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Identification and Characterization of a Rare Species of *Neurospora* Isolated from a Water Sample in China

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Abstract: An *Neurospora* isolate (ZZS4408) was obtained from a water sample of Xinye county, Henan, China. The isolate was identified as *Neurospora brevispora*, a rare species of *Neurospora* based on its morphological characteristics and ribosomal DNA internal transcribed spacer (rDNA-ITS) sequence. To clarify its biological characteristics, the relationship between growth of the isolate and environmental factors (temperature, pH and nutrition) was tested. The temperatures suitable for growth of the isolate were 28-37 °C with 31 °C as the optimum. The growth rates of hyphal tips reached to 19.1-42.5 (av.31.9) μm min^{-1} at 32 °C. The pHs suitable for vegetative growth were 5-7 with 5.5 as the optimum. Of the 10 carbon sources tested, two heterodisaccharides (sucrose and lactose) were most favorable for vegetative growth. Of the 7 nitrogen sources tested, D-alanine was the most favorable one for growth of *N. brevispora*. The vegetative growth of the fungus was more significantly influenced by the nitrogen sources compared to the carbon sources. *N. brevispora* was a fast-growing species, and could be considered a desirable material in fungal morphodifferentiation study and demonstration.
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1. Introduction

Neurospora is a genus of Ascomycete fungi with high academic and economical values, and consists of at least 26 species. As a well-known species in the genus, *Neurospora crassa* has been used as a model organism in biology (Chung et al., 2001; He and Liu, 2005), and has greater potential in the production of valuable metabolites such as cellulase (Feng et al., 2004), laccase (Chen et al., 2005), ethanol and xylitol (Zhang et al., 2003), carotenoids (Li et al., 2009), and highly reactive dietary fiber (Tu et al., 2008). Other *Neurospora* spp. such as *N. intermedia* (Chen et al., 2009) and *N. stophila* (Wu et al., 2008; Deng et al., 2009) also showed strong abilities in cellulose production.

Since 1998, the Neurospra International Symposium has been held every two years in California Asilomar Conference Center. The information presented in the symposium reflects the latest status in *Neurospora* research. The current researches on *Neurospora* were predominantly focused on *N. crassa* worldwide. Little is known in most of the other *Neurospora* spp., making the knowledge system dramatically unbalanced among different species in this genus. For most of the rare species such as *N. brevispora* (=*Galasinospora brevispora*), their biological characteristics are even unclear. Studies on these rare species of *Neurospora* might contribute to a better understanding on genetic diversity in *Neurospora*, and to a further utilization of the genus. In recent years, the authors isolated a rare species of *Neurospora* (isolate ZZS4408) from a river water sample in Henan, China. The goal of the present study is to identify and characterize the rare *Neurospora* species.

2. Material and Methods

The isolate (ZZS4408) of *Neurospora brevispora* was obtained from a river water sample of Xinye county, Nanyang city, China (112°35´E, 32°52´N), using a conventional dilution plate method. The water sample was spread on PDA plate (200 g potato, 20 g glucose, 16 g agar, 1000 ml water), and incubated at 28°C for 24h. A rapid-growing fungal colony (isolate ZZS4408) was obtained from the water sample and used for the present study.

The isolate ZZS4408 was grown on PDA (200 g potato, 20 g glucose, 17 g agar, 1000 ml water) plates at 28-32°C for 20 days to observe its morphological characteristics and to test its single hyphal growth rate. The determination of the single hyphal growth rate was conducted within 24h of colony development at 32°C under a microscope. Two photographs of a hyphal tip were taken at 10-15 min interval. The hyphal growth rates were

determined based on the hyphal length increased to the time elapsed. After sporulation occurred, the dimensions of the developed perithecia, asci and ascospores were separately measured.

