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Microbiological Research **163** (2008) 431–440

Biological and physiological characteristics of *Neotyphodium gansuense* symbiotic with *Achnatherum inebrians*

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Received 22 January 2005; received in revised form 19 July 2006; accepted 20 July 2006

KEYWORDS
Neotyphodium gansuense;
Endophyte;
Achnatherum inebrians;
Biology;
Physiology

Summary

Biological and physiological characteristics of *Neotyphodium gansuense* were compared with *Neotyphodium coenophialum* and *Epichloë festucae* at a range of temperatures and pH values, and on carbon and nitrogen amended media. *N. gansuense* was able to grow at 10–30 °C, but not at 5 °C, and slowly at 35 °C. The optimal temperature for both *N. gansuense* and *N. coenophialum* was 25 °C, but that of *E. festucae* was 20–25 °C. The optimal pH ranges for mycelial growth of *N. gansuense*, *N. coenophialum* and *E. festucae* were 5–9, 5–9 and 5-7, respectively. The *Neotyphodium* and *Epichloë* endophytes varied in their ability to grow on media containing different carbon and nitrogen nutrients. The preference of *N. gansuense* for carbon source was sucrose > glucose, lactose, sorbitol, inulin, maltose, mannitol, starch, fructose > xylose. Growth of all three endophytes tested was significantly improved by peptone, tryptone, casein, yeast extract and L-proline. Yeast extract, peptone, casein, tryptone, L-proline, potassium nitrate, ammonium oxalic acid and L-leucine significantly improved growth of *N. gansuense*. However, ammonium nitrite was not utilized at all by any tested endophyte. *N. gansuense* grew significantly better on potato dextrose agar (PDA) and oat meal agar (OMA) than on corn meal agar (CMA) and drunken-horse-grass agar (DA), and most slowly on water agar (WA) and saltwater nutrient agar (SNA).
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Introduction

Plant–microbial associations are important symbioses and may play an important role in structuring

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0944-5013/$ - see front matter © 2006 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2006.07.007

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plant communities and ecosystems (Saikkonen, 2000; Clay and Schardl, 2002; Rudgers et al., 2004; Saikkonen et al., 2004; Schardl et al., 2004; Müller and Krauss, 2005). Symbiotic interactions of green plants with bacteria and fungi are widespread, and include plants infected by nitrogen-fixing bacteria, mycorrhizal fungi or endophytic fungi (Clay, 1990; Clay and Schardl, 2002; Rudgers et al., 2004). Numerous studies have revealed a widespread mutualistic associations between grasses and endophytic fungi, which infect systemically and intercellularly within the vegetative and reproductive tissues of host plants (Siegel et al., 1987; Clay, 1990; Schardl and Phillips, 1997; Faeth, 2002). These endophytes mostly belong to the genera *Epichloë* and *Neotyphodium* of the Ascomycete family Clavicipitaceae or to their anamorphs (Clay, 1990; Schardl et al., 1994; Glenn et al., 1996; Craven et al., 2001; Schardl et al., 2004). However, interactions of *Epichloë* endophytes with their grass hosts span the symbiotic continuum from antagonism to mutualism (Schardl, 1996; Schardl et al., 2004).

Endophytic *Neotyphodium* and some *Epichloe* species are vertically transmitted via the seeds of infected plants and can confer biological protection from insects (Siegel et al., 1990; Latch, 1993), nematodes (Latch, 1993; Eerens et al., 1998), grazing livestock (Bacon et al., 1977; Fletcher and Harvey, 1981; Faeth and Bultman, 2002), wildlife (Conover, 2003a, b), fungal diseases (Welty et al., 1991; West and Gwinn, 1993; Nan and Li, 2000) and viral disease (West et al., 1990), together with enhanced drought tolerance (Bacon, 1993; West and Gwinn, 1993; Malinowski and Belesky, 2000), nutrient acquisition (Malinowski and Belesky, 2000) and competitive ability (Hill et al., 1991; Malinowski et al., 1997; Clay and Holah, 1999; Müller and Krauss, 2005). Although these advantages of endophytes are well-characterized in agronomic grasses, some research in natural systems has suggested that not all such associations are defensive mutualisms (Saikkonen et al., 1998; Faeth, 2002; Faeth and Sullivan, 2003; Müller and Krauss, 2005).

