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MOLECULAR CHARACTERIZATION OF FOUR MORPHOLOGICAL MUTANTS OF *NEUROSPORA CRASSA*

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Abstract

Four morphological mutants (*albino, ropy, conidial band* and *buff*) of *Neurospora crassa* were characterized based on morphological features and molecular markers. The mycelial color of mutants *buff, ropy* and *conidial band* were more or less orange like wild parents. *Albino* mutant showed colorless mycelium. The germination time of conidia of wild and other mutants ranged from 5 to 7 days while in *albino* it was 12 days. Esterase and acid phosphatase isozymes analysis of the *N. crassa* mutants clearly indicated that mutation altered the carotenoid biosynthesis pathway creating the *albino* mutant due to the effect UV light. Most of the mutants *viz*; *albino*, *conidial band* and *buff* showed characteristic RAPD banding profile. However, no band was found in wild *EmA*, *Ema* and mutant *ropy*. Highest number of RAPD bands were found in *albino*. The mutant *albino* showed very different morphological and molecular features from the rest specimens.

Introduction

Neurospora crassa is a non-toxic and filamentous fungus belongs to Ascomycetes. It grows in tropical and subtropical regions. It is rich in carotenoids showing bright orange color in the culture. It possesses two opposite mating types called "A" and "a" and has distinct male and female sexual structures[1-2].

Neurospora crassa has been using as a model organism because of its easy growing nature and has a haploid life cycle. The ascospores are linearly arranged that make genetic analysis simple. Wide range of diversity present among the mutants of *N. crassa*.

A number of molecular investigations and genetics of *N. crassa* have been studied intensively[3-6]. In Bangladesh, a good number of classical researches were done on *N. crassa* such as linkage mapping, induction of different morphological mutants by UV-irradiation, complementation, segregation pattern, enzyme activities, soluble protein estimation etc.[7-8] However, no molecular research on this materials has yet been initiated which may provide more information about the material.

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To assess the genetic diversity at the molecular level, isozyme techniques with polyacrylamide gel electrophoresis (PAGE) are being used as a powerful tool[9-16]. The electrical properties of enzymes in this process can be employed to obtain mobility values for characterization of any organism.

In addition, DNA finger printing by randomly amplified polymorphic DNA (RAPD) is a unique tool for characterizing germplasms authentically. RAPD markers generated by the polymerase chain reaction (PCR), has been widely used since late 1980s of the last century to assess intraspecific genetic variation on a molecular level[17-18].

Therefore, the aim of the present study was to characterize the UV induced mutants by PAGE and RAPD markers and to find the correlation between morphological and molecular data.

Materials and Methods

Two wild (*EmA, Ema*) and four induced morphological mutants (*albino, ropy, conidial band* and *buff*) of *Neurospora crassa* were investigated in this study. The wild type strains used in the study were received from Fungal Genetic Stock Centre, Department of Microbiology, University of Kansas Medical School, Kansas, USA. Vogel's minimal medium (VM)[19] was used for the maintenance of cultures.

Five-day-old conidia of *Ema* were irradiated with UV light having wave length of 254 nm. Radiation was given at a distance of 12 cm. Media supplemented with arginine, histidine, leucine, lysine and tryptophan were used for plating and isolating the irradiated conidia. The isolated conidial cultures were incubated at 25ºC. After 5 days they were observed and classified according to their morphological and nutritional requirements. Fresh conidia of 4 - 6 days old cultures of selected morphological mutants and *Ema* were inoculated at the centre of the VM plate for germination. Germination and radial growth of the conidia were observed under the microscope after 1 and 3 hrs intervals, respectively. Linear growth of morphological mutants and *Ema* were studied through inoculation of mutant and wild type *N. crassa* at one end of the Race Tube containing VM media solidified with 3% agar. Observations were recorded after 6 hrs interval up to 96 hrs.

