Identification and characterization of a new branching mutant of *Neurospora intermedia* from nature

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Neurospora is a tropical fungus which is found abundantly growing on burnt sugarcane, discarded corn cobs and other burnt vegetation. It is being used as a model organism for understanding growth and branching in fungi. We have isolated and characterized a naturally occurring branching mutant of *Neurospora intermedia* which may be useful for understanding growth and branching in fungi.

Introduction

Neurospora has been studied extensively to understand various phenomenon of fungal growth and morphogenesis. Fungal morphogenesis is a complex process which includes important aspects like polarized extension of the hyphal tip and hyphal branching. Observations at the tip have shown that growth vesicles arrive at the tip from proximal locations, and then seem to be distributed into the tip dome by the spitzenkorper. No specific mechanism has been defined for branching. Several theories have been proposed for branching that can be broadly divided into two groups. One group supports the mechanism involving origin of branch initiation factors at the hyphal tips (Bachewich and Heath, 1997; Kaminskyj and Heath, 1996; Riquelme and Bartnicki-Garcia, 2004; Watters and Griffiths, 2001). Other group suggests that the signals for branch initiation originate from within the colony or mycelial body (Prosser and Trinci, 1979; Trinci, 1974; Watters, 2006). Watters *et al.,* (2000) proposed that the branching initiation is not completely controlled by the tip but, to some extent by factors occurring at previous branch point. Trinci (1974) proposed that mutation or factor which reduces the maximum rate of tip extension without disturbing the rate of vesicle production would results in an increase in the frequency of branch initiation without reducing the overall rate of hypha formation. Recently it has been found that the fungal growth rate show relationship with the frequency of hyphal branching (Watters *et al.,* 2008, 2011). In this paper we describe a naturally occurring mutant of *N. intermedia* which shows defect in branching and the inheritance of this defect in the progeny.

Material and Methods

Fungal strains used and growth conditions

Tester strains used in the study *N. crassa* [74-OR23-IVA (FGSC 2489 *mat A), fl* (FGSC 4317 *mat A*), *fl* (FGSC 4347 *mat a*)], *N. intermedia* (FGSC 1766 *mat A* and FGSC 1767 *mat a*), *N. sitophila* (FGSC 2216 *mat A*) and *N. discreta* (FGSC 3228 *mat A*) were obtained from Fungal Genetic Stock Centre, Kansas City, USA. *N. intermedia* strain (RM126-3A) was obtained from Professor R. Maheshwari. Neurospora cultures were grown on Vogel's minimal media and incubated at 34±2 °C and all growth conditions were as described by Davis and de Serres (1970). The extension growth rate was determined by measuring the growth of the cultures in the race tubes (Ryan, 1950).

Isolation and identification of Neurospora

A naturally occurring mutant of Neurospora was isolated from visible colony growing on corn cob at Sanver in Madhya Pradesh, India, following the method of Perkins and Turner (1988). Its mating type was determined by making spot crosses with fluffy tester strains by using the method of Perkins *et al.*,

(1989). The species was determined as described by Perkins and Turner (1988) by observing fertility in crosses with tester strains.

Morphological studies

For studying the morphology, cultures were grown on Petri dishes containing Vogel's minimal media and incubated at 34±2 °C. The colony characteristics were recorded and details of hyphal growth were observed under microscope (40X, 100X and 400X magnification). The microscopic observations were taken after 18 - 20 h of growth. The culture was photographed at different magnifications and the photographs were used to determine branch angle, branching frequency, branching interval and distance between tip and first branch.

Determination of conidiation banding pattern

The conidiation pattern in response to light/dark cycles was studied in S9-5 by growing the culture in race tube containing Vogel's minimal agar medium and incubating at 34±2 ºC under 12 h light/12 h dark (LD) condition. The conidiation pattern under different conditions of light and temperature was studied in culture AM68-8 (progeny of S9-5) using the modified method of Sargent and Kaltenborn (1972). The culture was grown in race tubes and incubated at 25±2 ºC, 30±2 ºC and 34±2 ºC under 12 h light/12 h dark (LD), constant light (LL) and constant dark (DD) conditions. The light was provided by cool white fluorescent light (Surya, 8 Watt). The position of the growth front was marked at 24 h intervals. The period length of the conidiation bands was determined following the modified method of Feldman and Hoyle (1973). The time of each band was determined at the end of the experiment by measuring the position of the center of each band. The period lengths between successive bands were averaged to determine the period length of the culture.

