producing areas throughout China and was

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affecting production levels (Zhou et al., 1993; Zhu et al.,1999; Qin et al., 2000). In Europe, chestnut blight was first recorded in northern Italy in 1938, and is now widespread throughout most of Europe where it mainly affects the European chestnut (*Castanea sativa* Mill.) (Robin and Heiniger, 2001).

Chestnut blight causes severe damage to trees, which results in loss of production. However, at present there is no effective way of curing infected trees. Since the first report of chestnut blight, scientists have studied many aspects of the disease, such as the biology (Anagnostakis and Aylor, 1984; Zhou et al., 1996; Guerin et al., 2001; Ni et al., 2008; Zhao et al., 2008), genetic diversity (Milgroom et al., 1992; Xu et al., 2005; Breuillin et al., 2006; Piao et al., 2007; Xu et al., 2008; Adamcikova et al., 2009), water stress (McManus and Ewers, 1990; Gao and Shain, 1995), enzymes (Kim et al., 1992, 2002), secondary metabolism (Havir and Anagnostakis, 1983; Anagnostakis, 1992; Vannini et al., 1993), geographic environment and culture conditions (Cao, 2004; Zhu et al., 2009), control using antifungal compounds (Solel and Shain, 1992; Ni et al., 2008; Tang et al., 2008), and biological control (Robin and Heiniger, 2001).

Although much is now known about the disease and mixed medium of carbon, nitrogen sources and minerals was used for culture of conidia (Puhalla and Anagnostakis, 1971), there are no reports about the effect of different carbon sources, or nitrogen sources, or minerals sources on the mycelial growth of *C. parasitica*. We carried out the study to examine the relationship between mycelial growth and the availability of different carbon sources, or nitrogen sources, or different concentrations of minerals with the aim of determining whether mycelia could be grown more rapidly in the laboratory to aid *in vitro* studies of the fungus, and whether studies of mycelial growth could be explained phenomenon observed in the field to guide control of fungal disease spread with trees and hence reduce timber losses in application.

MATERIALS AND METHODS

Isolation and culture of strains of chestnut blight

We collected chestnut bark showing typical symptoms of infection by *C. parasitica* from Tongcheng, Jinshan and Luotian in primary chestnut production areas in the Hubei province of China. Three strains, JS-6, TC-4, LT-1, were isolated from the infected bark using a routine method (Fang, 1998): that is, bark samples showing symptoms of disease were first rinsed with distilled water. Sections of 2 mm × 2 mm were then excised from the bark and disinfected in 70% alcohol for 30 s and in 0.1% liquid mercury for 0.5 to 1.0 min. The bark sections were then washed three times with sterilized water for 1 min, and cultured on potato-dextrose agar (PDA) at 28°C for 3 days. The white mycelium that grew out from the bark was subcultured on PDA repeatedly to ensure there were no contaminants before inoculation onto PDA slopes in tubes, which were stored in a 4°C refrigerator.

Morphological, ribosomal DNA-internal transcribed spacer (ITS) (rDNA-ITS) sequencing and virulent verification of strains

PDA plates were inoculated with mycelium from the stored strains. Growing mycelia were colorless after 2 days, and light yellow, saffron yellow or colorless after 6 days. Hyphae grew close together, and were 1.1 to 4.5 µm in diameter. Conidiophores were round or oval. After 7 days in culture, strains JS-6 and TC-4 had changed from yellow, saffron yellow or white to a deeper yellow–orange colour. The culture of LT-1 remained white.

Genomic DNA was extracted using the CTAB method (Doyle and Doyle, 1987), and the internal transcribed spacer (ITS) region was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. The ITS sequences of strains were recorded in two directions and compared with ITS sequences of *C. parasitica* in the GenBank database. The sequences had 100% homology with accession numbers EF545115.1 and EF545114.1 (Table 1).

The morphological characteristics of the fungal colony and the molecular biology of the strains confirmed that the strains used in this study were *C. parasitica* (Murr.) Barr. belonged to the Ascomycota, Diaporthales. *C. parasitica* was previously known as *Endothia parasitica*, but was reclassified as *C. parasitica* by Barrm (1978). Based on the colony color and the presence of the canker sizes of the plots inoculated with three strains in culture, strain JS-6 and TC-4 were identified as virulent strains and strain LT-1 as a hypovirulent strain (Wu, 2009).