Mycelia were harvested and total genomic DNA was extracted by using CTAB method[20]. DNA concentration was quantified through spectrophotometer (Analylikjena, Specord 50, Germany). The A260/280 nm readings for DNA samples were 1.6 - 1.8. The PCR reaction mix for 25 µl containing template DNA (25 ng/µl) 2 µl, de-ionized distilled water 18.8 µl, Taq buffer A 10X (Tris with 15 mM $MgCl_2$) 2.5 µl, primer (10µM) 1.0 µl, dNTPs (2.5 mM) 0.5 µl, Taq DNA polymerase (5U/µl) 0.2 µl. PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature 94°C for 5 min, denature at 94°C for 1 min, annealing at 36°C for 30 sec, extension at 72°C for 3 min and final extension at 72°C for 5 min. Four random primers

were used for RAPD analysis- *viz.* Primer-10 (GCC ACG GAG A), Primer-11 (CAC GGC TGC G), Primer 12 (GTA TGG GGC T) and Primer-13 (GCG AAC CTC G).

The amplified products were separated electrophoretically on 1% agarose gel and DNA bands observed on UV-transilluminator and photographed by a gel documentation system. The PCR products were analyzed after gel electrophoresis. The photographs were critically discussed on the basis of presence (1), absence (0), size of bands and overall polymorphism of bands. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's gene diversity[21], genetic distance (D) and constructing a UPGMA (Unweighted pair group method of arithmetic means) Dendrogram among the mutants using computer program "POPGENE" (Version 1.31).

Enzyme location on polyacrylamide gel was carried out following standard method with slight modification[22]. For this purpose vertical polyacrylamide gel electrophoresis was carried out. Two separate methods of staining were used to detect the enzyme activity on the gels. Before loading, the samples were diluted in the ratio 2 : 1 with the sample buffer. Sample containing 250 μg of protein was applied during running of gels. After running, the gels were stained by different methods for two different types of enzyme localization. The incubation mixtures for esterase and acid phosphatase were used in different gels for the detection of two different isozymes. The banded gels were photographed quickly with digital camera.

Results and Discussion

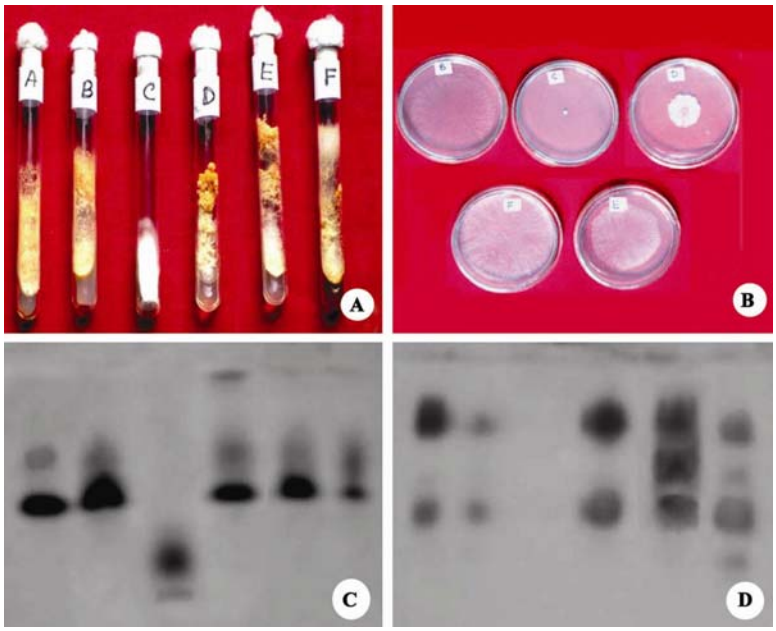
Four morphological mutants of *N. crassa* were able to produce from the wild *Ema* by using a common source and wave length of ultraviolet (UV) ray after exposing to different duration of time. The longest (90S) and shortest (45S) exposer time required in case of mutant *buff* and *albino*, respectively. Intermediate exposer time (60S) required for mutants *ropy* and *conidial band* (Table 1). This result suggested that duration of exposer time was a factor for developing different mutants.