Inheritance of the morphological defects

To study the inheritance of morphological defects the mutant was crossed with tester strains of *N. intermedia* (FGSC 1767 *mat a* or RM126-3A). Twenty viable progeny from each cross were screened for morphological defects to determine if they inherited the parental defect or not. To determine cytoplasmic (plasmid/ mitochondrial) or nuclear basis of inheritance reciprocal crosses were made.

Results and Discussion

Isolation, identification and characterization of S9-5

A Neurospora culture namely S9-5 was isolated from the visible conidial sample collected from Sanver (Madhya Pradesh). The culture was purified and it was identified as *N. intermedia* and its mating type was found to be *mat 'a'.* The culture has yellow coloured conidia and thus it is the yellow ecotype of *N. intermidia* which is predominantly present in Ujjain and areas in its vicinity (Mukati *et al.*, 2012). The morphology of the culture (S9-5) was studied under the microscope and the results show that it has multiple defects in growth and branching. S9-5 is a slow growing culture and the extension growth rate is shown in Figure 1. As seen from the graph the culture shows erratic growth. Initially the culture grows with an extension growth rate of approximately 2 mm/h. Thus, there is about two fold reduction in growth rate as compared to wild type *N. intermedia* (RM126-3A) which grows with the growth rate of 4.1 mm/h (Figure 1). During further incubation growth rate of S9-5 is further reduced and after few days only submerged growth takes place with highly reduced growth rate and then growth stops completely. The growth is completely stopped for 2 days after which the growth resumes again (Figure 1). Thus S9-5 is a stop start mutant.

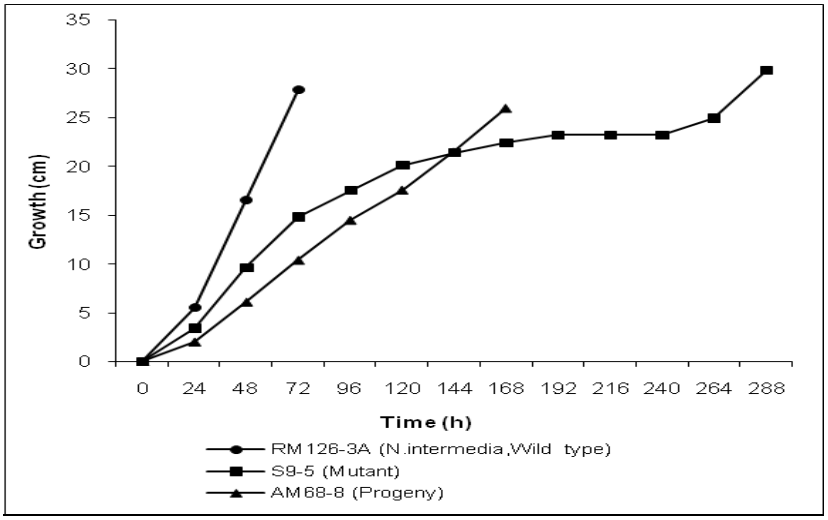

Figure 1: Extension growth rates of wild-type *N. intermedia* (RM126-3A), S9-5 and AM68-8.

When grown on a Petri dish, initially the culture forms a thick circular mycelial mat and then growth stops (Figure 2b). After some-time the growth resumes from a few points in the circular colony. At these points hyphae extend and grow in a feather-like pattern (Figure 2b). Surface growth takes place for few hours then short aerial hyphae are formed.