Despite the advantages of endophytes, they can have economic costs because some cause livestock problems throughout the world. *Neotyphodium coenophialum* and *Neotyphodium lolii* cause tall fescue toxicosis (Bacon et al., 1977) and ryegrass staggers (Fletcher and Harvey, 1981), respectively. In China, *Neotyphodium gansuense* is symbiotic with drunken horse grass (*Achnatherum inebrians*) (Li et al., 2004b), which is reported to be toxic to livestock. It is distributed throughout the arid, semi-arid, alpine and subalpine grasslands in Gansu, Xinjiang, Qinghai and Inner Mongolia of China (Shi, 1997; Li et al., 2004a). *N. coenophialum* is the most common endophyte symbiotic with *Festuca arundinacea* in North America, and it has been well studied (Siegel et al., 1987; Schardl and Siegel, 1993; Schardl and Phillips, 1997). *Epichloë festucae* is a parasitic or symbiotic fungus isolated from *Festuca rubra*, in which it can be transmitted horizontally or vertically (Schardl, 1996, 2001; Zabalgogeazcoa et al., 2005). So far, at least 15 species of *Neotyphodium* (Li et al., 2004b; Nan and Li, 2004; Schardl and Leuchtmann, 2004) and 10 of *Epichloë sensu stricto* have been described (White et al., 2000; Moon et al., 2004; Schardl and Leuchtmann, 2004). The majority of vertically transmitted endophytes that have been analyzed phylogenetically are hybrids with multiple sexual (*Epichloë*) species as ancestors (Schardl et al., 1994; Moon et al., 2002; Craven et al., 2001). At least eight *Neotyphodium* species have been inferred as hybrids evolving from *Epichloë* species (Moon et al., 2004). For example, *N. coenophialum* symbiotic with *Festuca arundinacea* is a hybrid of at least 3 *Epichloë* species: *E. festucae, E. typhina* and a relative of *E. baconii* (Craven et al., 2001; Moon et al., 2004). Hybrid mutualists may be better able to adapt to new hosts and possibly provide greater or more diverse benefits to host plants than non-hybrids (Schardl and Craven, 2003).

Identification and classification of *Neotyphodium* endophytes has been based primarily on morphological characteristics, including colony feature, growth rate, reverse pigmentation, shape and dimensions of conidia and conidiophores, as well as presence or absence of septa at the base of conidiophores (White et al., 1993; Craven et al., 2001). Schardl and co-investigators defined morphological, biological and phylogenetic species of these endophytes (Schardl and Craven, 2003) and identified new species that were the result of apparent hybridization between species of *Epichloe* (Schardl et al., 1994; Schardl and Craven, 2003; Moon et al., 2004). Thus, the utilization of interspecific hybridization and gene sequence data has become increasingly important for taxonomy of *Neotyphodium* species (Glenn et al., 1996; Schardl and Craven, 2003; Schardl and Leuchtmann, 2004).

There have been few reports of the biological and physiological characteristics of *Neotyphodium* and *Epichloë* endophytes. Numerous nitrogen sources and 31 carbon sources were tested for nutritional requirements of *N. coenophialum* by Kulkarni and Nielsen (1986). White and Morgan-Jones (1987) measured radial colony growth and sporulation of four *Neotyphodium* species at three different temperatures, and gave a key to four known *Neotyphodium* species based on morphological characteristics. Colony features of several

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Neotyphodium species on five sugar-based media were studied by Morgan-Jones et al. (1990) and the effect of various culture media, antibiotics, and carbon sources on growth parameters of *N. coenophialum* by Pope and Hill (1991).

N. gansuense is the first *Neotyphodium* species found in China (Li et al., 2004b). Although endophyte infections have been detected and the *Neotyphodium – A. inebrians* association investigated widely (Li et al., 2004a), very little is known about the biology and physiology of *N. gansuense* itself. The main aims of this research were to compare the biological and physiological characteristics of *N. gansuense* with *N. coenophialum* and *E. festucae* in vitro under various environmental conditions of temperature, pH, media, carbon and nitrogen sources.

Materials and methods

Biological materials

Thirteen isolates of *N. gansuense* were isolated from endophyte infected *Achnatherum inebrians* in China by Li et al. (2004b). One isolate of *N. coenophialum* (CBS772.95) and one isolate of *Epichloë festucae* (CBS761.95) were purchased from the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands.

