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Selection of Nitrate-nonutilizing Mutants of *Hypoxylon atropunctatum*, a Fungal Pathogen on Oak Species

Se-Chul Chun* and P. Fenn[1]

Pathogen Research Division, National Plant Quarantine Service, Anyang 430-016, Korea
[1]Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701, USA.

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Latent infection of healthy-appearing oaks of *Hypoxylon atropunctatum* complicates field studies by interfering with inoculation experiments to follow pathogenesis, fungal development and reproduction of this canker rot fungus. Mutants with unique and easily scorable phenotypes would be useful for inoculation studies. There is a broad range in the capacity of wild-type isolates to utilize nitrate as a sole nitrogen sources. Several types of nitrate-nonutilization mutants (*nit*1, Nit3, NitM) were selected from nitrate-utilizing wild-type isolates. Also, a few mutants of *Hypoxylon atropunctatum* were selected that could only grow poorly on basal medium supplemented with various nitrogen sources and even on yeast extract agar. These unknown mutants need to be characterized further. *Nit* mutants of *Hypoxylon atropunctatum* were readily selected, grew well and were recovered after inoculation into oak stems. These results suggest that *nit* mutants could be useful for inoculation studies in trees that contain latent infections.

Keywords **:** *Hypoxylon atropunctatum*, nitrate-nonutilizing (*nit*) mutant, oak, inoculation studies, canker rot.

Species of *Hypoxylon*, which occur primarily on hardwood trees, are ascomycetes in the sub-class Pyrenomycetes and family Xylariaceae (Webster, 1980). *H. atropunctatum* (Schwein.: Fr.) Cooke. occurs mainly on *Quercus* spp. but has been collected on species of *Acer*, *Fagus*, *Tilia*, *Malus*, *Ostrya* and *Platanus*. It has been reported only in the United States (Miller, 1961). Extensive oak decline and death have been reported to occur following severe droughts. Such incidences have been documented in areas within Pennsylvania (Fergus et al., 1956), West Virginia (Tryon et al., 1958), Florida, Mississippi, and Arkansas (Lewis, 1981; Bassett et al., 1982).

H. atropunctatum is known to be as an early colonizer of both declining and the dead trees (Tainter et al., 1983) but its role in tree decline and death is unclear. In addition, it has been shown to latently infect healthy-appearing oaks (Bassett and Fenn, 1984). This complicates field studies by interfering with inoculation experiments to follow pathogenesis, fungal development and reproduction of this canker rot fungus. Mutants with unique and easily scorable phenotypes would be useful for inoculation studies. Nitrate-nonutilizing mutants (*nit* mutants) have been selected from numerous fungi and have been used to test for vegetative compatibility in several species of pathogenic fungi (Puhalla, 1985; Correll et al., 1987; Brooker et al., 1991). *Nit* mutants also have been used to differentiate strains in the ubiquitous nonpathogenic portion of the *F. oxysporum* population (Correll et al., 1986). Nitrogen metabolism by species of *Hypoxylon* has not been investigated and *nit* mutants have never been selected from any species of this genus. The possibility of selecting *nit* mutants of *H. atropunctatum* that express easily observable phenotypes was the impetus for this research. The objectives were, to determine if nitrate-nonutilizing mutants could be selected from *H. atropunctatum*, to characterize these *nit* mutants, and to determine if *nit* mutants colonize oak stem tissues to a similar extent as wild-type isolates.

Materials and Methods

Media. Yeast extract glucose agar (YEGA) contained 1.5 g yeast extract (Difco), 10 g D-glucose and 11.5 g agar per liter of distilled water. The basal medium used in this study was the one described by Puhalla (1985) and Correll et al. (1987). Minimal medium (MM) was prepared by adding 4 g of $NaNO_3$ to 1 L of basal medium. *Nit* mutants were selected on agar minimal medium with chlorate (MMC), which contained 4 g of $NaNO_3$, and 15 g of $KClO_3$ in 1 L of basal medium. MMC did not contain asparagine as used by Correll et al. (1987).

