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INDUCTION OF MUTATION IN *NEUROSPORA CRASSA* USING NEEM (*AZADIRACHTA INDICA* A. JUSS) LEAF EXTRACT

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Key words: Morphological mutant, *Neurospora crassa*, *Azadirachta indica* A. Juss, Leaf extract

Abstract

Filamentous fungus has been used as a significant source of biotechnological applications. *Neurospora crassa*, a type of red bread mold, has been well recognized as a model system in fundamental scientific investigations. It can be effectively utilized as a valuable resource for molecular tools, and many mutations are available. Furthermore, *N. crassa* exhibits rapid growth and has no harmful properties. These characteristics indicate a significant, although unexplored, capacity of this fungus for use in biotechnological endeavors. The present investigation aimed to induce morphological changes in the *N. crassa* wild type by employing leaf extracts derived from *Azadirachta indica* A. Juss. To determine the mutagenic and growth-inhibitory effects of *A. indica* leaves, morphological mutants were identified and individually examined. Six morphological mutants such as *albino (al), cauliflower (clf), conidial band (co.band), ropy light (ro.lig), dirty (dir), and ropy (ro)* were obtained from the conidia of the wild-type *N. crassa* Ema (5297) strain, treated with *A. indica* A. Juss. leaf extracts. These mutants were compared to the wild-type, natural form of the organism in terms of their morphology, radial growth, and reproduction ability. Apparent variations were observed when a comparative study of Ema and the selected morphological mutants was undertaken. Linkage among the selected mutants in their specific linkage group was determined. *Ropy*, *dirty*, and *albino* were all linked to leu-3 (linkage group I), *conidial-band* and *ropy light* to trp-1 (linkage group III), and *cauliflower* to trp-4 (linkage group VII). Complementation was not observed in similar morphological *N. crassa* mutants.

Introduction

Plant extracts have antifungal effects because they are rich in bioactive substances[(1)]. The utilization of plant preparations for the treatment of fungal diseases has garnered significant interest in recent times. Presently, there is a particular emphasis on the identification of novel antifungal constituents derived from plants that do not pose any adverse impact on the environment, as well as on animal and human systems. Numerous researchers have documented the antifungal properties exhibited by plant extracts against various diseases affecting significant crops[(2-4)]. However, it is important to consider that the

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effectiveness of plant extracts as antifungal agents may vary depending on the specific fungal strain and the concentration of the extract used, making it necessary to conduct further research and testing before widespread implementation. Furthermore, the phytochemical properties of plant extracts also make them an appealing source for studying the mutagenic properties of *N. crassa*, as the chemical mutagens can penetrate the cells and change the DNA[5,6]. The use of artificial mutation makes it possible to generate a large number of mutants that cannot grow without adding a particular amino acid, vitamin, or other nutrient to the culture medium. The actual mutation is anticipated to occur at the structural level of the DNA. Westergaard and Mitchel (1947)[7] conducted the most extensive study of biochemical mutagens in *N. crassa*. They studied how biochemical mutagens interact with fungi, and their experiments showed that when exposed to certain compounds, the fungi could develop mutations.

Filamentous fungi play important roles in numerous biotechnological applications. *Neurospora crassa* the red bread mold has been widely recognized and utilized as a model organism in fundamental genetic investigations[8]. The filamentous fungus *Neurospora crassa* has long been established as a model organism in basic research covering a wide variety of scientific topics including circadian rhythms, genome evolution, cell fusion, cell polarity[9-12]. Furthermore, *Neurospora crassa* exhibits rapid growth and is devoid of any harmful properties [13, 14].

Beadle and Tatum's (1941)[15] attempt to expose *N. crassa* to x-rays, which resulted in mutation, combined genetics, and biochemistry. It enabled intensive use of *N. crassa* for the artificial induction of mutation and the investigation of the molecular mechanism of mutation[16-18]. Furthermore, this fungus can make a lot of identical, mostly haploid-nucleated conidia, which makes it possible to study spontaneous and natural mutation[19, 20]. Artificial mutations can be used to produce auxotrophic mutants.

Azadirachta indica A. Juss., commonly known as neem, is widely used in both conventional and modern medicine for the treatment of many infectious, metabolic, or cancerous diseases[21,22]. *A. indica* has been linked to a variety of pharmacological and biological effects, including antifungal, anti-inflammatory, and antibacterial properties[23]. Neem leaves contain Nimbinene, Nimbolide, Quercetin, β-sitosterol, Azadirachtin, and Nimbosterin[24]. The antifungal properties of *A. indica* leaf extracts were evaluated by Haque and Shamsi (1996)[25], and radial growth inhibition of *N. crassa* was observed, but the mutagenic effects of the plant extracts were not studied. The goal of the current study was to use leaf extracts from *A. indica* to cause morphological mutations in the *N. crassa* wild type. To ascertain the mutagenic and growth-inhibitory effects of *A. indica* leaves, morphological mutants were isolated and individually characterized. Additionally, a linkage map of the induced mutants was prepared along with their complementation studies.

Materials and Methods

Two opposite mating types known as Emerson 'a' (Ema) (5297) and Emerson 'A' (5296) and some markers were used for this investigation. *N. crassa* Ema (5297) was applied for treating conidia with the mutagen, i.e., leaf extracts of Neem. *N. crassa* EmA (5296) and Ema (5297) were used to examine the mating types of the mutants. Seven markers were used for the study of linkage (Table 1). The wild-type strains of *N. crassa* and markers were obtained from the Microbial Genetics Stock, Department of Botany, University of Dhaka, Bangladesh.