Pure cultures of *N. gansuense*, *N. coenophialum* and *E. festucae* were grown on potato dextrose agar (PDA) at 22 °C in the dark for 4 weeks as inocula. Mycelial plugs (6 mm in diam.) were taken from margins of actively growing colonies with a cork borer and transferred to a range of agar media in 90 mm diam. sterile polystyrene Petri dishes. Media were inoculated by placing plugs face down on each dish, 3 replications per isolate. Dishes were sealed with laboratory film (Parafilm^{TM}, USA) and colony diameters of all fungi were measured weekly for four weeks. Fungal growth was assessed as final colony diameter less the 6 mm mycelial plugs, and data from 13 isolates of *N. gansuense* were averaged in the results.

Effect of temperature on fungal endophytes

The inoculated dishes were incubated in the dark at temperatures of 5, 10, 15, 20, 25, 30 or 35 °C.

Effect of pH on fungal endophytes

Autoclaved PDA media were cooled to ~50 °C and 1 N NaOH or 1 N HCl were used to adjust the pH to 3, 5, 7, 9 or 11 before the media were poured into sterile Petri dishes. After inoculation with endophytes, the dishes were incubated in the dark at 22 °C.

Effects of carbon sources on fungal endophytes

Ten carbon compounds were assessed for their effects on mycelial growth. They were: dextrose (Tianjing Guangfu, China), lactose (Laiyang Chemical Industry, China), inulin (Shanghai Chemical, China), maltose (Beijing Aoboxing, China), xylose (Tianjing Guangfu, China), fructose (Tianjing Guangfu, China), sucrose (Tianjing Chemical, China), soluble starch (Laiyang Chemical, China), sorbitol (Tianjing Guangfu, China) and mannitol (Tianjing Guangfu, China).

Potassium nitrate was added to a basal medium ($MgSO_4$ 0.5 g, KH_2PO_4 0.5 g, agar 20 g, distilled water 1000 ml) to give a concentration of 2% and each carbon compound as appropriate was then added at a rate of 20 g/1000 ml before autoclaving. The control was basal medium plus potassium nitrate only. Inoculated dishes were incubated in the dark at 22 °C.

Effects of nitrogen sources on fungal endophytes

Eleven nitrogen compounds were assessed for their effects on mycelial growth: potassium nitrate (Xi'an Chemical, China), ammonium nitrate (Xi'an Chemical, China), ammonium nitrite (Tianjing Chemical, China), ammonium oxalic acid (Beijing Chemical Industry, China), peptone (Shanghai Donghai, China), tryptone (Beijing Shuangxuan, China), casein (Beijing Shuangxuan, China), yeast extract (Beijing Shuangxuan, China), urea (Tianjing Chemical, China), L-leucine (Tianjing Guangfu, China) and L-proline (Tianjing Guangfu, China).

Dextrose was added to the basal medium to a concentration of 2%. Each of the nitrogen compounds was added at a rate of 2 g/1000 ml before media were autoclaved. The control was basal medium plus dextrose only. Inoculated dishes were incubated in the dark at 22 °C.

Growth of fungal endophytes on various agar media

Six different media were assessed: PDA (fresh potato 200 g, dextrose 20 g, agar 17 g, distilled water 1000 *g*), drunken-horse-grass agar (DA: drunken-horse-grass meal 50 g, agar 17 g, distilled water

1000 *g*), oat meal agar (OMA: oat meal 30 g, agar 17 g, distilled water 1000 *g*), corn meal agar (CMA: corn meal powder 300 g, agar 17 g, distilled water 1000 *g*), water agar (WA: agar 20 g, distilled water 1000 *g*) and saltwater nutrient agar (SNA: KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4$ 0.5 g, KCl 0.5 g, dextrose 0.2 g, sucrose 0.2 g, agar 17 g, distilled water 1000 *g*). Peeled fresh potato, snipped drunken-horse-grass pieces, oat meal and corn meal were boiled in distilled water for 30 min, then filtered through 2-layer gauze. All agar media were autoclaved at 121 °C for 20 min, then 25 ml cooled (~50 °C), sterilized media were poured into individual Petri dishes. After 4 weeks incubation, colony features were described.

Results

Effect of temperature on fungal endophytes

The isolates of *N. gansuense*, *N. coenophialum* and *E. festucae* were able to grow at 10–30 °C, but all showed no growth at 5 °C and little growth of *Neotyphodium* species at 35 °C in cultures on PDA (Table 1). The 4-week-old colony diameters of *N. gansuense* and *N. coenophialum* at 25 °C were significantly ($p<0.05$) larger than those at other temperatures, whereas the diameters of *E. festucae* at both 20 and 25 °C were significantly ($p<0.05$) larger than those at other temperatures (Table 1).

