A NEW MUTANT OF *Neurospora crassa* (VU-82)
HAVING DEFECTS IN BRANCHING AND TIP GROWTH

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ABSTRACT

A growth defective mutant of *Neurospora crassa*, VU-82 was generated in the laboratory by mutagenizing conidia of well characterized strain of *N. crassa* (FGSC 2489) using Ethyl Methane Sulfonate (EMS) as mutagen. This mutant (VU-82) has slow growth rate (2.9 mm/h), shows predominant dichotomous branching, bursting of tips, hyperbranching and has irregularities in hyphal wall. Genetic studies show that these defects are heritable and are controlled by a single nuclear gene. This mutant may help in understanding mechanisms of branching and tip growth in fungi.

**Key Words**: *Neurospora crassa*, Mutagenesis, Ethyl methanesulfonate (EMS), Hyperbranching, Dichotomous branching, Bursting of tips

INTRODUCTION

Fungi are among the most biotechnologically useful group of organisms and are used for synthesizing a variety of economically important compounds like enzymes, chemicals, pharmaceuticals, foodstuffs etc. Yeast and some filamentous fungi have also been genetically modified for the production of heterologous gene proteins (recombinant proteins) and cheap biofuel. On the other hand fungi cause many diseases in plants, animals and human beings. Fungi are the most important group of plant pathogens which cause significant losses in crop yield worldwide. In humans, fungi cause many superficial and systemic acute infections like athlete’s foot and vaginitis. Invasive fungal infections like aspergillosis, candidiasis and histoplasmosis are major threat to immune-compromised cancer and HIV patients. Thus, fungi are beneficial as well as harmful to us. Fungal growth is crucial in all the above mentioned aspects of fungi. Many models have been proposed to explain fungal growth but many aspects like branching and tip growth are still not clearly understood. Fungal biologists all over the world are trying to understand the mechanisms of fungal growth. The deeper understanding of fungal growth mechanisms can be used in increasing the production of economically important compounds from fungi or in decreasing (or stopping) the fungal growth. The filamentous fungus *Neurospora crassa* has been used as a model microbe for understanding fungal growth using genetics, biochemistry and cell biology approaches. The prime requirement for such studies is the availability of novel growth defective mutants of fungi (like *Neurospora*) which can either be obtained from screening the natural populations of *Neurospora* or generated in laboratory by mutagenesis of well characterized culture using UV radiation or chemical mutagen like EMS. Study of these mutants may help in identifying new genes that are involved in fungal growth and biochemical studies may help in understanding the mechanism of fungal growth and branching.

AIMS AND OBJECTIVES

To mutagenize conidia of wild-type culture of *Neurospora crassa* using EMS and to isolate a morphological mutant which shows distinct heritable defects in growth.

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MATERIAL AND METHODS

Fungal strains and growth conditions

*Neurospora* tester strains used in the study, *N. crassa* 74-OR23-IVA (FGSC 2489) and ORS6a (FGSC 4200) were obtained from Fungal Genetic Stock Centre (FGSC), Kansas City, USA. Vogel’s minimal medium was used for growth and maintenance of *Neurospora* cultures. All growth conditions were as described by Perkins.  

Isolation of morphological mutant

Chemical mutagenesis was carried out using ethyl methanesulfonate (EMS) as chemical mutagen. Conidia were harvested from a seven days old culture of *N. crassa* wild-type strain 74-OR23-IV A (FGSC 2489). Conidial suspension was prepared in 0.05 M phosphate buffer, pH 7, containing $1.6 \times 10^8$ conidia per ml. Conidial concentration was determined by using Neubauer counting chamber. EMS was added to the conidial suspension to a final concentration of 0.1 M. Conidia were treated with EMS for different time periods i.e. 30 minutes, 1 h, 1.5 h and 2 h after which the conidial suspension was centrifuged and washed twice with phosphate buffer and finally stored in refrigerator at 4±2°C for further use. Mutant enrichment was done by cold treatment of mutagenized conidia at 7-8°C (in refrigerator) for 2 days. The filtrate containing enriched mutagenized conidia was plated on 1% sorbose containing Vogel's agar media. Plates were transferred to 25±2°C for 18 h. Well isolated colonies were picked and transferred to slants containing Vogel's minimal media.