Screening for ability to utilize nitrate. Twenty-six single ascospores or mycelial isolates (strains) which had been stored on YEGA slants at 4°C, were grown on MM at 28°C for 3 days. Three pieces of agar (2 mm square) with mycelium were transferred to 50 ml of liquid MM in cotton-plugged 250 ml flasks and incubated at 28°C to determine if any isolates utilize nitrate as a sole nitrogen source.

After they had grown on liquid MM for 21 days, mycelia were

*Corresponding author.
Phone) +82-31-445-1225, FAX) +82-31-447-0525
E-mail) sechulc@yahoo.com

harvested on to Whatman No.1 filter paper under vacuum and washed with distilled water. The washed mycelia were oven dried (70°C) for 24 hr and dry weights were measured. Three replicate were done for each isolate.

Selection of *nit* mutants. For each isolate, five agar blocks (about 2 mm^2) with mycelium on MM were transferred to plates of MMC. Ninety plates for wild-type isolate 86#5, 62 plates for wild-type isolate 86#2 and 39 plates for wild-type isolate #1 were used to select chlorate-resistant sectors. The plates were incubated at 28°C and examined twice a day for the appearance of fast-growing sectors from the initially restricted colony. Fast growing sectors were transferred to MM, and those that grew as sparse colonies with no aerial mycelium were considered as *nit* mutants. All *nit* mutants were resistant to chlorate and most showed wild-type growth on YEGA.

Phenotypic classification of *nit* mutants. The phenotypes of *nit* mutants were determined by culture on basal medium supplemented with five nitrogen sources (Correll et al., 1987): 1) MM as described above, 2) basal medium amended with 0.1 g/L of sodium nitrite, 3) basal medium amended with 0.2 g/L of hypoxanthine, 4) basal medium amended with 1 g/L of ammonium tartrate, and 5) basal medium amended with 0.2 g/L of uric acid. A mycelial block (about 2 × 2 mm) of each *nit* mutant on MM was plated on each of these five media. The plates were incubated at 28°C for 7 days and culture characteristics were scored compared to the wild-type isolates on the same media.

Preparation of stem segments and inoculation. The stems of Shumard oak (*Quercus shumardii* Buckl.) for the inoculation with wild-type isolates and their *nit* mutants were about 3 years old stump sprouts (9 mm in diameter) collected near Lake Wedington in the Ozark National Forest near Fayetteville, Arkansas, U. S. A. In the laboratory, leaves and side branches were removed, and the stems were cut into 18-cm-long segments. Stem segments were washed with distilled water and disinfected in 1% sodium hypochlorite (1 : 4 v/v Clorox, Clorox Co., CA) containing 0.02% Tween 20 for 20 minutes with periodic agitation, washed under a continuous stream of distilled water for 15 min, and laid on sterile paper towels in a transfer hood to dry. The cut surfaces were sealed with liquified paraffin wax. The stems were wounded (approximately 3 mm diameter and 2 mm deep) and inoculated with mycelium of wild-type isolates (86#5 and 86#2) and their *nit* mutants (two *nit1* and one Nit3 mutant of 86#5, and two *nit* 1 mutants of 86#2). Two-day-old cultures grown on YEGA were used. Inoculation sites were wrapped in two layers of sterile cheesecloth, moistened with about 0.2 ml of sterile distilled water and sealed with two layers of Parafilm (Dixie/Marathon Co., CT).

Growth of *nit* mutants in detached oak stems. Replicate stem segments wound inoculated with the five *nit* mutants were incubated at 25°C at low humidity, about 50% RH. Stem segments inoculated with wild-type isolates were incubated at both high humidity (about 100% RH) and low humidity. After 5.5 days incubation, pieces were plated on YEGA to recover the fungi to determine how far they had grown from the inoculation sites on stem segments. Two colonies of *H. atropunctatum* recovered from each stem were plated on basal medium amended with the various nitrogen sources to determine their phenotypes.