Table 1. Standard markers

Fungal genetic stock center number	Markers with their allele numbers	Mating type	Position of the gene	Linkage group
4002	leu-3 (R156)	A	IL	I
4034		A	IIR	II
3735		A	IIIR	III
3865	arg-5 (27947)	A	IVR	IV
2332	trp-1 (10575)	A	VR	V
4107	trp- 4 (Y2198)	A	VIR	VI
4091	trp-5 (420)	A	VIIR	VII
	trp-2 (75001)			
	arg-10 (B317)			

Culture media: Vogel's minimal medium (VM)[26] was used for culturing *Neurospora crassa* in the test tube and counting the spores on the plate. Amino acid supplements were used in the culturing medium according to the specific type of mutant. The supplemented media were named after the name of the amino acid used, e.g. Leucine medium, Tryptophan medium, etc. Solid VM was used for subculturing, and liquid VM was used for determining the mycelial growth of the organism. To restrict the overlapping colonial growth Sorbose minimal (SM) medium[27] was used for isolation plating. Westergaard's[7] crossing medium was used for different wild mutants crossing. It restricts vegetative growth but enhances sexual reproduction. In some cases, when the crosses were sterile, i.e., perithecia did not form, Suyama's crossing medium was used[28].

Preparation of leaf extracts of Azadirachta indica: 100 gm of *A. indica* leaves were taken and washed twice, once with tap water and twice with distilled water. After air drying, the leaves were cut into small pieces. After that, a mortar and pestle were used to grind the leaves. The paste was then collected in a falcon tube and centrifuged for ten minutes at 3000 rpm. The supernatant was collected using another tube. With sterile distilled water, various extract concentrations were prepared.

Treating of Ema with desired extracts of A. indica leaves: To obtain fresh culture, Ema was cultured three times at 5-day intervals in each case. A 5-day-old culture was used for

treating conidia. All the media, essential elements, and instruments were sterilized in the autoclave at 121 °C under 15 lb pressures for 20 minutes. The inoculation chamber, needle, centrifuge machine, etc. were sterilized with rectified spirit. 4 ml of (100%) *Azadirachta indica* A. Juss. leaf extract was taken into a centrifuge tube. One loop of conidia Ema was taken into the tube and shaken for a homogenous solution. The tube was kept for 6 h, 12 h, and overnight to allow mutations to occur. The solution was centrifuged for 10 minutes. After centrifugation, the solution above the conidia was poured out from the centrifuge tube. 8 ml of sterilized distilled water was added to the centrifuge tube and centrifuged for 10 minutes and the supernatant was poured out. The same procedure was repeated three times. 8 ml of distilled water was added with the treated conidia remaining at the bottom of the centrifuge tube and the tube was shaken well. The sterilized petri dishes were marked and 200µl of suspension was taken accordingly. 10 ml of molten SM media was added to petri dish and shaken gently to mix the suspension and media. The plates were kept inside the incubator at 25 °C for 24 hours for growth of conidia. A number of well separated colonies were isolated from each petri dish by cutting agar blocks from the conidial colony with an arrow shaped isolating needle and being inoculated into small tubes containing VM media. Precautions were taken so that only the distinctly separated growing conidial colonies were isolated. After 5 days, all the cultures were observed and classified by comparing their characters with wild type Ema. The conidial cultures that had any morphological variations were sub-cultured several times in small tubes and checked carefully whether any permanent morphological change had taken place. The mutants were selected for further analysis. Growth of mycelia, color of hyphae and conidia, etc. were considered while selection was made.

Study of mycelial weight: Selected mutants and Ema were inoculated in 50 ml of liquid VM medium in the flask. To prevent the formation of conidia, the flask was shaken continuously. Weight of the empty filter paper was taken. When the cultures were 72 hours old, flasks with culture were boiled in water for 2 minutes and filtered with filter papers of known weight. The filter papers with mycelia were dried in the air, and the weights of the filter papers with culture were measured.

Study of radial growth of different mutants: To study the radial growth of different mutants, 10 ml of molten VM media were taken on each of the sterilized petri dishes. When the medium became solid, the center of the Petri dish was marked. A fresh culture of the mutant was inoculated at that point with a sterilized needle, and Ema was also inoculated into another petri dish as the control plate. All the Petri dishes were kept in incubator at 25 °C. After 18, 24, 30 and 36 hours, the radial growth of mutants of each petri dish was measured in cm.

Table 2. Different concentrations of leaf extracts of *A. indica* solutions.

Amount of leaf (g)	Amount of crude extracts (ml)	Amount of sterilized distilled water (ml)	Concentration of the crude extract in the solution in %
100	10	0	100
	10	10	50
	10	20	33
	10	30	25
	10	40	20

Study the mutagenic effect of extracts of Azadirachta indica.: To test the mutagenic effects of leaf extracts of *A. indica* on the growth of different mutants with different concentrations of leaf extract was prepared (Table 2). 100 g leaf with 4.0 ml sterilized distilled water were added and grained. Finally, 10.0 ml of crude extract was obtained. From this crude extracts different concentrations of *A. indica* leaf extract solutions viz. 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml were taken to determine the mutagenic effect. Ema was also inoculated into another petridis as for control plate. All the petridishes were kept in an incubator at 25 °C. Observations were made after 6, 12, 18 and 24 hours. Lower radial growth indicated higher mutagenic effect (Table 3).

Table 3. Different concentrations of *A. indica* leaf extract solutions.

Amount of leaf (g)	Amount of crude extracts (ml)	Amount of sterilized distilled water (ml)	Concentration of the solution taken (ml)	% of the leaf extract in the solution
100	10	4	1.0	6
			2.0	12
			3.0	18
			4.0	24

Determination of mating type: For determination of mating type of six morphological mutants of *N. crassa*-Ema, EmA and the mutants were sub-cultured 3 times on VM media. Five days old cultures were used for determination of mating type. The mating type of selected mutants was determined by crossing them with both Ema and EmA. The result of the crosses was observed for 5-30 days. If the perithecia were formed with Ema, the mating type of the mutant is 'A' and if the perithecia were formed with EmA, mating type of the mutant is 'a'.

Study of fertility: Six morphological mutants were crossed with marker of 7 linkage groups. The crosses were made in Westergaard crossing medium with required supplements to study the fertility of the mutants.