Growth and morphology

The extension growth rate of the mutant and wild-type cultures was measured using race tubes as described by Mukati et al.  

Inheritance of the morphological defects

To study the inheritance of morphological defects the mutant was crossed with wild-type tester strain *Neurospora crassa* ORS6a (FGSC 4200) and random ascospores analysis was done. Twenty viable progeny from each cross were screened for morphological defects to determine if they inherited the parental defect or not. Inheritance of mutant phenotype and wild-type phenotype in 1:1 ratio in cross indicates that mutant phenotype may be due to mutation in single locus. To determine cytoplasmic (plasmid / mitochondrial) or nuclear basis of inheritance reciprocal crosses were made.  

RESULTS AND DISCUSSION

Mutagenesis and isolation of growth defective mutant

Conidia of seven day old culture of *N. crassa* 74-OR23-IVA (FGSC 2489) were mutagenized using EMS and after mutant enrichment conidial suspension was placed on 1% sorbose containing medium. Well isolated 156 colonies were obtained which were picked and transferred to Vogel's medium slants. All the 156 cultures were screened for morphological defects. From this screen, it was found that VU-82 had distinct growth defects.

Extension growth

Extension growth rate of VU-82 was measured by growing the culture in race tube at 25±2°C and 34±2°C and wild-type *N. crassa* (FGSC 2489) was used as control (Fig. 1). Results are graphically presented in Fig. 2. At 34±2°C the growth rate of VU-82 is 2.9 mm/h which is about 1.7 fold reduced as compared to wild-type culture which has growth rate of 5 mm/h (Fig. 2). At 25±2°C the growth rate of VU-82 is 2.2 mm/h which is about 1.7 fold reduced as compared to wild-type culture which has growth rate of 3.8 mm/h (Fig. 2). Thus VU-82 is a slow growing mutant. At 25±2°C both VU-82 and wild-type strain show reduced growth rates i.e., there is about 1.3 fold reduction in growth rates of both the cultures at 25±2°C as compared to their growth at 34±2°C. This can be explained as due to the fact that at 34±2°C, is the optimum temperature for growth of *Neurospora*, where the metabolic activities are fast while at 25±2°C the metabolic activities are reduced and thus growth rate also reduces.
Colony morphology
The strain VU-82 and wild-type *N. crassa* (FGSC 2489) were grown in petri dish at 25±2°C and their colony morphology was observed (Fig. 3). It can be seen that in petri plate wild-type *N. crassa* (Fig. 3a) completely covers the whole plate after 24 h and show conidiation at the margin. On the other hand VU-82 initially show only surface growth but later, long aerial hyphae form and start conidiating so the colony appears small conidiating with irregular margins (Fig. 3b).
Hyphal morphology

Hyphal morphology of VU-82 and wild-type *N. crassa* cultures were studied under microscope at different magnifications i.e., 40X, 100X and 400X. Results are shown in Fig. 4. As can be seen in Fig. 4 (a-c), wild-type *N. crassa* grow straight, has normal tapering dome shaped tips and shows lateral branching. In VU-82, at early phase of growth (about 18 h growth), hyphae remain thin, mostly unbranched and show clockwise curling (Fig. 4(d)). Later on (after about 22 h) erratic branching occurs. Most of the tips become dichotomously branched (Fig. 4(e-g) and Fig. 4(i)). Initial event of formation of dichotomous branch is rapidly (soon) followed by another event of dichotomous branching. Three-four such branching events at the apical region results in the formation of many branches close to one another. All the apical branches then grow with similar rates due to which apical region appears like broom (Fig. 4(e)). Hyphae no longer grow straight at this point but meander and show irregularities in hyphal wall (Fig. 4(g)). It seems that due to some defect in delivery of secretory vesicles to the tip region the extension rate of tip is slow. However, vesicles are continuously formed and transported to the apical region. The accumulation of secretory vesicles in the apical region triggers the initiation of new branch points thus apex become hyperbranched. Tips in which apical branches fail to arise usually burst (Fig. 4(h)).