Table 1. Comparative morphological features of *Neurospora crassa*.

Features	Name of the specimens				
	Wild	*albino*	*ropy*	*conidial band*	*buff*
Color of mycelia	Orange	Colorless	Orange	Orange	Buff
Structure of mycelia	Wild type	Mycelia and conidia cannot be distinguished	Pink, rope like	Formed band at the tip of the growth	Buff and fibrous
Dry weight (g)	0.8958	0.25235	0.3141	0.73595	1.2495
Conidial germination (hrs)	6	12	7	6	5

The mutants *ropy*, *conidial band* and *buff* were more or less looked like wild in respect of color of mycelia (*i.e.* orange), conidial germination after inoculation and dry weight

measured after 72 hrs of inoculation (Fig. 1A, B). However, the *albino* mutant was totally different from the rest in this regards (Table 1). The orange color of mycelium is due to presence of carotenoid. The mutation in *ropy*, *conidial band* and *buff* did not change the biosynthesis pathway of carotenoid. The lack of orange color in *albino* clearly indicated that mutation affected somehow in the carotenoid biosynthesis pathway which prevents the synthesis of this pigments resulting the formation of colorless mycelia in *albino* mutant (Fig. 1A, B).



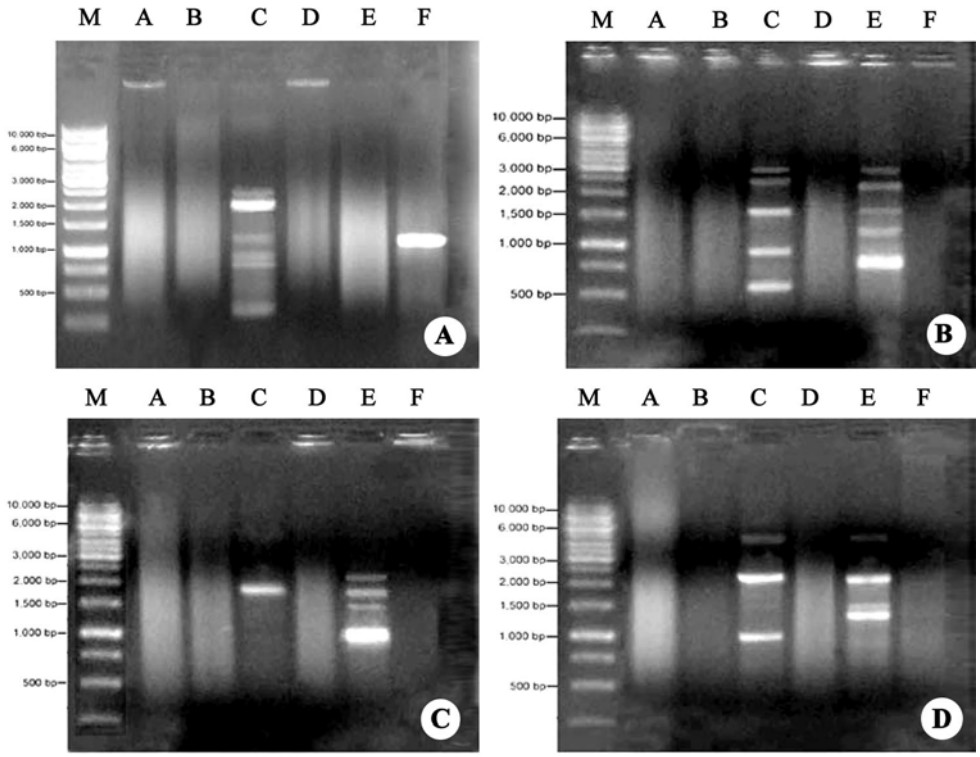
Figs 1A-B. Photograps of wild type and mutants of *Neurospora crassa.* A. Morphological features. B. Conidial germination and radial growth. C-D. Isozyme analysis of the mycelia extract of *Neurospora crassa.* C. Esterase activity following Polyacrylamide gel electrophoresis (PAGE). D. Acid phosphatase activity following polyacrylamide gel electrophoresis (PAGE). In all cases, A = *EmA*, B = *Ema*, C = *albino*, D = *ropy*, E = *conidial band*, F = *buff*.