The colony diameters of *E. festucae* at 10–25 °C were significantly ($p<0.05$) larger than those of *N. gansuense* and *N. coenophialum*, but the diameter of *N. coenophialum* at 30 °C was significantly ($p<0.05$) smaller than those of *N. gansuense* and *E. festucae*. (Table 1).

Effect of pH on fungal endophytes

The growth of *N. coenophialum* and *E. festucae* at the pH conditions tested showed a similar trend to that of *N. gansuense* in cultures on agar plates. The 4-week-old colony diameters of all three fungi were significantly ($p<0.05$) larger at pH 5–9 than those at pH 3 or 11, but there were no significant differences between pH 5, 7 and 9, although the fastest growth of *N. gansuense*, *N. coenophialum* and *E. festucae* was at pH 7, 9 and 5, respectively (Table 2). *N. coenophialum* showed a little growth at pH 3 but not at pH 11. Thus, the optimal pH value for mycelial growth of *N. gansuense*, *N. coenophialum* and *E. festucae* was 5–9, 5–9 and 5–7, respectively (Table 2).

The colony diameters of *N. gansuense* at pH 5–11 were significantly ($p<0.05$) larger than those of *N. coenophialum*, but significantly ($p<0.05$) smaller than those of *E. festucae*. *N. gansuense* colonies at pH 3 grew significantly ($p<0.05$) better than those of *N. coenophialum*, whereas *E. festucae* did not grow at this pH. At pH 11, the growth rates of *E. festucae* > *N. gansuense* > *N. coenophialum* were significantly ($p<0.05$) different. The results suggest that *N. gansuense* and *E. festucae* grow over a relatively wide pH spectrum from slightly acid to alkaline conditions, but *N. coenophialum* is more limited (Table 2).

Effects of carbon sources on fungal endophytes

Neotyphodium and *Epichloë* endophytes differed in their abilities to grow on media containing different carbon nutrients (Table 3). Growth of *N. gansuense* was significantly ($p<0.05$) better on most carbon compounds compared with the

Table 1. Colony diameter (mm) of *Neotyphodium gansuense*, *N. coenophialum* and *Epichloë festucae* after four weeks growth at different temperatures

Temperature (°C)	*Neotyphodium gansuense*		*N. coenophialum*		*Epichloë festucae*		LSD ($p<0.05$)
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	
5	0.0	0.00	0.0	0.00	0.0	0.00	0
10	0.0–7.8	2.47 ± 2.39	2.0–3.5	2.75 ± 0.61	6.0–9.0	7.33 ± 1.25	2.3999
15	4.0–21.0	9.62 ± 5.05	9.0–10.0	9.50 ± 0.41	24.0–34.0	27.33 ± 4.71	7.8150
20	13.5–29.5	18.47 ± 4.90	16.0–17.5	16.75 ± 0.61	39.5–41.5	40.67 ± 0.85	1.5126
25	15.0–29.3	25.79 ± 7.84	20.0–21.0	20.50 ± 0.41	40.5–45.5	42.17 ± 2.36	7.9049
30	0.0–31.3	18.77 ± 10.46	5.5–8.0	6.75 ± 1.02	20.5–22.5	21.33 ± 0.85	11.289
35	0.0–4.5	1.16 ± 1.39	0.0–1.5	0.50 ± 0.71	0.0	0.00 ± 0.00	1.7427
LSD ($p<0.05$)		1.9889		1.3134		4.4973	

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Table 2. Colony diameter (mm) of *Neotyphodium gansuense*, *N. coenophialum* and *Epichloë festucae* after 4 weeks growth at 22 °C on different pH media

pH value	*Neotyphodium gansuense*		*N. coenophialum*		*Epichloë festucae*		LSD ($p<0.05$)
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	
3	0.0–12.7	4.37 ± 3.05	1.0	1.00	0.0	0.00	1.4371
5	14.0–42.2	25.15 ± 8.45	8.0–12.0	9.69 ± 1.7	39.0–42.5	40.80 ± 1.43	4.2247
7	15.3–38.2	26.40 ± 7.60	10.0–12.0	11.00 ± 0.82	35.0–40.0	38.30 ± 2.36	6.2344
9	10.3–37.3	23.82 ± 8.19	12.0–13.0	12.33 ± 0.62	35.0–36.5	35.50 ± 1.08	4.5152
11	8.7–29.0	17.16 ± 7.61	0.0	0.00 ± 0.00	24.5–26.0	25.50 ± 0.71	2.2759
LSD ($p<0.05$)		5.6859		1.9368		3.0351	