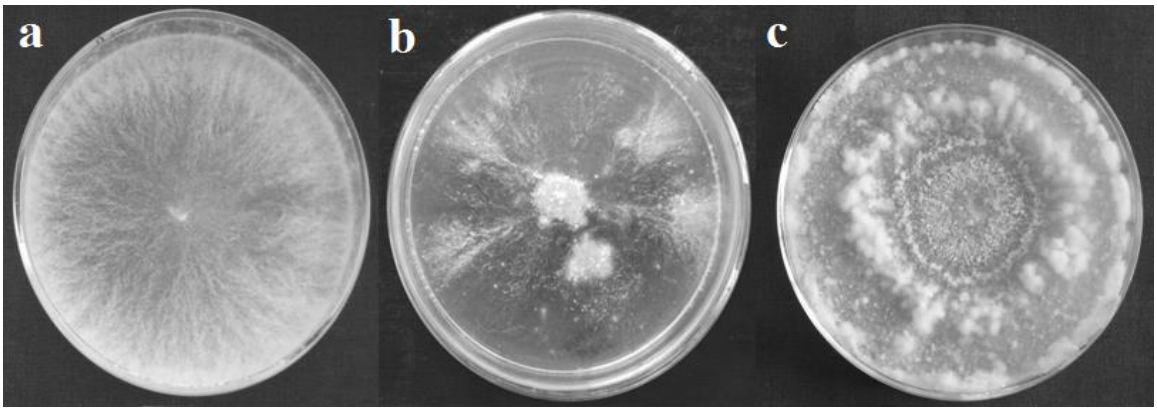

Figure 2: Morphology of wild-type and morphological mutants growing on Vogel's minimal agar media at 34±2 °C temperature. (a) Wild-type (*N. intermedia*, RM126-3A), (b) S9-5 and (c) AM68-8.

When observed under microscope the culture shows defects in branching (Figure 3). During initial growth of culture branching occurs at almost right angles (Figure 3d). The lateral branches are short and show infrequent branching or remain unbranched. In later phase of growth branching frequency increases (Figure 3e and 3f). New branches arise from sites that are very close to the hyphal tips (Figure 3i) and at many places dichotomous branching (Figure 3f and 3g) is also seen. At some points the tip becomes swollen (Figure 3h). The hyphae do not grow straight but take an undulated pattern (Figure3j).

S9-5 showed conidiation bands when it was grown in race tube at 34±2 ºC under 12 h light/12 h dark (LD) conditions. The period length of the bands was found to be 25.6±0.2 h. The wild-type control (RM126-3A) did not show conidiation bands under these conditions (Figure 4).