The isolate ZZS4408 was incubated on PDA plates for 2 days, and its mycelial plugs (6 mm diameter) were cut out from actively growing colonies. A mycelial plug was transferred to PDB liquid medium (the same components as PDA except agar), and incubated at 28°C for 7 days. The resultant mycelia were harvested and rinsed with sterile water to remove the residual medium components, and used for genomic DNA extraction after being dried at 80°C for 12h.

The genomic DNA of isolate ZZS4408 was extracted using the liquid nitrogen method (Lu et al., 2011). The dry mycelial sample was placed in a sterile mortar and rapidly ground in liquid nitrogen to obtain mycelial powder. The mycelial powder was transferred to a sterilized centrifuge tube with 800 µl lysis buffer, and bathed in hot water (65°C) for 2h, in which the tube was turned reversely once every 10 min. Subsequently the DNA solution was centrifuged at 4°C under 12 000 rpm for 10 min. The supernatant was added to an equal volume of the mixture of four organic solvents (phenol: chloroform : isoamyl alcohol = 25 : 24 : 1). After being gently blended, the DNA solution was centrifuged at 4 °C under 12000 rpm for 10 min. The extraction process was repeated three times. Two volumes of precooled ethanol and 1/10 volume of 3 mol/L sodium acetate solution were added into the DNA solution and well shaken. After being maintained at -20°C for 1.5h， the DNA solution was centrifuged at 4°C under 12000 rpm for 10 min. After removing the supernatant, the precipitate was rinsed three times with 75% alcohol by centrifugation as mentioned above. The DNA sample was dissolved in 25 µl ddH_2O and maintained at -20 °C for 30 min prior to electrophoresis analysis.

The DNA samples were diluted properly with PCR buffer according to the brightness of the genomic DNA bands in electrophoresis (Qi et al., 2009). The PCR reaction system consisted of 1 µl genomic DNA, 15 µl mixture (PCR buffer, dNTP, *Taq* DNA polymerase, $MgCl_2$), 1.2µl of each of 10 pmol/L ITS4 (5'-tcctccgcttattgatatgc-3') and 10 pmol/L ITS5 (5'-ggaagtaaaagtcgtaacaagg-3'), and ddH_2O was added to a final volume of 30 µl. PCR program was set as follows: predenaturing at 95°C for 5 min, subsequently denaturing at 94 °C for 40 s, annealing at 55°C for 40 s, and 30 cycles of extension at 72°C for 30 s followed by extension at 72°C for another 10 min. PCR products were analyzed with 1% agarose gel electrophoresis, and send to Takara Biotechnology (Dalian) Co., Ltd. for sequencing (both-strand sequenced) after purification. Sequences homology comparison was conducted using the BLAST program (http://blast.ncbi.nlm.nih.gov/ Blast. cgi). Related rDNA-ITS sequences of other *Neurospra* spp. from GenBank database were used as reference sequences. A phylogenetic tree was constructed with MEGA4 software (Tamura et al., 2007) to determine the taxonomic hierarchy of isolate ZZS4408 under 1000 bootstrap replicates.

To clarify the effects of temperatures on vegetative growth of isolate ZZS4408, the isolate was grown on PDA plates at 25°C for 2 days. Mycelial plugs (6 mm diameter) were cut out with a sterilized punch from the actively growing edge of the fungal colonies, and transferred on PDA plates. After being grown at a serial of temperatures (4°C, 7°C, 10°C, 13°C, 16°C, 19°C, 22°C, 25°C, 28°C, 31°C, 34°C, 37°C, 40°C, 43°C) for 3 days，the diameters of the developed colonies were cross-measured. Three replicates were set up for each treatment.

To know the effects of pHs on vegetative growth of isolate ZZS4408, the pHs of PDB were adjusted to different levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 8.0, 8.5, 9.0) with NaOH/HCl. Mycelial plugs of isolate ZZS4408 were separately inoculated in 100 ml PDB with different pHs. After being incubated at 25 °C for 6 days, the resultant mycelia were harvested, and dried overnight at 60 °C. The dry weights of the mycelia were separately measured (Lu et al., 2001). Three replicates were set up for each treatments.