Table 3. Colony diameter (mm) of *Neotyphodium gansuense*, *N. coenophialum* and *Epichloë festucae* after 4 weeks growth at 22 °C on different carbon sources

Carbon source	*Neotyphodium gansuense*		*N. coenophialum*		*Epichloë festucae*		LSD ($p<0.05$)
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	
Control	5.0–12.7	8.92 ± 2.53	0.5–1.0	0.83 ± 0.24	16.0–17.0	16.67 ± 0.47	1.5671
Fructose	7.2–20.3	13.08 ± 4.00	1.0	1.00 ± 0.00	14.0–16.0	14.67 ± 0.94	1.7004
Glucose	6.0–19.8	14.65 ± 3.51	1.0	1.00 ± 0.00	14.5–19.0	16.17 ± 2.01	2.9492
Inulin	9.5–17.8	14.26 ± 2.79	1.5–2.5	2.00 ± 0.41	21.5–23.0	22.17 ± 0.62	1.2906
Lactose	9.7–16.7	14.47 ± 3.02	2.0	2.00 ± 0.00	24.0–34.0	30.00 ± 4.32	6.1502
Maltose	8.8–21.7	14.23 ± 3.32	1.0–2.0	1.33 ± 0.47	17.0–21.0	19.33 ± 1.70	2.8546
Mannitol	6.5–19.7	13.86 ± 3.52	1.5–2.0	1.83 ± 0.24	14.0–19.5	16.17 ± 2.39	3.7135
Sorbitol	10.7–19.3	14.33 ± 2.53	2.0–2.5	2.17 ± 0.24	15.0–18.5	16.50 ± 1.47	2.3084
Starch	7.2–17.7	13.71 ± 3.03	1.0	1.00 ± 0.00	16.0–17.0	16.67 ± 0.47	1.5654
Sucrose	13.3–21.3	16.86 ± 2.06	1.5–2.0	1.83 ± 0.24	18.0–20.5	19.50 ± 1.08	2.2260
Xylose	0.5–9.7	2.89 ± 2.78	0.0	0.00 ± 0.00	3.0–10.0	7.17 ± 3.01	4.3685
LSD ($p<0.05$)		2.1164		0.5439		4.0846	

control. The only exception was *N. gansuense* on xylose. However, *N. coenophialum* grew significantly ($p<0.05$) better on sorbitol, inulin, lactose, mannitol and sucrose than that on control. The diameter on lactose medium of *E. festucae* was significantly ($p<0.05$) greater than those on other carbon media. The preference of *N. gansuense* for carbon source was sucrose > glucose, lactose, sorbitol, inulin, maltose, mannitol, starch, fructose > control > xylose, and that of *N. coenophialum* was sorbitol, inulin, lactose, mannitol, sucrose > maltose, fructose, glucose, starch, control > xylose. Only lactose and inulin significantly ($p<0.05$) improved the growth of *E. festucae* over the control. Of the 11 compounds tested, the best carbon source for *N. gansuense*, *N. coenophialum* and *E. festucae* was sucrose, sorbitol and lactose, respectively. Xylose was little utilized by either *Neotyphodium* or *Epichloë* endophytes (Table 3).

E. festucae grew faster than *N. gansuense* and *N. coenophialum* on all carbon sources. On inulin, lactose, maltose, starch, sucrose and control, *N. gansuense* grew significantly ($p<0.05$) slower than *E. festucae*. However, on all carbon sources, *N. gansuense* and *E. festucae* grew significantly ($p<0.05$) faster than *N. coenophialum* (Table 3).

Effects of nitrogen sources on fungal endophytes

The abilities of *Neotyphodium* and *Epichloe* endophytes to grow varied on media with different nitrogen sources (Table 4). Compared with the controls, growth of all three endophytes tested was significantly ($p<0.05$) improved by addition of peptone, tryptone, casein, yeast extract and L-proline. Potassium nitrate, ammonium oxalic acid, peptone, tryptone, casein, yeast extract, L-leucine and L-proline significantly ($p<0.05$) improved growth of *N. gansuense*. Tryptone, yeast extract and L-proline were better utilized by *N. coenophialum*, but urea and ammonium nitrite were not used at all. The best nitrogen sources for

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Table 4. Colony diameter (mm) of *Neotyphodium gansuense*, *N. coenophialum* and *Epichloë festucae* after 4 weeks growth at 22 °C on different nitrogen sources