Different carbon sources used in culture media

To assess the effect of different carbon sources on mycelial growth, the cultures were grown in a Czapek Dox liquid medium (2.00 g KNO_3, 1.00 g KH_2PO_4, 0.50 g KCl, 0.50 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 30.00 g sucrose, 1000.00 g distilled water) (Fang, 1998); however, the 30.00 g of sucrose in the Czapek Dox liquid medium was replaced by 30.00 g of one of six different carbon sources (glucose, soluble amylum, maltose, D-galactose, D-xylose, D-lactose). Czapek Dox liquid medium that lacked a carbon source was used as a control. The culture flasks were inoculated with a plug of mycelia 6-mm in diameter. Each treatment had three replicates. The flasks of culture medium were incubated on an orbital shaker (120 rpm) at 25°C for 6 days. The mycelia was then filtered from the liquid medium with filter paper and dried in an 80°C oven to constant weight, and weighed on electric scales (precision 0.0001 g).

Different nitrogen sources used in culture media

To assess the effect of different nitrogen sources on mycelial growth, the cultures were grown in a Czapek Dox liquid medium; however, the 2.00 g of KNO_3 used as a nitrogen source in the Czapek Dox liquid medium was replaced with 2.00 g of one of six different nitrogen sources [NH_4NO_3, $Ca(NO_3)_2$, NH_4Cl, $CO(NH_2)_2$, glycine, peptone]. Czapek Dox liquid medium that lacked a nitrogen source was used as a control. The culture flasks were inoculated with a plug of mycelia 6-mm in diameter. Each treatment had three replicates. The flasks of culture medium were incubated on an orbital shaker (120 rpm) at 25°C for 7 days. The mycelia was then filtered from the liquid medium with filter paper and dried in an 80°C oven to constant weight, and weighed on electric scales (precision 0.0001 g).

Different minerals used in culture media

To assess the effect of different minerals on mycelial growth, the

Table 1. Homology comparison of ITS sequences of isolated strains with strains of *C. parasitica* in GenBank.

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Max identity
AY141873.1	*C. parasitica*	904	904	92	0.0	95
AY309482.1	*C. parasitica*	1125	1125	93	0.0	99
EF545115.1	*C. parasitica*	1127	1127	100	0.0	99
EF545114.1	*C. parasitica*	1018	1018	100	0.0	99

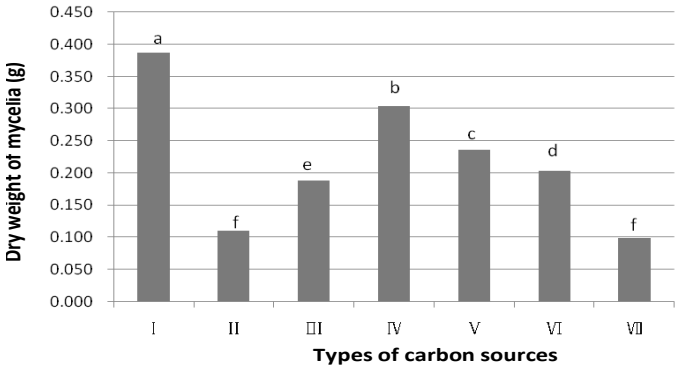

Figure 1. Mean dry weight of mycelia of three stains produced when cultured for six days in liquid medium containing different carbon sources: I, glucose; II, soluble amylum; III, maltose; IV, D-galactose; V, D-xylose; VI, D-lactose; VII, none (control). Different letters (a,b,c,d,e,f) above the bars indicate significant differences between treatments (p = 0.05).

cultures were grown in Czapek Dox liquid medium to which a primary element (Ca, B, Mn or Zn) used by chestnut trees during the growing period (Chen, 2003) had been added. Three different concentrations of the minerals were tested: 200, 400 and 600 mg/L. Czapek Dox liquid medium that lacked any of the selected primary elements was used as a control. The culture flasks were inoculated with a plug of mycelia 6-mm in diameter. Each treatment had five replicates. The flasks of culture medium were incubated on an orbital shaker (120 rpm) at 25°C for 7 days. The mycelia was then filtered from the liquid medium with filter paper and dried in an 80°C oven to constant weight and weighed on electric scales (precision 0.0001 g).

Statistical software

Mycelial growth under the different treatment regimes was recorded and analysed using SAS software (V8.1). The minimum significant method of residual (least significant difference, LSD) was compared among treatments, and Excel software was used for drawing plots.

RESULTS

Effects of different carbon sources on mycelial growth

The three strains of *C. parasitica* were all able to use the six different carbon sources tested in the different treatments. The mean dry weight of mycelia of the three strains that was produced using the different carbon sources was significantly different from each treatment and significantly greater than that produced by the control treatment except for the soluble amylum treatment (Figure 1). The greatest amount of mycelial growth was achieved when glucose was used as the carbon source (0.3955 g dry weight after culture for 6 days), followed by D-galactose, D-xylose, D-lactose and maltose. The least mycelial growth (0.1106 g dry weight) occurred when soluble amylum was used as the carbon source.