A basic medium (BM: 2g D-alanine, 1g KH_2PO_4, 0.5g KCl, 0.5g $MgSO_4 \cdot 7H_2O$, 0.01g $FeSO_4$, 30 g sucrose, 17g agar, water 1000 ml) was used for evaluating the effects of nutrition on vegetative growth of isolate ZZS4408. To determine the effects of carbon sources on vegetative growth of isolate ZZS4408, the sucrose of BM medium was substituted with equal amounts of glucose, fructose, lactose, galactose, D-mannose, xylitol, maltose, L-arabinose and D-sorbitol, respectively. To determine the effects of nitrogen sources on vegetative growth of isolate ZZS4408, the D-alanine of BM medium was substituted with equal amounts of glycine, carbamide, L-cystine, L-histidine, $NaNO_3$ and NH_4Cl, respectively. The media were sterilized using a conventional method. Mycelial plugs of isolate ZZS4408 were prepared as mentioned above, and inoculated on the BM plates containing different carbon/nitrogen sources. The plates were incubated at 25°C for 3 days prior to measuring the diameters of developed colonies. Three replicates were set up for each treatment.

Data obtained from the experiments were subjected to analysis of variance (ANOVA) using a SPSS statistical software (version 17.0, IBM Corporation, New York, USA). Multiple

comparisons of means were performed using ONE-WAY ANOVA at two significance levels (P=0.05 and P=0.01).

3. Results

A-Identification of isolate ZZS4408

Isolate ZZS4408 grew rapidly on PDA plates. Within 3 days of incubation on the plates at 28 °C, the colony diameters were greater than 7 cm. The growth rates of eight randomly selected hyphal tips were 19.1-42.5 (mean 31.9) μm min^{-1} at 32 °C. Before the colonies covered the PDA plates, they barely produced aerial mycelia. As the colonies fully covered the plates, aerial mycelia vigorously occurred, with white to dark gray color (Figure 1-A). The diameters of hyphae were 3.0-16.0μm. The mycelia overgrew on the inner glass surface of the Petri dish far away from the PDA plates. After 10 days of incubation, black perithecia developed on the inner surface of the Petri dish as well as on the PDA plates. The perithecia were spherical, oval or flask-shaped (Figure 1-B), with a dimension of 117.6-454.9μm×156.0-498.0μm. Ascospores were found in 60-day-old asci, but not in 14-day-old ones. The asci were rod-shaped with a dimension of approximately 160μm×16μm, containing eight linearly aligned ascospores within an ascus (Figure 1-C). The ascospores were oval-shaped, unicellular, black-brown with a dimension of 18.7-22.7μm×13.1-15.4 μm (mean 20.9μm×14.3μm)(Figure 1-D).

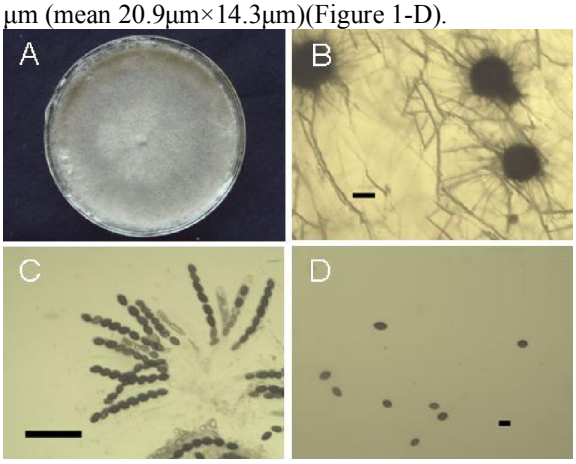

Figure 1. Morphological characteristics of isolate ZZS4408 (A: a colony incubated at 31°C on PDA plate for 14 days; B: perithecia developed on BM plate after 15 days of incubation at 28°C; C: eight linearly aligned ascospores within an ascus developed on BM plate after 15 days of incubation at 28 °C; D: ascospores on BM plate after 15 days of incubation at 28 °C. The scale bars represent 100 μm and 20 μm for B and C, respectively)