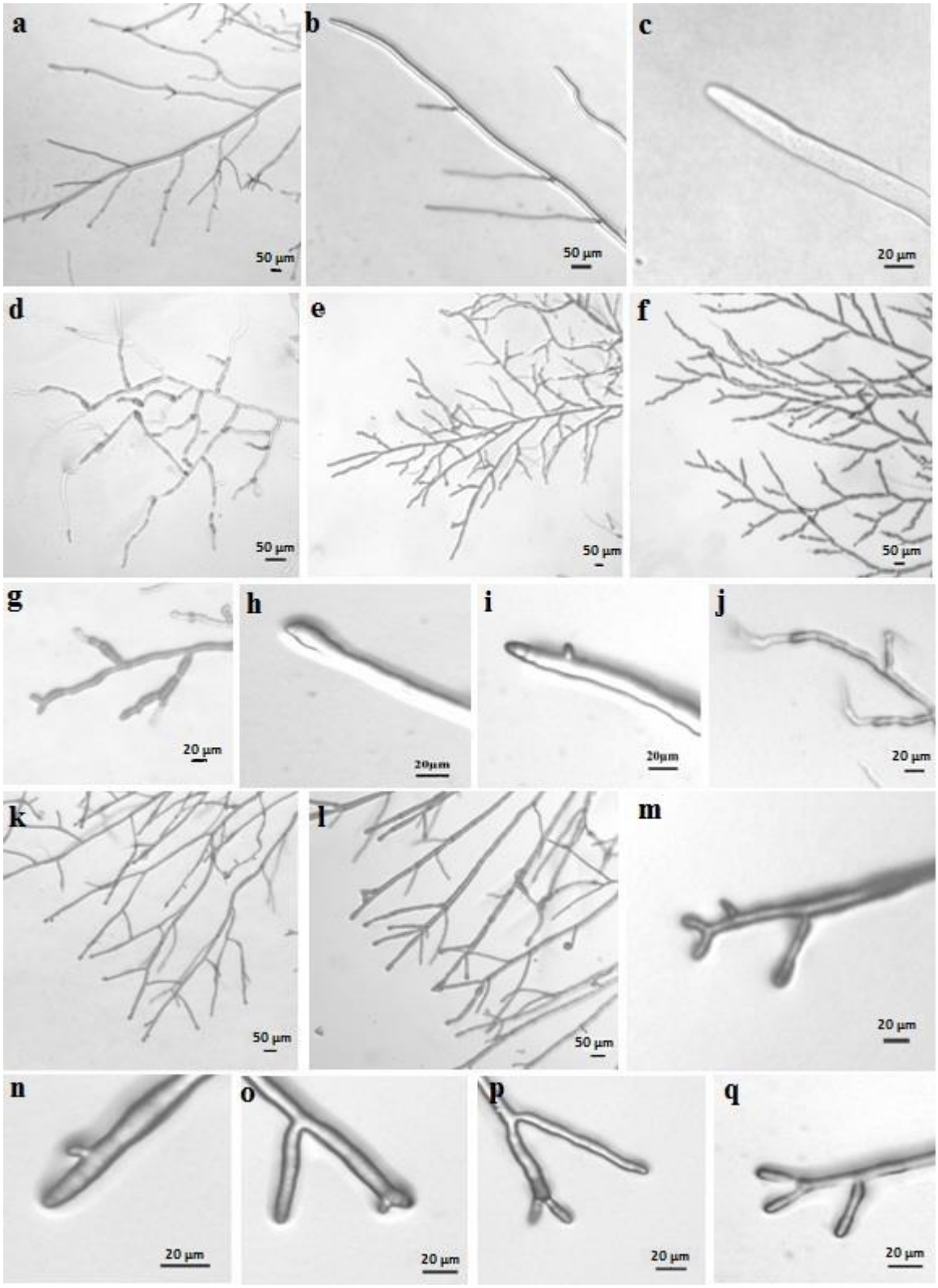

Figure 3: Hyphal morphologies of wild-type strain of *Neurospora* (a-c), S9-5 (d-j) and AM68-8 (k-q) at 34±2 °C. Fungi were grown on Vogel's minimal agar medium for 24 h and images were taken at 40X, 100X and 400X magnification.

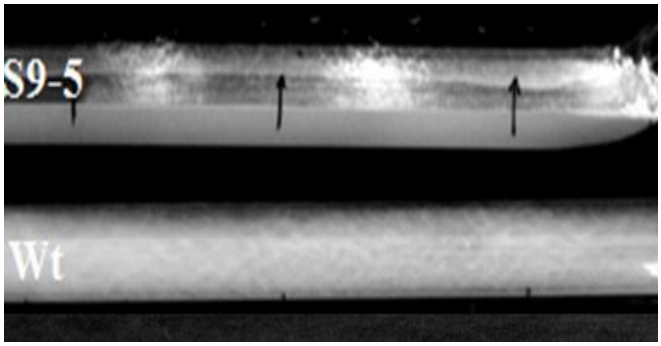

Figure 4: Conidiation bands in cultures incubated at 34±2 °C under 12 h light/ 12 h dark (LD) conditions.