Detection of linkage group: Detection method of linkage group of 6 morphological mutants induced with leaf extracts of *A. indica* is described below:

The mutants and markers were sub-cultured 3 times at 5 days interval to improve fertility. Mutants were crossed with seven markers in Westergaard's crossing medium with required supplement and incubated at 25 °C. The fertile cultures formed perthecia after 1st week of the crosses and shedded spores within 15-30 days. One drop of sterilized water was taken on the 10 ml SM medium in a petri dish. A loop of spore was taken from the desired cross with the help of a sterilized needle and was transferred to the water drop on the plate and uniformly spread on it. The name of the cross was written on the upper lid of the petri dish. The prepared petri dish was given a heat shock at 58 °C for 50 minutes and was then incubated at 25 °C for 12-16 hours. After incubation period the spreaded plate was examined under stereoscopic microscope and the germinating spores, growing spores, non-growing spores were counted. Growing spores were isolated with the help of isolating needle in the VM supplemented tubes. The tubes were incubated at 25 °C for growth. After 4 days isolates were analyzed. The ratio between the mutant isolates and wild type isolates were calculated. For linkage study the isolates were tested on SM.

Complementation test: Both small and large tubes of VM medium were used for complementation tests. Two mutants of the same mating type were tested for complementarity. Many conidia were taken from a fresh culture of one mutant that was five days old and placed inside the tube. Similarly, a few conidia from the other mutant were taken and put on top of the conidia of the former mutant. As a control, they were additionally placed separately in two different tubes. These tests were done twice. First, the tubes were incubated for 96 hours at 25 °C. The six morphological mutants of *N. crassa* induced with leaf extracts irradiation were tested for complementation in a solid VM medium. The tests were observed on the 2nd, 4th, 7th, 14th, and 21st days.

Determination of Genetic Map of different mutants: Linkage maps indicate the relative distances between genes in a linkage group. These distances are calculated as a percentage of crossing over for a single group of chromosomes. One map unit corresponds to the linear distance over which one percent of crossings occur. In order to calculate the map distance of genes on a chromosome, the recombination frequency, or percentage of crossing over, of each gene locus should have been determined. A centrimorgan (cM) is the unit of chromosome length for which crossing over/recombination frequency is 1 percent. Genetic map distances among the different mutants were carried out using the following formula.

$$\text{Distance} = \frac{R_1+R_2}{\text{Total Progenies}} \times 100 \text{ (cM)}$$

Here, R_1 = Wild, R_2 = Recombinants

Results and Discussion

Induction of mutation in N. crassa with treatment of leaf extracts of A. indica: 100% leaf extracts derived from *Azadirachta indica* A. Juss. were selected as a therapeutic approach for treating Ema. The duration of treatment was 6 hours, 12 hours, and 24 hours. The leaf extract of *A. indica* had a notable mutagenesis impact on *N. crassa*, resulting in the generation of distinct groups of mutants. From 61 mutants, the conidial band showed the lowest frequency (8.33%), and the dirty showed the highest frequency (28.33%). The mutants induced by *A. indica* leaf extract in *N. crassa* were classified, characterized, and named based on their observed frequencies and characteristics, as shown in Table 4. A visual representation of the mutants can also be seen in Figure 1. The results indicate that the extract had a significant mutagenic effect on the fungus, generating different groups of mutants with varying frequencies of the conidial band and dirty phenotypes. It is also proposed that the length of the exposer period played a role in the development of distinct mutants. The same finding has been established by Akter *et al.* (2014)[29].

Table 4. Classification and Nomenclature of *A. indica* leaf extract induced mutants of *N. crassa*.

Group	Name of the mutant	Characteristics	No. of mutant	Frequency (%)
A	*Albino (al)*	Both conidia and mycelia are colourless.	8	13.33
B	*Cauliflower (clf)*	Growth is checked. Conidia forming cauliflower like structure.	12	20
C	*Conidial band (con.band)*	Dense conidial growth forms a band shaped structure at the top. Colour of the conidia is pink or orange.	5	8.33
D	*ropy light (ro.lig)*	Both conidia and mycelia are whitish.	7	11.66
E	*dirty (dir)*	Conidial lump scattered throughout the body of the culture tube.	17	28.33
F	*ropy (ro)*	Mycelia form rope like structure and conidia pink.	11	18.33

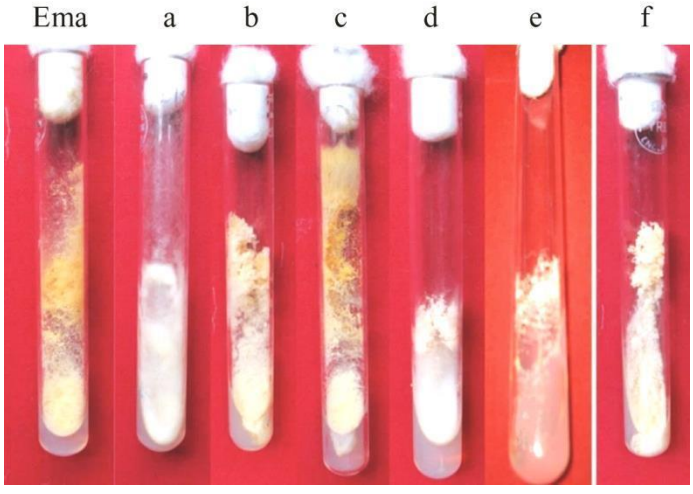

Fig. 1. Ema and morphological mutants induced with leaf extracts of A. indica- a. albino (al), b. cauliflower (clf), c. conidial band (con.band), d. ropy light (lig), e. dirty (dir) and f. ropy (ro).

Dry mycelial weight of selected morphological mutants and Ema: Differences have been identified in the dry weights of selected mutants and Ema. Table 5 presents the observed differences in the dry weight of mycelial growth among the mutants with Ema following a 72-hour culture period. It came to light that specific mutant with Ema varied in their dry weight. These variations in dry weight suggest that the mutants may have different growth rates or metabolic activities compared to Ema. Further investigation is needed to determine the underlying factors contributing to these differences and their potential implications for the overall performance of the mutants.