The rDNA-ITS sequence of isolate ZZS4408 was amplified using the primers ITS4 and ITS5. PCR products were visualized by 1% agarose gel electrophoresis (Figure 2). After purification and sequencing, a 545-bp rDNA-ITS fragment (GenBank accession number: JN003623) was obtained. In the established phylogenetic tree based on rDNA-ITS sequences of *Neurospora* spp, isolate ZZS4408 clustered with *Neurospora brevispora* (= *Gelasiaospora brevispora*) (Figure 3) as well as the isolate IR353 (Li et al., 2011). The phylogenetic tree indicated that both isolates ZZS4408 and IR353 belonged to *N. brevispora*.

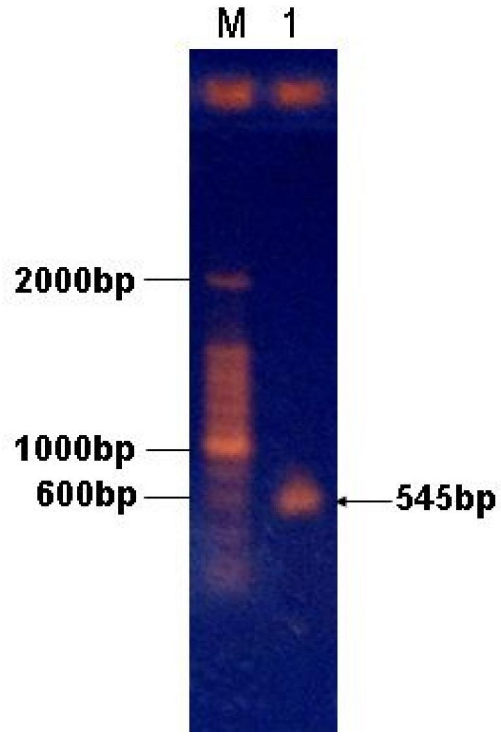

Figure 2. Electrophoretogram for PCR products of the rDNA-ITS of isolate ZZS4408 (1: isolate ZZS4408; M: DNA marker)