Inheritance of morphological defects

S9-5 was crossed with tester strain of *N. intermedia* namely RM126-3A to see the inheritance of defects in progeny. Reciprocal crosses were set up in order to see whether the defects in S9-5 were due to mutations in nuclear gene or due to presence of some cytoplasmic factor like plasmid. Random ascospore analysis (Davis, 2000) was done after one month. Twenty viable progeny from each cross were studied. Each progeny was examined for colonial and hyphal characters. The results show that conidiation banding pattern and frequent dichotomous branching are inherited in the progeny in both the crosses. The inheritance of both characters is almost same in reciprocal crosses so it can be inferred that these characters may not be controlled by cytoplasmic factor/gene. Conidiation banding pattern and dichotomous branching are not inherited together so they may be controlled by different nuclear genes. From these crosses single progeny namely AM68-8 was chosen for further study.

Characteristics of AM68-8

The extension growth rate of AM68-8 was measured and results are shown in Figure 1. It can be seen that the growth rate is approximately 1.6 mm/h thus there is about 2.5 fold reduction in growth as compared to the wild type (RM126-3A) parent. However its growth rate is not erratic as it was in the parent S9-5.

When grown on solid Vogel's minimal media in Petri dish, then initially for about 18 – 20 h only surface growth occurs and few aerial hyphae are formed. During further incubation vigorous aerial hyphae formation takes place which lasts for few hours then again surface growth takes place. When Petri dish was observed from above the aerial hyphae gives the appearance of ring (Figure 2c).

The culture was examined under the microscope to study the branching pattern. It was observed that the culture has clear dichotomous branching at the tip (Figure 3k and 3l). However, when the initial emergence of new branch was examined, three types of patterns of branch initiation were seen. (i) A new branch arises below the tip but extremely near to the tip (Figure 3n and 3o). Both tip and new branch grows almost at equal rate and it appears that branching is dichotomous (Figure 3p). (ii) Tip bifurcates into two branches and branching is dichotomous in true sense (Figure 3m and 3q). (iii) Multiple branches arise from tip (Figure 3l). But the overall culture appears to be dichotomously branched (Figure 3k and 3l). All the three types of branching are equally prevalent in the culture. AM68-8 is also female sterile i.e. it is infertile when used as a female parent in a genetic cross.

AM68-8 was grown in race tubes and incubated at 25±2 ºC, 30±2 ºC and 34±2 ºC under 12 h light/12 h dark (LD), constant dark (DD) and constant light (LL) conditions and the conidiation banding pattern was recorded. Wild-type RM126-3A was used as control. The results are shown in Table 1 and Figure 5. AM68-8 shows conidiation bands with period length of 25.1±0.2 h and 25.3±0.2 h at 30±2 ºC and

34±2 ºC respectively under all three light conditions. The period length under all three conditions did not change much. Interestingly, AM68-8 did not show any conidiation band at 25±2ºC under all the conditions of light. In the control (RM126-3A) conidiation bands with period length of 21.3±0.2 h were seen under constant dark condition at 25±2 ºC, 30±2 ºC and 34±2 ºC, but conidiation bands were absent under 12 h light/12 h dark (LD) and constant light (LL) conditions at all the temperatures.

Table 1. Occurrence of conidiation bands in AM68-8 and RM126-3A at different temperature and light conditions.

S. No.	Culture No.	Temperature								
		25±2ºC			30±2ºC			34±2ºC		
		LD	DD	LL	LD	DD	LL	LD	DD	LL
1	AM68-8	-	-	-	+	+	+	+	+	+
2	RM126-3A	-	+	-	-	+	-	-	+	-

LD = 12 h light/ 12 h dark condition, DD = constant dark condition, LL = constant light condition, + = indicates presence of conidiation bands, - = indicates absence of conidiation bands.

Figure 5: Conidiation bands in race tubes under constant dark conditions (DD). (a) AM68-8 at 25±2 ºC, (b) AM68-8 at 30±2 ºC, (c) AM68-8 at 34±2 ºC and (d) RM126-3A at 25±2 ºC.