Table 5. Mycelial growth of selected mutants after 72 hours.

Name of the cultures	Mean weight of the empty filter paper (gm) ± SD	Mean weight of the filter paper with dried mycelia (gm) ± SD	Mean weight of the dried mycelia (gm) ± SD
Albino	1.002 ± 0.000	1.430 ± 0.000	0.429 ± 0.001
cauliflower	1.002 ± 0.000	1.123 ± 0.003	0.131 ± 0.003
Conidial band	1.002 ± 0.000	1.410 ± 0.005	0.424 ± 0.005
ropy light	1.002 ± 0.000	1.198 ± 0.024	0.119 ± 0.024
ropy	1.002 ± 0.000	1.501 ± 0.003	0.489 ± 0.004
dirty	1.003 ± 0.001	1.380 ± 0.002	0.371 ± 0.002
Ema	1.002 ± 0.000	1.364 ± 0.007	0.460 ± 0.036

Values are represented as mean ± SD where n =3

Study of radial growth of morphological mutants: Radial growth of the wild-type Ema and selected mutants was studied after 18 to 36 hours (Fig. 2). After 24 hours, wild-type Ema (5297)

grew up to 3.73 cm, whereas mutant albino grew up to 4.5 cm, ropy grew up to 1.5 cm, dirty grew up to 3.17 cm, conidial band grew up to 3.07 cm, light grew up to 1.8 cm, and cauliflower grew up to 1.53 cm. These results indicate that the mutant albino strain exhibited the highest radial growth rate among all the tested strains after 24 hours. However, it is worth noting that the wild-type Ema strain still displayed a considerable growth rate compared to the other mutant strains, except for the albino mutant. The results suggest that the mutant albino strain has a significantly faster growth rate compared to the other mutant strains, as evidenced by its larger size after 24 hours. This indicates that the mutation in the albino strain may have led to an enhanced ability to proliferate and expand radially. It would be interesting to further investigate the genetic factors responsible for this increased growth rate in the albino mutant and compare it to the wild-type strain for a better understanding of the underlying mechanisms.

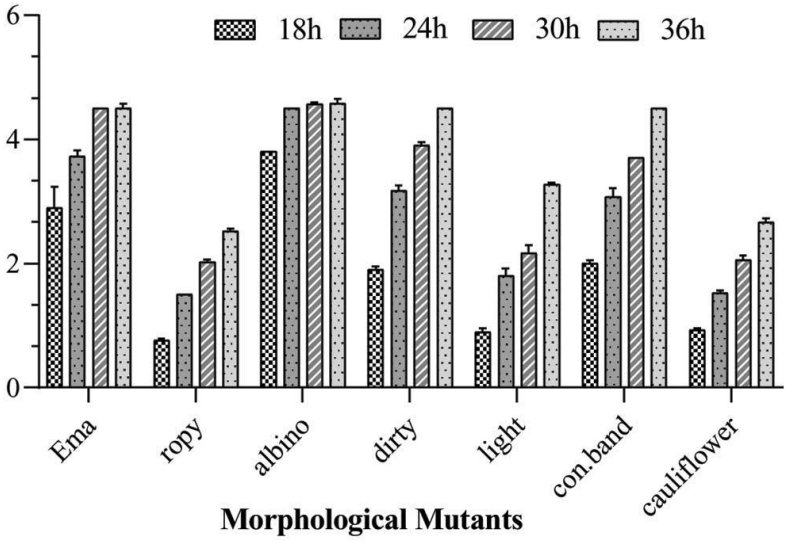

Fig. 2. Radial growth of selected mutants and Ema on VM media.

Mutagenic effect of leaf extracts (A. indica) on different mutants: To test the mutagenic effects of leaf extracts of *A. indica* on the growth of different mutants with different concentrations of leaf extract was prepared. Leaf extracts of *A. indica* showed significant mutagenic effects on different mutants of *N. crassa*. The effect was determined by measuring the radial growth of mycelia of different mutants. The results are shown in fig. 3 (a-f). It was observed that 1 ml concentration of leaf extract caused highest radial growth and lowest radial growth was observed at 4 ml concentration as compared to control plate. Highest mutagenic activity was observed to be caused by 24% leaf extract after 24 hrs on *albino, ropy, conidial band, ropy light, cauliflower, dirty* mutants where the radial growths were 1 cm, 0.34 cm, 0.67 cm, 0.3 cm, 0.3 cm, 1 cm respectively representing the lowest growth in each case. These findings suggest that the concentration of leaf extract has a significant impact on radial growth and mutagenic activity. Specifically, higher concentrations of leaf extract leads to decreased

radial growth and increased mutagenic activity. These results highlight the potential of the leaf extract as a powerful mutagenic agent for inducing genetic mutations in fungi.

Fertility of selected mutants from the crosses with Ema and EmA: Selected mutants were crossed with Ema and EmA separately and were thereafter studied for a duration ranging from 4 to 28 days. The mutants did not show variations in their mating types. However, different mutants behaved differently; most were fertile, while some were poor in their fertility (Table 6). The results of this study indicate that the observed variations in fertility among the mutants may be influenced by factors other than mating types. Additionally, exploring other genetic and environmental factors that could contribute to the observed variations would provide a more comprehensive understanding of their fertility characteristics.