Effects of different nitrogen sources on mycelial growth

The mean dry weight of mycelia of the three strains produced using the different nitrogen sources was significantly different from each treatment and was significantly different from the control except for the treatment that used $CO(NH_2)_2$ as the nitrogen source (Figure 2). The greatest amount of mycelial growth was achieved using $Ca(NO_3)_2$ as the nitrogen source (0.4982 g dry weight), followed by peptone, NH_4Cl, glycine and $CO(NH_2)_2$. The least mycelial growth occurred when NH_4NO_3 was used as the nitrogen source: the dry weightwas even lower than that produced by the control.

Effects of different minerals on mycelial growth

The addition of one of the minerals used by the chestnut tree during the growing period to the liquid culture medium

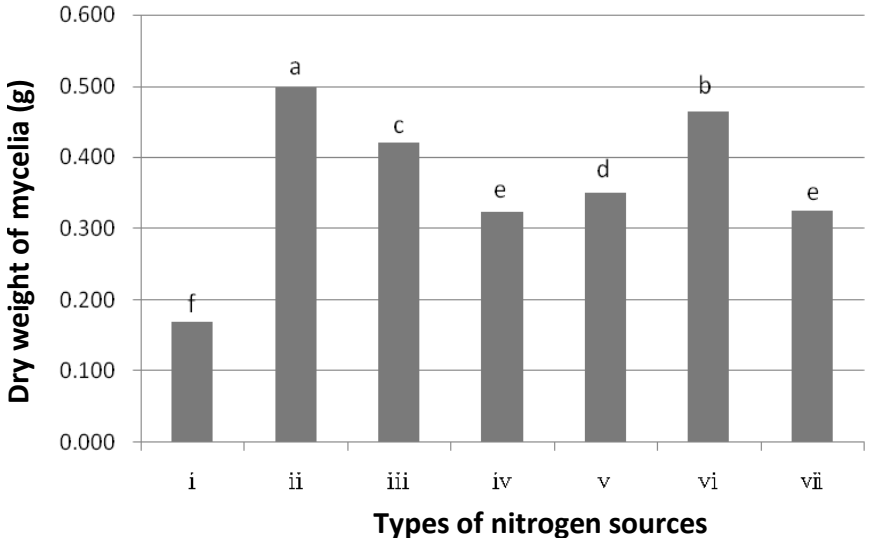

Figure 2. Mean dry weight of mycelia of three stains produced when cultured for seven days in liquid medium containing different nitrogen sources: i, NH_4NO_3; ii, $Ca(NO_3)_2$; iii, NH_4Cl; iv, $CO(NH_2)_2$; v, glycine; vi, peptone; vii, none (control). The different letters (a,b,c,d,e,f) above the bars indicate significant differences between treatments (p = 0.05).

Table 2. Dry weight of mycelia produced when cultured for seven days in liquid medium containing different minerals.

		Dry weight of mycelia (g)		
Mineral compound	**Concentration (mg/L)**	**JS-6 strain**	**TC-4 strain**	**LT-1 strain**
$MnCl_2 \cdot 4H_2O$	0	0.3923^{AB}	0.4433^{A}	0.2742^{A}
	200	0.3413^{AB}	0.4010^{AB}	0.1367^{B}
	400	0.2002^{B}	0.4008^{AB}	0.2034^{AB}
	600	0.4133^{A}	0.3529^{B}	0.1201^{B}
$Na_2B_4O_7 \cdot 10H_2O$	0	0.3923^{AB}	0.4433^{B}	0.2742^{B}
	200	0.1702^{B}	0.4766^{B}	0.3565^{AB}
	400	0.2004^{B}	0.5833^{A}	0.3702^{AB}
	600	0.4521^{A}	0.5528^{A}	0.4564^{A}
$ZnSO_4 \cdot 7H_2O$	0	0.3923^{A}	0.4433^{A}	0.2742^{C}
	200	0.4003^{A}	0.3433^{A}	0.4566^{A}
	400	0.4403^{A}	0.2677^{A}	0.3852^{B}
	600	0.4856^{A}	0.2367^{A}	0.4011^{AB}
$CaCl_2 \cdot 4H_2O$	0	0.3923^{C}	0.4433^{B}	0.2742^{A}
	200	0.5862^{A}	0.5631^{A}	0.2333^{A}
	400	0.5107^{B}	0.5524^{A}	0.2922^{A}
	600	0.4326^{C}	0.5910^{A}	0.1416^{B}

Different letters next to the dry mycelial weights listed by column for each mineral treatment indicate mean significance differences between treatments (p = 0.01) using the Duncan multiple range tests.