Inheritance of morphological defects

AM68-8 was crossed with tester strain of *N. intermedia* (FGSC # 1767) to see the inheritance of defects. Reciprocal crosses were not possible as the mutant culture is female sterile. Random ascospore analysis (Davis, 2000) was done after one month. Twenty viable progeny were studied. Each progeny was examined for colonial and hyphal characters. It was seen that 9 progeny had hyphal morphology similar to the parent AM68-8 (dichotomous branching pattern) whereas 11 cultures had wild-type hyphal morphologies. This indicates that about 50% of the progeny inherited the dichotomous branching pattern and this character may be controlled by a single gene. All the progeny were examined for the presence of conidiation bands under 12 h light/ 12 h dark conditions at 34±2 ºC. The results show that 8 progenies had distinct conidiation bands whereas 12 progeny did not show conidiation bands. This also indicates that about 50% of the progeny inherited the conidiation banding pattern. The further analysis showed that 7 progeny inherited both dichotomous branching and

conidiation banding pattern and 2 progeny had dichotomous branching but no conidiation bands while 1 progeny had conidiation banding pattern and wild-type hyphal morphology. These results indicate that most of the progenies inherit both the characters together i.e., dichotomous branching pattern and conidiation banding pattern but few progeny inherit only one character i.e., either dichotomous branching or conidiation banding pattern. Thus both the characters are not always inherited together. These results suggest that these two characters may be controlled by two different genes which may be present very close to each other.

Effect of temperature on growth and branching

The culture was grown at 25±2 ºC and 34±2 ºC temperatures to determine if there is any difference in growth rate and morphology with change in temperature and to understand the phenomenon of branch initiation. The extension growth rate was determined by growing the cultures in race tubes and at both the temperatures the growth rate was almost the same (1.7 mm/h at 25±2 ºC and 1.6 mm/h at 34±2 ºC), however, as compared to the wild type there was about 2.5 fold reduction in growth rate at both the temperatures. This shows that there is no significant effect of temperature on growth rate of this mutant. The culture was grown in Petri dish at both the temperatures (25±2 ºC and 34±2 ºC) for 18 h and the difference in branch angle, branching frequency and branching interval were recorded at this stage of growth. The distance between tip and new branch point was 235 µm at 25±2 ºC and 130µm at 34±2 ºC. It was observed that at 25±2 ºC branch interval was 194 µm which was reduced to 166 µm at 34±2 ºC. The branch angle was 44º and 53º at 25±2 ºC and 34±2 ºC respectively. The frequency of branching was 0.553/100µm and 0.623/100µm at 25±2 ºC and 34±2 ºC respectively. Thus the branching frequency and branching angle are higher at 34±2 ºC while distance between tip and new branch point and branching interval are higher at 25±2 ºC, while the growth rate remains almost the same at both the temperatures. As a result at 34±2 ºC temperature the culture appears hyperbranched and shows predominantly dichotomous branching, whereas, at 25±2 ºC the culture shows more spreading growth. These results are consistent with the results of Watters and Griffith (2001), who grew various colonial mutants at different temperatures and found that at reduced temperature the branching interval, is increased. Our mutant shows differences in branching frequency, branch angle, branch interval and distance between tip and new branch point with change in temperature without showing much change in growth rate. It is proposed that this could be because at higher temperature the metabolic growth rate is high due to which large numbers of secretory vesicles reach the tip but due to some defect (mutation) these vesicles are not incorporated at the tip and are utilized for lateral branch formation. As a result branch frequency increases at high temperature i.e. 34±2 ºC. At lower temperature, there may be reduction in metabolic (synthetic) activities due to which fewer secretory vesicles reach the tip which are utilized for tip extension leaving fewer vesicles for branch initiation. As a result the branch interval and the distance between the tip and new branch point increased at 25±2 ºC whereas branching frequency decreased. It is still not clear why the angle of branching has changed. However, it was reported by Simonin *et al.,* (2012) that during initial phase of growth (when colony diameter was 2.5 mm) the hyphae display a branching angle of ~90º whereas the mature hyphae (when colony diameter was 5 mm) display branching angles of ~50º i.e. hyphal architecture changes with colony age. Thus change in branch angle may be related to change in physiology of the hyphae. This mutant may be useful for future research.

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