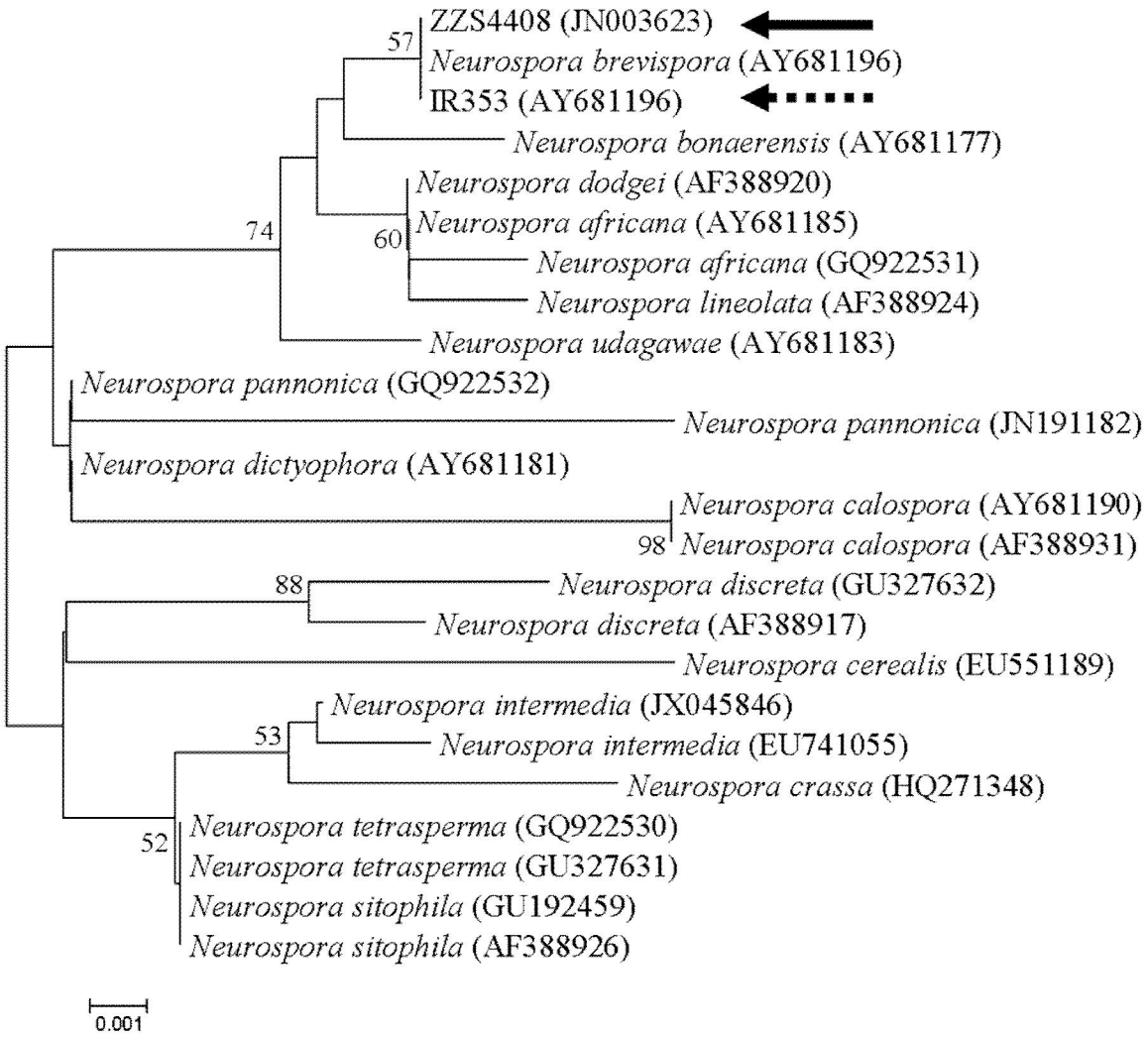

Figure 3. A rDNA-ITS-based phylogenetic tree of *Neurospora* spp. showing the position of isolate ZZS4408. The numbers in parentheses represent the accession numbers of the *Neurospora* spp. in GenBank. The numbers in each branch points denote the percentages supported by bootstrap (values lower than 50% were not shown). The scale bar represents 0.001 substitutions per nucleotide position. The real line arrowhead indicates the isolate ZZS4408. The broken line arrowhead indicates the isolate IR353 (Li et al., 2011)

Effects of environmental factors on the growth of isolate ZZS4408

The temperature range for the growth of isolate ZZS4408 was 10-40°C (Table 1). The mean diameters of colonies developed at temperatures 28-37 °C for 5 days were greater than 80 mm. Although no difference was observed among the temperatures at $P=0.05$ level, the maximum growth occurred at 31 °C with a mean colony diameter of 89.7mm. Therefore, the temperatures suitable for active growth of isolate ZZS4408 could be considered as 28-37°C with 31°C as the optimum (Table 1). Taking the peak growth temperature 31°C as a dividing line, significant difference was observed in the variation of colony diameters between the low temperature side and high temperature one: colony diameters gradually raised with the increase of temperatures in the former, and sharply decreased with the enhancement of temperatures in the latter. At the temperatures lower than 31 °C, the increase of 1°C came with an increased mean diameter of 3.74 mm, while at the temperatures higher than 31°C, the increase of 1°C came with a reduced mean colony diameter of 7.48 mm. As the temperature raised from 40 °C to 43 °C, the mean colony diameter sharply declined from 73.6 mm to 0 mm.