Measurement of relative water content. Samples containing two or three pieces of stem tissues with both xylem and phloem were cut from two different locations of each stem segment. The tissue pieces were approximately 3 mm wide and 2 mm deep. These were weighed and placed in a 5-cm petri dish containing 10 ml of distilled water and soaked at room temperature (19-20°C) for 24 hr. After excess surface water was removed by blotting, the saturated weight was taken and the pieces were dried at 60°C for 24 hr when the dry weight was measured. The samples from each stem were averaged to give the relative water content of each stem segment. Initial relative water content before incubation as previously and the final relative water content after incubation were determined.

Results

Growth of wild-type isolates on liquid MM. Of the 26 isolates grown on liquid MM for 21 days, 18 used nitrate poorly, two were intermediate and six used nitrate well as a sole nitrogen source (Fig. 1). Three of the latter isolates (86#2, 86#5 and #1) were used to select *nit* mutants by their resistance to chlorate.

Selection and classification of *nit* mutants. Chlorate-resistant sectors first appeared usually 4 or 5 days after the fungi were plated on MMC. Chlorate resistant sectors did not develop if asparagine was added to MMC. All chlorate resistant sectors showed sparse growth on MM. While wild-type isolates showed abundant mycelial development with aerial hyphae on MM after 7 days, *nit* mutants showed thin hyphae, sparse mycelium, and little or no aerial

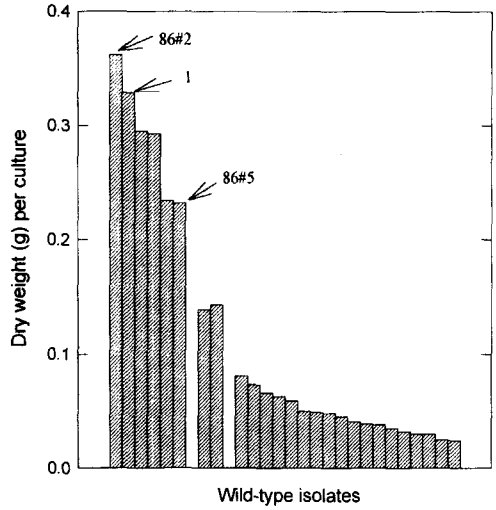

Fig. 1. Growth of *Hypoxylon atropunctatum* wild-type isolates. Three pieces of agar (2 mm square) with mycelium from each isolate were transferred to 50 ml of liquid MM in cotton-plugged 250 ml flasks and incubated at 28°C. Mycelia were harvested on to Whatman No. 1 filter paper under vacuum and washed with distilled water. Dry weights of the oven dried mycelia at 70°C for 24hr were measured.

Table 1. Phenotypic classification of nitrate nonutilizing (*nit*) mutants from *Hypoxylon atropunctatum* by growth on basal medium supplemented with various nitrogen sources

Mutant Designation	Phenotype on various nitrogen sources[a]				
	Nitrate	Nitrite	Ammonium	Hypoxanthine	Uric Acid
Wild type	+	+	+	+	+
nit1	−	+	+	+	+
Nit3	−	−	+	+	+
NitM	−	+	+	−	+

[a] + = abundant mycelial development with aerial hyphae.
− = thin hyphae, sparse mycelium, little or no aerial hyphae.