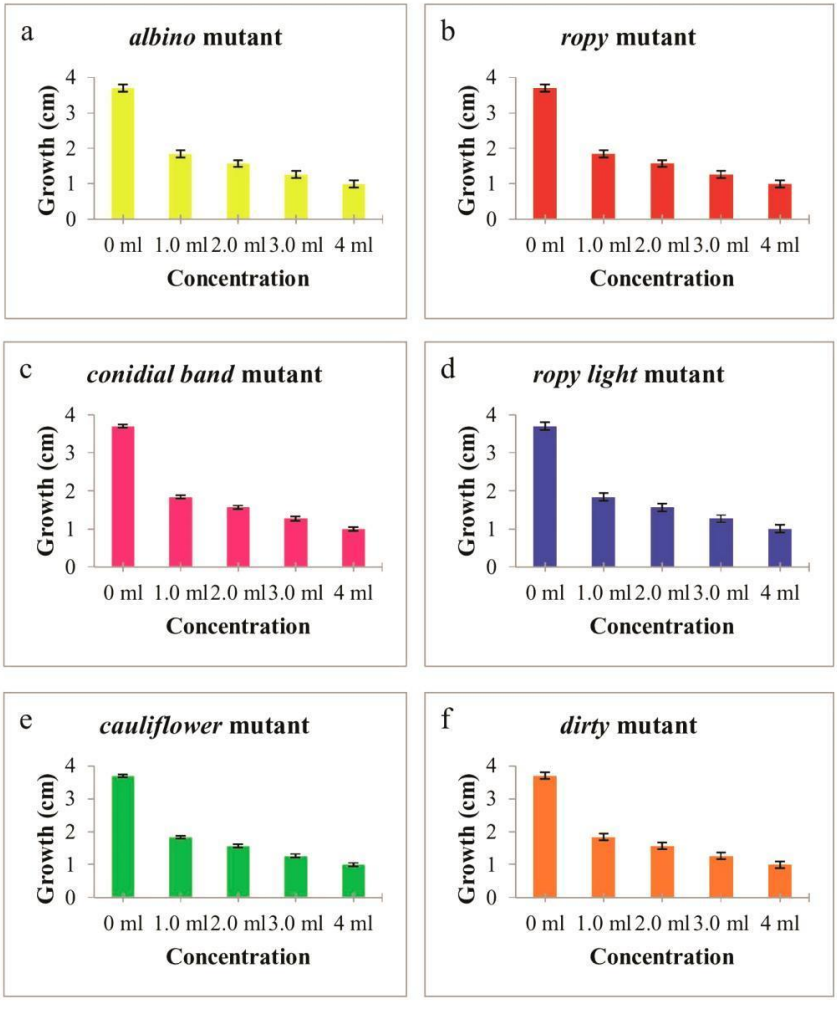

Fig. 3 (a-f): Graphical representations of radial growths of *albino, ropy, conidial band, ropy light, cauliflower, dirty* mutants on VM containing *Azadirachta indica* leaf extracts (after 24 hours).

Table 6. Mating types and fertility of the selected mutants.

Designation of the cross	Whether perithecia formed Yes/No	Days of initiation of perithecia	frequency of perithecia	Size of perithecia	Mating type	Whether spore shed Yes/No	Shedding days	Fertility
ropy × *Ema*	No	-	-	-	a	-	-	Fertile
ropy × EmA	Yes	6	Few	Medium		Yes	13	
dirty× Ema	No	-	-	-		-	-	Poor
dirty × EmA	Yes	14	Few	Large	a	Yes	27	fertile
conband ×Ema	No	-	-	-		-	-	Poor
conband × EmA	Yes	7	Many	Medium	a	Yes	22	fertile
light × Ema	No	-	-	-		-	-	Fertile
light × EmA	Yes	13	Few	Medium	a	Yes	21	
clf × Ema	No	-	-	-		-	-	Fertile
clf × EmA	Yes	6	Many	Large	a	Yes	15	
al × Ema	No	-	-	-		-	-	Poor
al × EmA	Yes	12	Few	Medium	a	Yes	24	fertile

Fertility of mutants with markers of seven linkage groups: Six morphological mutants were crossed with markers of 7 linkage groups to study the fertility of the mutants. The results from the crosses with the markers from the seven linkage groups revealed exciting fertility patterns among the different morphological mutants. The *ropy* mutant showed fertility in crosses with five linkage groups I, III, V, VI, and VII out of the seven. This observation suggests a possible genetic link between the morphological mutants of the individual and the markers present in the specific linkage groups. However, the *ropy* mutant displayed reduced fertility when crossed with markers from linkage groups II and IV. Similar observations were made for the *dirty*, *conidial band*, *light*, *cauliflower*, and *albino* mutants. These findings are summarized in Table 7, suggesting a possible disruption in the genetic interactions between these specific linkage groups and the mutants.

Study of linkage: A linkage study of *ropy* (498a), *dirty* (213a), *conidial band* (118a), *light ropy* (285a), *cauliflower* (112a) and *albino* (136a) were conducted by crossing them with seven markers leu-3 (R156)A, arg-5 (27947)A, trp-I (106)A, trp-4 (Y2198)A, trp-5 (A420)A, trp-2 (75001)A and arg-10 (B317)A-representative of all seven linkage groups (I – VII). The data regarding the crosses are presented in table 8. The results of the crosses revealed that the traits ropy, dirty and *albino* were found to be linked with the marker leu-3 of linkage group I. Furthermore, it was shown that the traits *conidial band* and *ropy light* exhibited significant links with the marker trp-I of linkage group III. In contrast, c*lf* strongly correlated with the marker trp-4 of linkage group IV.

Determination of Genetic Map of different mutant: Genetic maps of the mutants with their marker were constructed. Genetic map of *ropy* 498a mutant with its marker leu-3 (R 156)A of linkage group I gave a distance of 34.84 CM. Distance of dirty 213a with leu-3 is 27.66 CM and distance of albino 136a with its marker leu-3 is 22.62 CM. Mutant *con-band* 118a was linked with trp-I (106)A of linkage group-III and distance between them is 32CM and genetic map of ropy light 285a mutant with its marker trp-I (106) gave a distance of 21.74 CM. Mutant *clf* 112a was linked with trp-4 (Y2198) A of linkage group- IV and distance between them is 24.14 CM (Table 9).