Nitrogen source	*Neotyphodium gansuense*		*N. coenophialum*		*Epichloë festucae*		LSD ($p<0.05$)
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	
Control	0.0–11.3	4.98 ± 2.67	0.0–0.5	0.17 ± 0.24	2.5–4.5	3.33 ± 0.85	2.6287
Ammonium nitrate	0.0–14.3	7.95 ± 5.96	1.0–2.0	1.33 ± 0.47	1.5–3.0	2.33 ± 0.62	4.4969
Ammonium nitrite	0.0	0.00 ± 0.00	0.0	0.00 ± 0.00	0.0	0.00 ± 0.00	0.0000
Ammonium oxalic acid	6.0–21.5	11.73 ± 4.40	0.0–1.0	0.50 ± 0.41	5.0–7.0	6.33 ± 0.94	4.2169
Casein	19.7–23.8	22.12 ± 1.82	4.0–9.5	7.50 ± 2.48	23.0–27.5	25.50 ± 1.87	4.6801
L-leucine	2.8–21.2	10.65 ± 5.13	0.5–1.5	1.00 ± 0.41	3.0–4.0	3.33 ± 0.47	1.9205
L-proline	13.0–25.5	19.59 ± 2.68	12.0–17.0	13.83 ± 2.25	12.5–20.0	16.50 ± 3.08	5.5503
Peptone	12.0–29.8	22.88 ± 4.97	2.0–6.0	4.67 ± 1.89	18.5–20.5	19.83 ± 0.94	5.5759
Potassium nitrate	10.3–20.2	14.50 ± 2.74	1.0–2.0	1.33 ± 0.47	10.0–11.0	10.50 ± 0.41	0.9255
Tryptone	17.3–25.5	21.95 ± 2.33	10.0–12.0	11.17 ± 0.85	14.5–18.5	17.33 ± 2.46	4.2153
Urea	0.0–16.7	6.71 ± 4.75	0.0	0.00 ± 0.00	3.0–4.5	3.83 ± 0.62	1.3351
Yeast extract	16.7–28.2	23.83 ± 3.58	11.5–13.0	12.17 ± 0.62	21.0–22.0	21.50 ± 0.41	3.6198
LSD ($p<0.05$)		3.9305		2.4364		2.8504	

N. gansuense were yeast extract, peptone, casein, tryptone and L-proline. Ammonium nitrate, urea, ammonium nitrite and L-leucine were not good sources for growth of *N. gansuense*, *N. coenophialum* and *E. festucae*. However, ammonium nitrite was not utilized at all (Table 4).

Overall on all nitrogen sources, *N. gansuense* grew faster than *N. coenophialum* but slower than *E. festucae*. Almost all nitrogen sources significantly ($p<0.05$) improved growth of *N. gansuense* more than that of *N. coenophilaum*, but only potassium nitrate, ammonium oxalic acid, L-leucine, ammonium nitrate and urea significantly ($p<0.05$) improved growth of *N. gansuense* more than those of *E. festucae* (Table 4).

Growth of fungal endophytes on various agar media

The colony upper surface of *N. gansuense* was white, centrally raised, dense and cottony on PDA, OMA and DA, but sparse on CMA, WA and SNA; the reverse was brown to dark brown on DA, yellowish brown to brown on PDA and OMA, but white to yellowish brown on CMA, WA and SNA. The colony upper surface of *N. coenophialum* was compact and felty on PDA and DA, sparse on OMA, CMA and WA, but with no aerial mycelium growth on SNA after 4 weeks incubation; the reverse had similar pigmentation to *N. gansuense* except that there was sometimes fracturing on PDA. However *E. festucae* was a relatively faster growing fungus with white, dense, cottony colonies on PDA and DA, but sparse ones on OMA, CMA, WA and SNA; the reverse was brown to dark brown on PDA and DA, and white to pale yellow on OMA, CMA, WA and SNA.

After 4 weeks incubation on PDA, OMA, DA, CMA, WA and SNA media dishes, *N. gansuense* had grown significantly ($p<0.05$) better on PDA and OMA than on CMA and DA, which supported better growth than WA and SNA. *E. festucae* showed a similar trend except that growth was significantly ($p<0.05$) better on CMA than on DA. For *N. coenophialum* growth rate was in the order PDA > CMA > OMA > DA > WA > SNA ($p<0.05$) with no growth on SNA. (Table 5).

N. gansuense and *N. coenophialum* had significantly ($p<0.05$) slower growth than E. festucae on each medium tested. However, *N. gansuense* grew significantly ($p<0.05$) faster than *N. coenophialum* on poor nutritional media, such as DA, WA and SNA (Table 5).