Besides mycelium color, albino mutants were different from the wild and other mutants in two more parameters: (i) time of conidial germination after inoculation was nearly double in *albino* and (ii) lowest dry weight after 72 hrs of incubation found in *albino* (Table 1). The delayed germination (Fig. 1B) in *albino* may be due to change in the DNA responsible for mitotic growth.

Generally mutants are less vigor than the wild since the mutants usually carrying recessive allele[23]. In this experiment, an exceptional case was found in *buff* mutant. The dry weight of the mutant at 72 hrs of incubation was higher than wild (Table 1). The reason for this exception was not clear, however the mutation may change the respective recessive allele somehow.

The mutant *ropy* and *conidial band* differed from wild in respect of conidial structure. The *ropy* mutant produced rope like conidia while a clear band was formed at the tip of the conidia in conidial band mutants (Table 1). In contrast, the mycelium and conidial structure in *albino* and *buff* were like wild. This result revealed that mutation occurred in in the gene responsible for conidial structure *ropy* and *conidial band* mutant while, this gene remained unaffected in the mutant *albino* and *buff*.

Activities of two isozyme systems *viz.* esterase and acid phosphatase were investigated in six specimens of *Neurospora crassa*. In esterase, more or less two common bands were found in all specimens except *albino* (Fig. 1C). Presence of common bands in five specimens indicated the regular esterase activity. However, *albino* showed two unique esterase bands at the lower region of the profile (Fig. 1C). This result point out that mutation in *albino* may change the product of esterase activity into smaller molecular weight thus these two bands found at the lower region of profile. In acid phosphatase, no band was found in *albino* whereas different banding patterns were observed in the rest five specimens (Fig. 1D) suggested that mutation affected the activity of acid phosphatase in *albino*.



Figs. 2A-D. RAPD analysis: A. Primer sequence GCC ACG GAG A. B. CAC GGC TGC G. C. GTA TGG GGC T. D. GCC ACG GAG A. M = 1 Kb DNA ladder, A = EmA, B = Ema, C = albino, D = ropy, E = conidial band, F = buff.

Four primer combinations were used for RAPD analysis of six *Neurospora crassa* specimens. No RAPD band was found in wild *EmA*, *Ema* and mutant *ropy*. Mutant *albino*, *conidial band* and *buff* showed different RAPD bands. Although these 3 mutants showed some common bands in different primer combinations, each specimen showed characteristic DNA finger printing patterns. Highest number of bands were found in *albino* for this 4 primer combinations followed by *conidial band* mutants (Figs 2A-D).

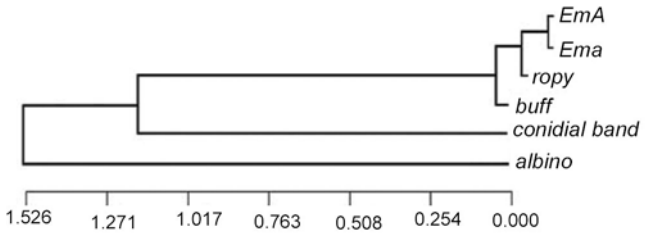


Fig. 3. UPGMA dendrogram based on Nei's genetic distance summarizing the data on differentiation between six specimens of *Neurospora crassa*, according to RAPD analysis.

With the help of RAPD scoring, a dendogram was made on the basis of genetic distance[21]. The cluster analysis clearly indicated that the *albino* mutant was totally different from the rest five specimens (Fig. 3). On the basis of genetic distance this mutant was separated initially into different clusters. The other five samples however, placed in the same cluster different from that of *albino*. This differentiation was correlated with the morphological characteristic and isozyme activities. Therefore, a combination of classical and molecular data it was possible to characterize different *Neurospora crassa* mutants.

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