Table 2. Frequency and phenotypes of nitrate nonutilizing (*nit*) mutants recovered from wild-type isolates plated on chlorate-amended minimal medium

Isolate[a]	Sectors per colony[b]	Number of potential *nit* mutant examined	*nit* mutant classes[c] (number)			
			nit1	Nit3	NitM	Unknown[d]
86#5	0.245	110	64	29	0	17
86#2	0.240	75	55	15	2	3
1	0.150	29	24	4	0	1

[a] See Figure 1.
[b] Mean frequency of chlorate-resistant sectors per colony.
[c] *nit* phenotypes determined by growth on basal medium amended with different nitrogen sources (see Table 1).
[d] Poor growth on any of nitrogen sources tested.

hyphae. Two hundred and fourteen prospective *nit* mutants from chlorate-resistant sectors were cultured on basal medium supplemented with one of five nitrogen sources to determine their phenotypes (Correll et al., 1987; Brooker et al., 1991) (Table 1). The frequency of *nit1* mutants were much higher than that of Nit3 or NitM. Of 110 potential *nit* mutants examined from wild-type isolate 86#5, 64 were *nit1* mutants, 29 were Nit3, and 17 were unknown. No NitM was selected. Of 75 potential *nit* mutants from wild-type isolate 86#2, 55 were *nit1* mutants, 15 were Nit3, 2 were NitM and Nit3 were unknown. Of 29 potential *nit* mutants from wild-type isolate #1, 24 were *nit1*, 4 were Nit3 and 1 was unknown, no NitM was selected (Table 2).

Growth of *nit* mutants in detached oak stem segments. After inoculation into oak stem segments, all *nit* mutants grew similarly to their parent wild-type isolates as the relative water content of the tissues decreased under incubation at low relative humidity (Table 3). The mean growth (14.0 cm) of all *nit* mutants of 86#5 and 86#2 was not significantly different from that (14.6 cm) of their wild-type isolates (*t* test, P=0.05). The average initial relative water content of stem segments inoculated with wild-type isolates and incubated at high humidity (about 100%) was 79.3% and the final relative water content was 74.5%. The average initial relative water content of stem segments inoculated with *nit* mutants and incubated at low humidity was 79.3% and the final relative water content was 60.2% (Table 3). In all cases, the two colonies recovered from each inoculated stem gave the expected phenotypes when cultured on the different nitrogen sources.

Table 3. Growth and recovery of *Hypoxylon atropunctatum* wild-type isolates and *nit* mutants from detached oak stems

Isolate or mutant[b]	Growth[a] (cm)		Recovery of *nit*[e] mutant
	High RH[c]	Low RH[d]	
86#5 wild-type	5.5	15.0	
86#5 *nit1*		13.5	+
86#5 *nit1*		12.8	+
86#5 Nit3		14.6	+
86#2 wild-type	6.0	14.3	
86#2 *nit1*		14.6	+
86#2 *nit1*		14.3	+
wild-type[f]		14.6	
nit mutants		14.0	

[a] The isolates or mutants were inoculated in each stem.
[b] Growth after 5.5 days under ca. 100% RH or ca. 50% RH.
[c] Initial relative water content = 79.3%, final = 74.5%.
[d] Initial relative water content = 79.3%, final = 60.2%.
[e] Two colonies recovered from each inoculated stem were checked for proper phenotype on the different nitrogen sources. Two stems were used for each isolate or mutant.
[f] The mean growth of all *nit* mutants of 86#5 and 86#2 was not significantly different from that of their wild-type isolates (*t* test, P = 0.05).

Discussion

Of the 26 wild-type isolates of *H. atropunctatum*, most did not utilize nitrate well (Fig. 1). It is generally considered that fungi inhabiting and reproducing on wood do not utilize nitrate well as a sole nitrogen source. Woody tissues usually contain only 0.03-0.10% N by weight, whereas herbaceous tissues typically contain 1-5% N (Allison and Murphy, 1963; Crook and Holden, 1948). Wood with such a high C : N ratio would be very low in N, and most saprophytic microorganisms would grow poorly on it, but wood-destroying fungi are well adapted to wood as a substrate despite its meager N content (Cowling and Merrill, 1966). *H. atropunctatum* isolates appear to vary widely in their capacity to utilize NO_3. Whether this is actual or an artifact of culture storage needs to be examined. All isolates used had been stored for four or more years and many may have lost their ability to utilize NO_3.