Table 1. Effects of temperatures on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different at $P=0.05$ and $P=0.01$, respectively,)

Temperature(°C)	Colony diameter(mm)
4	0 .0 Aa
7	0 .0 Aa
10	7.7 Bab
13	12.5 Cb
16	25.0 Db
19	40.6 Ec
22	64.6 Fd
25	68.8 FGd
28	87.6 He
31	89.7 He
34	85.6 He
37	83.8 He
40	73.6 Dd
43	0.0 Aa

Vegetative growth of isolate ZZS4408 was significantly influenced by pHs (Table 2). The isolate ZZS4408 could grow at pHs 4-9 as indicated by mycelial dry weights, wherein a greater mycelial dry weight was found at pHs 5-7 (greater than 0.16 g) and the maximum mycelial dry weight occurred at pH5.5 (0.993 g). The mean mycelial dry weight was less than 0.12g in the other pH treatments. Therefore, pHs 5-7 could be considered as the favorable pH range for vegetative growth of isolate ZZS4408 with pH5.5 as the optimum.

Table 2. Effects of pHs on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different

pH	Dry mycelial weight (g)
4.0	0.055 ABa
4.5	0.106 Ba
5.0	0.177 CDb
5.5	0.993 Ec
6.0	0.197 CDb
6.5	0.168 Cb
7.0	0.227 Db
7.5	0.111 BCa
8.0	0.073 ABa
8.5	0.066 ABa
9.0	0.038 Aa

at $P=0.05$ and $P=0.01$, respectively)

The effects of carbon sources on vegetative growth of isolate ZZS4408 was shown in Table 3. Among 10 carbon sources tested, fast growth occurred on the medium plates containing heterodisaccharides (sucrose/lactose) with a colony diameter of greater than 80 mm, compared to the other carbon sources with a reduced colony diameter. Slow growth occurred on the plates containing galactose as carbon source with a colony diameter less than 70 mm. Moderate growth occurred on the plates containing the other carbon sources with colony diameters of 70-80 mm. In general, the disaccharides (especially heterodisaccharide) was more beneficial to the vegetative growth of isolate ZZS4408 compared to the monosaccharides. Among the 10 carbon sources tested, the deviation between the maximum mean colony diameter and minimum mean colony diameter was of 17.4 mm.

The effects of nitrogen sources on the vegetative growth of isolate ZZS4408 were shown in Table 4. Among 7 nitrogen sources tested, D-alanine treatment had the largest mean colony diameter (87.7 mm) compared with the other nitrogen sources, followed by the L-histidine and L-cysteine treatments with colony diameters of 70-80 mm. The colony diameters on the plates containing the other nitrogen sources were less than 70 mm. The difference between the maximum mean colony diameter and the minimum one reached up to 55.3 mm.

Table 3. Effects of carbon sources on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different at $P = 0.05$ and $P = 0.01$, respectively)

Carbon source	Colony diameter(mm)
Sucrose	85.2 Dc
Lactose	82.7 CDc
Glucose	70.7 ABab
Fructose	72.6 ABab
Xylitole	74.5 Bab
L-arabinose	76.5 Bb
D-sorbitol	78.5 BCbc
Galactose	67.8 Aa
D-mannose	75.0 Bb
Maltose	75.1 Bb

Table 4. Effects of nitrogen sources on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different at $P = 0.05$ and $P = 0.01$, respectively)

Nitrogen source	Colony diameter(mm)*
D-alanine	87.7 Ed
Carbamide	32.4 Aa
L-histidine	70.9 CDc
Glycine	54.5 Bb
$NaNO_3$	69.5 CDc
L-cystine	77.0 Dcd
NH_4Cl	65.7 Cbc

4. Discussions

Dowding established the genus *Gelasinospora* in 1933. The ascospores with surface depression were considered as the principal morphological characteristic of *Neurospora* (Dowding, 1933). Thereafter, *Gelasinospora* was once accepted as a valid genus and used for descriptions of new fungal species (Alexopoulos and Sung, 1950; Khan and Krug, 1989). In 2004, García et al. systematically conducted a comparative study between *Neurospora* spp. and *Gelasinospora* spp. using molecular and morphological methods, and found that no differences existed in the cell wall patterns of ascospores as well as in specific DNA sequences between *Gelasinospora* and *Neurospora* spp., indicating that both *Gelasinospora* and *Neurospora* might be the same taxon. As *Neurospora* was established earlier than *Gelasinospora*, the latter was treated as a synonymy of *Neurospora* (García et al., 2004).