Discussion

Neotyphodium endophytes have been detected from 67 native grass populations belonging to 25 species of 13 genera in China. Some, such as *A. inebrians* and some species of *Elymus*, *Festuca* and *Hordeum* had high infection rates of nearly 100% (Nan and Li, 2000). The symbiotic association of *Neotyphodium* and *A. inebrians* is distributed widely in China from 74°49′–105°54′ E, 35°01′–47°58′N, from 1200 to 3580 m altitude, and 127–558 mm rainfall (Li et al., 2004a). Compared with non-endophytic *A. splendens*, endophytic *A. inebrians* can grow in areas with less rainfall

Table 5. Colony diameter (mm) of *Neotyphodium gansuense*, *N. coenophialum* and *Epichloë festucae* after 4 weeks growth at 22 °C on different media.

Media	*Neotyphodium gansuense*		*N. coenophialum*		*Epichloë festucae*		LSD ($p<0.05$)
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	
CMA	10.8–26.7	20.45 ± 5.00	20.5–22.0	21.17 ± 0.76	26.0–31.5	29.00 ± 0.29	3.2964
DA	6.5–35.5	19.95 ± 7.54	9.5–11.0	10.17 ± 0.76	23.5–24.0	23.67 ± 2.78	6.0140
PDA	12.5–34.7	28.91 ± 6.22	28.0–29.0	28.67 ± 0.58	39.0–41.5	40.00 ± 1.32	5.5574
OMA	14.7–34.7	27.21 ± 10.51	12.0–14.0	13.17 ± 1.04	39.0–39.5	39.17 ± 0.29	5.6799
SNA	4.2–13.5	8.66 ± 3.46	0.0	0.00 ± 0.00	3.0–7.0	5.33 ± 2.08	5.5574
WA	2.0–12.0	5.46 ± 3.36	2.0–4.0	2.83 ± 1.04	6.0–13.0	9.00 ± 3.61	1.9406
LSD ($p<0.05$)		5.5309		1.3907		3.7738	

and lower accumulated temperatures (Nan and Li, 2000) and under harsh conditions such as arid or semi-arid and alpine or subalpine grasslands (Li et al., 2004a). These stress tolerances are probably imparted to *A. inebrians* by *Neotyphodium* endophytes. This is the first report on biological and physiological characteristics of *N. gansuense* since it was named by Li and Nan (Li et al., 2004b).

The optimal temperature for mycelial growth of the isolates of both *N. gansuense* and *N. coenophialum* was 25 °C, and for *E. festucae* was 20–25 °C. There was little or no growth at temperatures below 5 °C or higher than 35 °C (Table 1). Other researchers have found similar results. White and Morgan-Jones (1987) found that thirteen isolates of *N. coenophialum, N. huerfanum, N. typhinum* and *N. starrii* endophytes grew best at 25 °C, and Latch et al. (1984) found that 25 °C was the optimal temperature for growth of *N. lolii, N. coenophialum* and *E. typhina*.

Our research showed that the optimal pH values for mycelial growth of *N. gansuense*, *N. coenophialum* and *E. festucae* was pH 5–9, pH 5–9 and pH 5–7, respectively (Table 2). These results agree with those of Bacon and White (1994) who found that, growth of *Neotyphodium* and *Epichloë* endophytes was best at pH 6 and 6.5. However, *N. gansuense* and *N. coenophialum* grew slowly at pH 3 and 11, and *E. festucae* at pH 3 (Table 2). *N. gansuense* was relatively tolerant of both alkaline and acid conditions while *E. festucae* was only tolerant of alkaline ones. This study suggests that *A. inebrians* infected with *N. gansuense* may grow better on soil stressed by acidity and salinity than uninfected plants.