No chlorate-resistant sector of *H. atropunctatum* was found if MMC contained asparagine which is often required to keep other fungi alive on MMC (Correll et al., 1987).

In the present work, the amount of nitrate were increased from 2 g/L to 4 g/L in the MMC as reported by Correll et al. (1987) and asparagine was not added. Nitrite was very toxic to all *nit* mutants when used at 0.5 g/L as was added by other researchers (Correll et al., 1987; Brooker et al., 1991), however, when nitrite was decreased to 0.1 g/L, all *nit* mutants grew except for Nit3.

All of the chlorate-resistant sectors from the three wild-type isolates were unable to use nitrate as a sole nitrogen source and are assumed to be *nit* mutants (Table 1). Most of *nit* mutants were *nit1* and few were Nit3 or NitM, therefore, selection of *nit* mutants in *H. atropunctatum* was similar to the other filamentous fungi which have been studied (Table 2). No chlorate-resistant, nitrate-utilizing mutant (*crn* mutant) was selected, which is similar to the studies of *Colletotrichum* (Brooker et al., 1991) and *Neurospora* (Tomsett and Garrett, 1980) but different from *Fusarium* (Klittich and Leslie, 1989) and *Aspergillus* (Cove, 1976). Correll et al. (1987), working with *Fusarium*, suggested that chlorate-resistant sectors might be homokaryotic or heterokaryotic. Individual microconidia from homokaryotic sectors were chlorate-resistant, nitrate-utilizing mutants (*crn* mutants) and microconidia recovered from heterokaryotic sectors were often a mixture of *nit* mutant conidia, wild-type conidia, and/or *crn* mutant conidia (Correll et al., 1987).

The frequencies of recovery of *nit* mutants of *H. atropunctatum* (range 0.15-0.25 sectors per colony on MMC) (Table 2) were much lower than from *Fusarium* (range 0.33-0.96) or *Colletotrichum* (range 1.1-1.25) (Correll et al., 1987; Brooker et al., 1991) indicating that *H. atropunctatum* may be genetically more stable than these other fungi. These fungi are considered to be genetically unstable in culture but the cause of instability is unknown (Correll et al., 1987; Klittich and Leslie, 1988; Brooker et al., 1991). Klittich and Leslie (1988) suggested that instability in *Fusarium moniliforme* might be associated with a transposable element. Transposon movement has been associated with high mutation frequencies in a number of eukaryotic organisms, including yeast (Roeder et al., 1980), *Drosophila* (Green, 1980; Engels, 1983), and maize (Lillis and Freeling, 1986).

A few chlorate-resistant sectors of *H. atropunctatum* were selected that grew poorly on basal medium supplemented with the five different nitrogen sources and on YEGA (Table 2). Chlorate is considered to cause mutations of *nit* loci and at loci which are unrelated to nitrate anabolism (Brooker et al., 1991). Alternatively, these mutants may be mutated at loci such as those described by Cove (1976 and 1979) in *Aspergillus* in which chlorate may cause a general cessation of nitrogen metabolism rather than a simple inactivation of the nitrate assimilation system.

The growth of *nit* mutants in oak stem segments held under low humidity was very similar to that of the wild-type parent isolates indicating that these mutants respond similarly to stress in the host tissue (Table 3). Therefore, it appears that *nit* mutants have phenotypes that could be used for inoculation studies in healthy and stressed trees.

In summary, there is a broad range in the capacity of wild-type isolates to utilize nitrate as a sole nitrogen source. Several types of *nit* mutants (*nit1*, Nit3, NitM) were selected from nitrate-utilizing wild-type isolates. Also, a few mutants of *H. atropunctatum* were selected that could only grow poorly on basal medium supplemented with various nitrogen sources and even on YEGA. These unknown mutants need to be characterized further. *Nit* mutants of *H. atropunctatum* were readily selected, grew well and were recovered after inoculation into oak stems. These results suggest that *nit* mutants could be useful for inoculation studies in trees that contain latent infections.

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