Complementation test: Complementation tests were made on Vogel's minimal medium in small and big tubes. The six morphological mutants were tested for complementation in solid medium. The tests were observed on 3, 5, 7, 15 and 21 days. Complementations of different and similar morphological mutants were done to see whether the mutants were allelic or not (Fig. 4).

Table 7. Study the fertility test of six morphological mutants of *N. crassa* with markers of 7 linkage groups.

Name of the mutant	Designation of the cross	Linkage group	Whether perithecia formed Yes/No	Days of initiation of Perithecia	Frequency and size of the Perithecia	Whether spore shed	Shedding days	Fertility
Dirty	*dirty*×lue-3A	I	Yes	13	Few, Medium	Yes	26	Poor fertile
	dirty× arg-5A	II	Yes	8	Few, Small	Yes	27	Poor fertile
	dirty× trp-1A	III	Yes	10	Many, Medium	Yes	22	Fertile
	dirty× trp-4A	IV	Yes	12	Few, Medium	Yes	26	Poor fertile
	dirty× trp-5A	V	Yes	9	Few, Medium	Yes	21	Fertile
	dirty× trp-2A	VI	Yes	8	Many, Medium	Yes	20	Fertile
	dirty× arg-10A	VII	Yes	7	Many, Medium	Yes	21	Fertile
Conidial band	*con band* × lue-3A	I	Yes	8	Many Small	Yes	19	Fertile
	con band × arg-5A	II	Yes	9	Few Medium	Yes	26	Poor fertile
	con band × trp-1A	III	Yes	8	Many Small	Yes	20	Fertile
	con band × trp-4A	IV	Yes	10	Few Large	Yes	25	Poor fertile
	con band × trp-5A	V	Yes	8	Some Large	Yes	18	Fertile
	con band × trp-2A	VI	Yes	9	Few Medium	Yes	20	Fertile
	con band × arg-10A	VII	Yes	8	Some Small	Yes	21	Fertile
Ropy light	*ropy light* ×lue-3A	I	Yes	15	Few, Medium	Yes	27	Poor Fertile
	ropy light × arg-5A	II	yes	8	Many, Medium	Yes	18	Fertile
	ropy light × trp-1A	III	Yes	8	Many, Medium	Yes	18	Fertile
	ropy light × trp-4A	IV	Yes	9	Few, Large	Yes	25	Poor Fertile
	ropy light × trp-5A	V	Yes	8	Many, Medium	Yes	18	Fertile
	ropy light × trp-2A	VI	Yes	15	Few, Medium	Yes	26	Poor Fertile
	ropy light × arg-10A	VII	Yes	8	Many, Medium	Yes	20	Fertile
	clf ×lue-3A	I	Yes	8	Many, Medium	Yes	21	Fertile

Cauliflower	*clf* × arg-5A	II	yes	11	Few, Medium	Yes	27	Poor Fertile
	clf × trp-1A	III	Yes	7	Few, Large	Yes	16	Fertile
	clf × trp-4A	IV	Yes	10	Few, Small	Yes	25	Poor Fertile
	clf × trp-5A	V	Yes	7	Few, Medium	Yes	17	Fertile
	clf × trp-2A	VI	Yes	12	Few, Medium	Yes	26	Poor Fertile
	clf × arg-10A	VII	Yes	8	Many, Medium	Yes	18	Fertile
Albino	*al* ×lue-3A	I	Yes	10	Many, Medium	Yes	18	Fertile
	al × arg-5A	II	Yes	16	Many, Medium	Yes	22	Fertile
	al × trp-1A	III	Yes	12	Few, Small	Yes	21	Poor Fertile
	al × trp-4A	IV	Yes	12	Many, Medium	Yes	21	Fertile
	al × trp-5A	V	Yes	15	Many, Medium	Yes	21	Fertile
	al × trp-2A	VI	Yes	18	Few, Small	Yes	27	Poor Fertile
	al × arg-10A	VII	Yes	8	Many, Medium	Yes	18	Fertile
Ropy	*ropy* ×lue-3A	I	Yes	8	Many, Medium	Yes	16	Fertile
	ropy× arg-5A	II	Yes	9	Many, Small	Yes	18	Poor fertile
	ropy× trp-1A	III	Yes	8	Many, Small	Yes	17	Fertile
	ropy× trp-4A	IV	Yes	12	Many, Medium	Yes	26	Poor fertile
	ropy× trp-5A	V	Yes	10	Few, Small	Yes	27	Fertile
	ropy× trp-2A	VI	Yes	13	Few, Medium	Yes	25	Fertile
	ropy× arg-10A	VII	Yes	11	Many, Large	Yes	20	Fertile

Table 8. Linkage of *ropy* (498a), *dirty* (213a), *conidial band* (118a), *ropy light* (285a), *cauliflower* (112a) and *albino* mutant (136a).

Marker used	Linkage group	Crossing name	Total isolates	Progenies	% of the progenies	Inference on linkage
leu-3A	I	*ro* × leu-3A	89	a. Wild=14	a. 15.73	Linked with leu-3A of linkage group-I
				b. leu-3A=30	b. 33.70	
				c. *ro*=28	c. 31.46	
				d. *ro*+leuA=17	d. 19.10	
arg-5A	II	*ro* × arg-5A	94	a. Wild= 21	a. 22.34	Not linked with linkage group-II
				b. arg-5A=21	b. 22.34	
				c. *ro*=28	c. 29.78	
				d. *ro*+arg-5A=24	d. 25.53	
trp-1A	III	*ro* × trp-1A	82	a. Wild=20	a. 24.39	Not linked with linkage group-III
				b. trp-1A=35	b. 42.68	
				c. *ro*=11	c. 13.41	
				d. *ro*+trp-1A=16	d. 19.51	
trp-4A	IV	*ro* × trp-4A	84	a. Wild=23	a. 27.38	Not linked with linkage group-IV
				b. trp-4A=23	b. 27.38	
				c. *ro*=11	c. 13.09	
				d. *ro*+trp-4A=27	d. 32.14	