![Fig. 4](image_url)
It may be possible that due to failure in the formation of new branch points, accumulated secretary vesicles in the apical region create pressure and thus tip bursts. Thus by mutagenesis of conidia of well characterized strain of *N. crassa* (FGSC 2489), we have generated an interesting mutant, VU-82 which has slow growth rate, shows predominant dichotomous branching, hyperbranching, bursting of tips and has irregularities in hyphal wall.

**Inheritance of mutant phenotype**

To determine whether the defects in VU-82 were inherited in the progeny or not VU-82 was crossed with wild-type tester strain of *N. crassa* (FGSC 4200). Reciprocal crosses were made to distinguish if the mutant phenotype was due to mutation in nuclear gene/mitochondrial DNA or due to presence of some cytoplasmic factor like plasmid). In cross # AM232, VU-82 was used as male parent whereas *N. crassa* (FGSC 4200) was used as female parent. This cross was successful. Whereas in cross # AM233, VU-82 was used as female parent and *N. crassa* (FGSC 4200) was used as male parent but the cross was not successful. Thus, from this it appears that VU-82 has one more interesting character i.e., it is sterile when used as female parent, which means it has some defect in the formation, structure or functioning of protoperithicia but it can be used as fertile male parent. Random ascospores analysis was done from cross # AM232 and thirty random ascospores were picked from the cross out of which twenty five ascospores germinated. So, ascospores viability was 83.3%. Morphological characters of twenty viable progeny were studied. Each progeny was grown in petri plate for 18–20 h at 25±2°C and observed under microscope and screened for defects in hyphal morphology. Extension growth rate of each progeny was also determined. The results are shown in Fig. 5 and summarized in Table 1.

**Fig. 5**: Inheritance of mutant phenotype of VU-82. Male and female parents are shown in upper part of the figure. Viable progeny are shown in middle part and bottom part shows progeny having morphology similar to wild-type parent and mutant parent.
Table 1: Inheritance of mutant phenotype of VU-82 (hyperbranching, dichotomous branching, bursting of tips, irregularities in hyphal wall and slow growth rate)

<table>
<thead>
<tr>
<th>S/N</th>
<th>Cross number and parents</th>
<th>Ascospore viability (% germination)</th>
<th>Analysis of 20 viable progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cultures showing hyphal morphology and growth rate similar to mutant parent</td>
</tr>
<tr>
<td>1</td>
<td>AM232 N. crassa (FGSC 4200) ♀ X VU-82 ♂</td>
<td>83.3%</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>AM233 VU-82 ♂ X N. crassa (FGSC 4200) ♀</td>
<td>Cross did not work and ascospores were not formed.</td>
<td>-</td>
</tr>
</tbody>
</table>

Results show that defects in hyphal morphology (i.e., predominant dichotomous branching, hyperbranching, bursting of tips and irregularities in hyphal wall) and growth rate, similar to mutant parent (VU-82) were present in twelve progeny. Whereas, eight progeny had hyphal morphology and extension growth rate similar to wild-type parent (N. crassa FGSC 4200) i.e., they had lateral branching pattern and normal dome shaped tips. From the above results, it is clear that defects of VU-82 are heritable and they are inherited as such in about 50% of the progeny. Thus, it appears that there is mutation in a single nuclear gene (and not due to cytoplasmic factor like plasmid or mutation in mitochondrial DNA) in VU-82 which leads to defects in growth and morphology because the inheritance of wild-type gene and mutant gene is 1:1 and not biased towards the female parent.12,13

CONCLUSION

We have isolated a new mutant of N. crassa by mutagenizing the conidia of wild-type culture obtained from FGSC. The mutant culture, VU-82 shows hyperbranching, dichotomous branching, bursting of tips, irregularities in hyphal wall and slow growth rate. The defects in growth and morphology are heritable and due to mutation in a single nuclear gene. Further, biochemical and genetic studies of this mutant may help in identifying gene responsible for these defects and understanding mechanisms of fungal growth and branching.

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