This study showed that growth of *N. gansuense* was best on the carbon sources sucrose, glucose, lactose, sorbitol, inulin, maltose, mannitol, starch and fructose, whereas growth of *N. coenophialum* was best on sorbitol, inulin, lactose, sucrose and mannitol (Table 3). Kulkarni and Nielsen (1986) studied carbon nutritional requirements of *N. coenophialum* and found that some hexoses (fructose, glucose, mannose), disaccharides (sucrose and trehalose) and an oligosaccharide (raffinose) were utilized as carbon sources. In a defined medium consisting of mineral salts, the preference for carbon source utilized by *N. coenophialum* was in the order mannitol > fructose > mannose = sucrose > glucose (Kulkarni and Nielsen, 1986) whereas in a potato broth medium it was mannitol > glucose > sucrose > mannose > fructose (Pope and Hill, 1991). Our results agree with those reports that mannitol, sucrose, fructose and glucose are good carbon sources for *N. gansuense* and *N. coenophialum*. However, we found no extra growth on fructose and glucose media for *E. festucae* compared with its control (Table 3). White et al. (1993) found that stromata-forming isolates of *N. typhinum* utilizing both fructose and glucose, and grew significantly better on these carbon sources than isolates from plants without stromata ($p<0.05$). However, *N. coenophialum* did not grow on xylose medium and growth of *N. gansuense* and *E. festucae* was poor (Table 3). A similar lack of growth on xylose media of the endophytes *N. chilense*, *N. coenophialum*, *N. chisosum*, *N. huerfanum* and *N. typhinum* was found by White and Morgan-Jones (1987).

Our research showed that most nitrogen sources tested could be utilized by *N. gansuense*, *N. coenophialum* and *E. festucae*. The best nitrogen sources for endophyte growth were, yeast extract, peptone, casein, tryptone and L-proline. Urea supported a little growth but ammonium nitrite supported none (Table 4). Kulkarni and Nielsen (1986) obtained similar results from screening numerous nitrogen sources. They found that tryptone, yeast extract and peptone were well-utilized, and inorganic ammonium (ammonium chloride, ammonium nitrate) were as effective for *N. coenophialum* growth as amino acids,

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such as L-arginine, L-asparagine, L-cysteine, L-glutamine, L-proline and L-serine. Results of both this paper and Kulkarni and Nielsen (1986) clearly showed urea was hardly utilized by *Neotyphodium* or *Epichloë* endophytes. The lack of urea utilization in these endophytes could be due to the absence of an uptake system or to the absence of urease (Kulkarni and Nielsen, 1986). No growth at all on ammonium nitrite is because nitrite is highly toxic to endophytes (Chung and Schardl, 1997).

From this trial, *N. gansuense* grew best on PDA and OMA and well on CMA and DA, whereas *N. coenophialum* grew preferentially on PDA > CMA > OMA (Table 5). A previous study obtained similar results for *N. coenophialum*, but *N. lolii* grew better on OMA than on PDA and CMA (Latch et al., 1984). There are no reports to date of evaluation of the growth features of *Neotyphodium* or *Epichloë* endophytes on media made from host plant tissue. *N. gansuense* grew slower on DA than on PDA, but faster than on WA and SNA (Table 5). Normally, a variety of agar and liquid media are used to isolate, culture and subculture *Neotyphodium* and *Epichloë* endophytes (Parrott, 1994). PDA supplemented with the antibiotics streptomycin and chloramphenicol (ABPDA) is used for grass endophyte isolation (Bacon, 1977) while PDA, MEA, OMA and CMA are used for mycelial growth and sporulation (Latch et al., 1984; White and Morgan-Jones, 1987; Parrott, 1994).

Among the three endophyte species tested in this paper, *Epichloë* species grew faster under all growth conditions than *Neotyphodium* species, and *N. coenophialum* had slower growth than *N. gansuense* (Tables 1–5). There are many reports that *Epichloë* is fast-growing (Leuchtmann et al., 1994; Zabalgogeazcoa et al., 1999; White et al., 2000), whereas *N. coenophialum* is relatively slow-growing (Morgen-Jones and Gams, 1982; Latch et al., 1984; White and Morgan-Jones, 1987).

There are larger standard deviations of means for *N. gansuense* than for the other two fungi and this reflects the greater number of isolates of this species. Genetic and phylogenetic characteristics of *N. gansuense* are being assessed and will be compared with details of *Neotyphodium* and *Epichloë* species. Distinct differences exist between *N. gansuense* in Gansu and "*N. inebrians*" in Xinjiang (Li et al., 2004b; Moon et al., 2004; Schardl and Leuchtmann, 2004), thus the diversity of *Neotyphodium* isolates from different geographical populations and their ecological roles is worthy of further study.

Acknowledgements

We wish to thank professor Christopher Schardl, professor Peter Long, professor Volk Paul and two anonymous reviewers for reviewing the manuscript. This research was financially supported by the National High Technology Research and Development Program of China (2004AA244080), Gansu Middle and Young Scientist Foundation (3ZS041-A25-003), National Nature Science Foundation of China (30070546) and Interdisciplinary Innovation Research Fund for Young Scholars of Lanzhou University (LZU200316).

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