trp-5A	V	*ro* × trp-5A	81	a. Wild=21 b. trp-5A=23 c. *ropy*=22 d. *ropy*+trp-5A=15	a. 25.93 b. 28.39 c. 27.16 d. 18.51	Not linked with linkage group-V
trp-2A	VI	*ro* × trp-2A	86	a. Wild=21 b. trp-2A=22 c. *ro*=19 d. *ro*+trp-2A=24	a. 24.42 b. 25.58 c. 22.09 d. 27.9	Not linked with linkage group-VI
arg-10A	VII	*ro* × arg-10A	78	a. Wild=25 b. arg-10A=15 c. *ro*=26 d. *ro*+trp-1A=12	a. 32.05 b. 19.23 c. 33.33 d. 15.38	Not linked with linkage group-VII
leu-3A	I	*dir* × leu-3A	94	a. Wild=18 b. leu-3A=32 c. *dir*=36 d. *dir*+leuA=8	a. 34.04 b. 38.30 c. 19.15 d. 8.51	Linked with leu-3A of linkage group-I
arg-5A	II	*dir* × arg-5A	87	a. Wild= 25 b. arg-5A=20 c. *dir*=28 d. *dir*+arg-5A=14	a. 28.74 b. 22.98 c. 32.18 d. 16.09	Not linked with linkage group-II
trp-1A	III	*dir* × trp-1A	70	a. Wild=15 b. trp-1A=16 c. *dir*=18 d. *dir*+trp-1A=21	a. 22.86 b. 25.71 c. 21.43 d. 30	Not linked with linkage group-III
trp-4A	IV	*dir* × *trp*-4A	98	a. Wild=25 b. trp-4A=21 c. *dir*=24 d. *dir*+trp-4A=28	a. 21.43 b. 24.49 c. 25.51 d. 28.57	Not linked with linkage group-IV
trp-5A	V	*dir* × trp-5A	88	a. Wild=18 b. trp-5A=25 c. *dir*=29 d. *dir*+trp-5A=16	a. 20.45 b. 28.40 c. 32.95 d. 18.18	Not linked with linkage group-V
trp-2A	VI	*dir* × trp-2A	96	a. Wild=28 b. trp-2A=20 c. *dir*=22 d. *dir*+trp-2A=26	a. 29.17 b. 20.83 c. 22.91 d. 27.8	Not linked with linkage group-VI
arg-10A	VII	*dir* × arg-10A	95	a. Wild=24 b. arg-10A=22 c. *dir*=23 d. *dir*+trp-1A=26	a. 25.26 b. 23.16 c. 24.21 d. 27.37	Not linked with linkage group-VII
leu-3A	I	*con band* × leu-3A	94	a. Wild=27 b. leu-3A=21 c. *con band* =15	a. 28.72 b. 22.34 c. 15.95	Not linked with leu-3A of linkage group-I

				d. *con band* +leuA=31	d. 32.97	
arg-5A	II	*con band* × arg-5A	89	a. Wild= 12 b. arg-5A=31 c. *con band* =29 d. *con band* +arg-5A=17	a. 13.48 b. 34.83 c. 32.58 d. 19.10	Not linked with linkage group-II
trp-1A	III	*con band* × trp-1A	75	a. Wild=11 b. trp-1A=24 c. *con band* =27 d. *con band* +trp-1A=13	a. 14.66 b. 32.00 c. 36.00 d. 17.33	Linked with linkage group-III
trp-4A	IV	*con band* × trp-4A	77	a. Wild=17 b. trp-4A=9 c. *con band* =23 d. *con band* +trp-4A=28	a. 22.07 b. 11.68 c. 29.87 d. 36.36	Not linked with linkage group-IV
trp-5A	V	*con-band*× trp-5A	90	a. Wild=22 b. trp-5A=30 c. *con band* =22 d. *con band* +trp-5A=16	a. 24.44 b. 33.33 c. 24.44 d. 17.77	Not linked with linkage group-V
trp-2A	VI	*con band* × trp-2A	87	a. Wild=20 b. trp-2A=22 c. *con band* =19 d. *con band* +trp-2A=26	a. 22.99 b. 25.29 c. 21.84 d. 29.89	Not linked with linkage group-VI
arg-10A	VII	*con band* × arg-10A	92	a. Wild=26 b. arg-10A=27 c. *con band* =23 d. *con band* +trp-1A=26	a. 28.26 b. 29.34 c. 25.00 d. 28.26	Not linked with linkage group-VII
leu-3A	I	*ro lig* × leu-3A	92	a. Wild=23 b. leu-3A=26 c. *ro lig*=21 d. *ro lig*+leuA=22	a. 25.00 b. 28.26 c. 22.82 d. 23.91	Not linked with leu-3A of linkage group-I
arg-5A	II	*ro lig* × arg-5A	85	a. Wild= 21 b. arg-5A=27 c. *ro lig*=23 d. *ro lig*+arg-5A=14	a. 24.71 b. 31.76 c. 27.05 d. 16.47	Not linked with linkage group-II
trp-1A	III	*ro lig* × trp-1A	92	a. Wild=9 b. trp-1A=35 c. *ro lig*=37 d. *ro lig*+trp-1A=11	a. 9.78 b. 38.04 c. 40.21 d. 11.95	Linked with linkage group-III