produced different mycelial growth rates depending on the mineral and the concentration (Table 2). Culture medium with 600 mg/L of MnCl2•4H2O reduced the mycelial growth of strains TC-4 or LT-1 significantly compared with the mycelial growth in the control flasks, whereas culture medium with 600 mg/L of Na2B4O7•10H2O showed increased mycelial growth of strains TC-4 or LT-1 compared with the control flasks. Culture medium with ZnSO4•7H2O showed increased mycelial growth of strain LT-1 at all three concentrations. Culture medium with 200 and 400 mg/L of CaCl2•4H2O showed increased mycelial growth of strains JS-6 or TC-

4, but had no effect on the mycelial growth of strain LT-1.However, culture medium with 600 mg/L of $CaCl_2 \cdot 4H_2O$ reduced the mycelial growth of strain LT-1.

DISCUSSION

When soluble amylum was the only available carbon source, mycelial growth was not significantly different from that of the control, whereas the other carbon sources had positive effects on the growth of mycelia (D galactose > D-xylose > D-lactose > maltose). The results indicate that *C. parasitica* uses monosaccharides such as glucose most efficiently, followed by disaccharides such as lactose and then amylose such as amylum. A possible reason for this phenomenon is that mycelia digest simple carbon sources more easily than complex carbon sources. To grow cultures of *C. parasitica* more rapidly in the laboratory, carbon sources such as glucose should be used in the culture medium.

All the nitrogen sources tested effected mycelial growth except the culture medium containing $CO(NH_2)_2$, which showed the same mycelial growth rate as the mycelia in the control flask. Salts with the same NO_3^- in the culture liquid appeared to show the opposite effect, that is, mycelial growth was significantly faster in culture medium containing $Ca(NO_3)_2$ than in medium where NH_4NO_3 was the nitrogen source.

This phenomenon suggests that both the nitrogen source and the cation of the salts had an effect on mycelial growth. For culturing mycelia, $Ca(NO_3)_2$ is the better nitrogen source. Griffin (1992) found that many chestnut trees at two sites in Virginia (USA) did not sprout after dying from chestnut blight disease. Both of these sites had much higher nitrogen levels than sites with good survival rates, and the site with the poorest survival rate had high levels of calcium in the soil. The results observed by Griffin (1992) in his field investigation correspond to the findings in this study that high nitrogen and calcium concentrations increase mycelial growth. These findings from the laboratory and the field could give some guidance as to the most appropriate fertilizer to use for chestnut production. Field studies are further needed to compare whether applying $CO(NH_2)_2$ or NH_4NO_3 as a fertilizer in chestnut stands rather than $Ca(NO_3)_2$ would reduce the damage caused by chestnut blight disease.

The addition of minerals that are needed by the chestnut tree during the growing period to the liquid culture medium had different effects on mycelial growth. The treatment with added $MnCl_2 \cdot 4H_2O$ significantly reduced mycelial growth of strains TC-4 or LT-1 compared with the control treatment. This is not surprising because the manganese ion is a component of some fungicides. The treatment with 600 mg/L of $Na_2B_4O_7 \cdot 10H_2O$ significantly increased mycelial growth of strains TC-4 or LT-1. Generally, boron is required for cell elongation, hence, and enhances mycelial growth. The treatment only with added $ZnSO_4 \cdot 7H_2O$ had a positive effect on the growth of strain LT-1. The treatment with added $CaCl_2 \cdot 4H_2O$ was as effective at increasing the mycelial growth of strains JS-6 or TC-4 as the treatment with $Ca(NO_3)_2$. It seems that calcium has a positive effect on mycelial growth. Just as the evidence, Karamushka and Gadd (1994) have shown that *Saccharomyces cerevisiae* reproduction occurs more rapidly when calcium has been added to the culture medium, and a high level of mortality has been found among chestnut trees growing in soils containing high levels of calcium (Griffin, 1992). At concentrations of 200 or 400 mg/L, the presence of $CaCl_2 \cdot 4H_2O$ in the liquid medium did not significantly affect the mycelial growth of strain LT-1; however, at 600 mg/L mycelial growth was inhibited. The inhibition of this hypovirulent strain at 600 mg/L might explain why chestnuts growing in soils with high calcium levels show high levels of mortality as a result of infection by *C. parasitica* if high levels of calcium in the soil enhances the mycelial growth of virulent strains and controls the mycelial growth of hypovirulent strains. Perhaps hypovirulent strains use a different mechanism to absorb minerals compared with that used by the virulent strains. To increase the rate of culture development of virulent strains in the laboratory, we suggest adding $CaCl_2 \cdot 4H_2O$ and $Ca(NO_3)_2$ to the culture medium. To reduce the damage caused by chestnut blight disease, soils with high calcium content should be avoided for chestnut production, and calcium fertilizers should not be applied.

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