Gelasiaospora brevispora (= *Neurospora brevispora*) was reported as a new species of *Gelasiaospora* (Khan and Krug, 1989). Li et al. (2011) obtained isolate IR353 (GenBank accession number: AB640864) from a wild cardamine sam in Funiu Mountain, Henan, China, and treated it as a member of *G. udagawae*. In the phylogenetic tree established in the present study, both isolates IR353 and ZZS4408 clustered with *N. brevispora* (GenBank accession number: AY681196), clearly separating from *N. udagawae* (= *G. udagawae*, accession number: AY681183), and could be regarded as the same species (Figure 3). In the phylogenetic tree established by Li et al. (2011), only 9 isolates were used for establishing the tree. To establish the phylogenetic tree in the present study, twenty-two isolates of *Neurospora* spp. were used as the reference isolates besides the isolate ZZS4408 (Figure 3). The inadequateness of reference isolates of *Neurospora* spp. used in the phytogenetic tree established by Li et al. (2011) might lead to the isolate IR353 misidentified as *G. udagawae*. The river from which the isolate ZZS4408 was obtained, originated from the Funiu Mountain. It was reasonable to speculate that *N. brevispora* might be transmitted from the Funiu Mountain to the county through the river originating from the mountain.

Although a few researchers identified isolates of *N. brevispora* (Li et al. 2011; Khan and Krug, 1989), so far little is known on their biological characteristics. The temperatures suitable for the growth of isolate ZZS4408 were 28-37°C with 31 °C as the optimum (Table 1). Both the Funiu Mountain and the Xinye county are located in the northern subtropical and warm temperate transition zone in which the summer temperatures frequently fluctuates between 28-33°C, fully meeting the needs for active growth of *N. brevispora* in this season. Among the pHs tested, the fungus showed the highest mycelial dry weight at pH5.5 (Table 2), indicating that the acidic environment was more suitable for active growth of *N. brevispora*, which was agreed with the other fungi (Lu et al., 2011; Wang et al., 2013).

In the present work, the growth rates of isolate ZZS4408 were significantly enhanced by heterodisaccharides (sucrose, lactose) compared to its constituent monosaccharides (glucose, fructose, galactose) (Table 3). The results suggested that two pathways metabolizing two different constituent monosaccharides might be activated in the case of utilizing heterodisaccharides as carbon sources, resulting in more active growth compared to the monosacchardes. Among the 10 carbon sources tested, the deviation between the maximum mean colony diameter and the minimum mean colony diameter was 17.4 mm (Table 3). On the other hand, among the 7 nitrogen sources tested, the deviation between the maximum mean colony diameter and the

minimum mean colony diameter reached up to 55.3 mm (Table 4). The influence of the nitrogen sources on the growth of isolate ZZS4408 was significantly greater than that of the carbon sources. This was agreed with *Athelia rolfsii* causing sesame southern blight (Wang et al., 2013), and might be due to the differences in the basic biological functions between carbon and nitrogen sources. The former mainly act as an energy supplier while the latter as a supplier of nitrogen for synthesis of biologically important macromolecules such as proteins and DNAs. Based on the biological characteristics of the isolate ZZS4408 in the present study, *N. brevispora* might be considered a desirable fungal species in morphodifferentiation study due to its rapid growth feature. Our results enhance the understanding on biological characteristics of *N. brevispora*.

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