trp-4A	IV	*ro lig* × trp-4A	77	a. Wild=17 b. trp-4A=23 c. *ro lig*=22 d. *ro lig*+trp-4A=15	a. 22.07 b. 29.87 c. 28.57 d. 19.48	Not linked with linkage group-IV
trp-5A	V	*ro lig* × trp-5A	79	a. Wild=18 b. trp-5A=20 c. *ro lig*=22 d. *ro lig*+trp-5A=19	a. 22.78 b. 25.31 c. 27.84 d. 24.05	Not linked with linkage group-V
trp-2A	VI	*ro lig* × trp-2A	84	a. Wild=23 b. trp-2A=19 c. *ro lig*=27 d. *ro lig*+trp-2A=15	a. 27.38 b. 22.61 c. 32.14 d. 17.85	Not linked with linkage group-VI
arg-10A	VII	*ro lig* × arg-10A	88	a. Wild=22 b. arg-10A=17 c. *ro lig*=29 d. *ro lig*+trp-1A=20	a. 25.00 b. 19.31 c. 32.95 d. 22.72	Not linked with linkage group-VII
leu-3A	I	*clf* × leu-3A	88	a. Wild=25 b. leu-3A=27 c. *clf*=23 d. *clf*+leuA=13	a. 28.42 b. 30.69 c. 26.14 d. 14.78	Not linked with leu-3A of linkage group-I
arg-5A	II	*clf* × arg-5A	95	a. Wild= 24 b. arg-5A=29 c. *clf*=28 d. *clf*+arg-5A=14	a. 25.26 b. 30.53 c. 29.48 d. 14.74	Not linked with linkage group-II
trp-1A	III	*clf* × trp-1A	105	a. Wild=8 b. trp-1A=38 c. *clf.*=45 d. *clf*+trp-1A=14	a. 7.62 b. 36.20 c. 42.86 d. 13.34	Not linked with linkage group-III
trp-4A	IV	*clf* × trp-4A	87	a. Wild=03 b. trp-4A=35 c. *clf*=31 d. *clf*+trp-4A=18	a. 3.45 b. 40.23 c. 35.64 d. 20.69	Linked with linkage group-IV
trp-5A	V	*clf* × trp-5A	82	a. Wild=22 b. trp-5A=25 c. *clf*=22 d. *clf*+trp-5A=13	a. 26.83 b. 30.49 c. 26.83 d. 15.86	Not linked with linkage group-V
trp-2A	VI	*clf* × trp-2A	92	a. Wild=26 b. trp-2A=22 c. *clf*=21 d. *clf*+trp-2A=23	a. 28.26 b. 23.91 c. 22.82 d. 25.00	Not linked with linkage group-VI

arg-10A	VII	*clf.* × arg-10A	93	a. Wild=33 b. arg-10A=25 c. *clf*=19 d. *clf*+trp-1A=16	a. 35.48 b. 26.89 c. 20.44 d. 17.21	Not linked with linkage group-VII
leu-3A	I	*al* × leu-3A	84	a. Wild=11 b. leu-3A=30 c. *al*=35 d. *al*+leuA=8	a. 13.10 b. 35.72 c. 41.67 d. 9.53	Linked with leu-3A of linkage group-I
arg-5A	II	*al* × arg-5A	91	a. Wild= 27 b. arg-5A=20 c. *al*=30 d. *al*+arg-5A=14	a. 29.67 b. 21.98 c. 32.97 d. 15.39	Not linked with linkage group-II
trp-1A	III	*al* × trp-1A	90	a. Wild=23 b. trp-1A=4 c. *al*=25 d. *al*+trp-1A=18	a. 25.55 b. 26.66 c. 27.77 d. 20.00	Not linked with linkage group-III
trp-4A	IV	*al* × trp-4A	99	a. Wild=29 b. trp-4A=25 c. *al*=29 d. *al*+trp-4A=16	a. 29.29 b. 25.26 c. 29.29 d. 16.17	Not linked with linkage group-IV
trp-5A	V	*al* × trp-5A	103	a. Wild=32 b. trp-5A=26 c. *al*=23 d. *al*+trp-5A=12	a. 31.07 b. 25.25 c. 22.34 d. 11.66	Not linked with linkage group-V
trp-2A	VI	*al* × trp-2A	85	a. Wild=26 b. trp-2A=18 c. *al*=18 d. *al*+trp-2A=23	a. 30.59 b. 21.18 c. 21.18 d. 27.06	Not linked with linkage group-VI
arg-10A	VII	*al* × arg-10A	82	a. Wild=21 b. arg-10A=16 c. *al*=31 d. *al*+trp-1A=14	a. 25.61 b. 19.52 c. 37.81 d. 17.08	Not linked with linkage group-VII

The results were negative when similar morphological mutant were used but positive positive when different morphological mutants were used.

Table 9. Distance of the mutant with the marker.

Mutant used	Marker Used	Linkage group of the marker	Distance of and mutant with marker
ropy 498a	leu-3 (R156)A	I	*ropy* leu-3 34.84 CM
dirty 213a	leu-3 (R156)A	I	*dirty* leu-3 27.66 CM
albino 136a	leu-3 (R156)A	I	*albino* leu-3 22.62 CM
con-band 118a	trp-1 (106A)	III	*con.band* trp-1 32 CM
ropy light 285a	trp-1 (106A)	III	*ropy light* trp-1 21.74 CM
clf 112a	trp-4 (Y2198)A	IV	*clf* trp-4 24.14 CM

	con.band 255	*dir* 403	*ro* 412	*clf* 127	*ab* 201	*ro lig* 171
conidial band 255	◎	●	●	●	●	●
	dirty 403	◎	●	●	●	●
		ropy 412	◎	●	●	●
			clf 127	◎	●	●
				albino 201	◎	●
					ropy light 171	◎

● = Complementation positive, ◎ = Complementation negative

Fig. 4. Complementation matrix of selected mutants in solid VM media.

Treatment with leaf extracts of *Azadirachta indica* successfully induced mutations on *Neurospora crassa* wild type (Ema). 6 groups of morphological mutants with different mutation frequencies were obtained. The mutants showed variation with Ema in their morphology, weight of mycelia, radial growth, fertility and mating type. Linkage studies are a valuable method for investigating whether genes in microorganisms, plants, or animals are connected or independently distributed from parents to offspring. These findings revealed that neem leaf extracts has considerable effect for the production of mutation in *Neurospora crassa* which can be used for further biotechnological research.

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