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# Isolation, Characterization of *Neurospora crassa* Mutants Exhibiting Growth Inhibition and Mutagenesis of this Fungus by the Leaf Extracts of *Leucas aspera* (Willd) Link.

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## Abstract

Ten *Neurospora crassa* mutants obtained by chemical mutagenesis were screened in *Leucas aspera* (willd) Link. (Bengali name- swetadrone) plants. The aqueous extracts of the leaves of *L. aspera* showed significant inhibition of growth and mutagenic effect on *N. crassa*. Results showed that 1 ml extract reacted with the test organism of *N. crassa* very rapidly. During the period of 24 hours, it produced only 2.6 cm linear vegetative growth of the mycelia. Whereas, 1 ml and 0.50 ml extracts exhibited mild reactions (3.20 and 3.90 cm. respectively). The linear growth decreased with the increase of the concentrations of extracts. Conidia of Ema (5297) of *N. crassa* were treated for 3-4 hours in 100% concentration of the extracts. 100% extracts produced ten types of mutants, namely- *dirty, vigorus, ropy, albino, mat, plug, check, fluppy, cauliflower* and *conidial brand.*

**Key words**: *Neurospora crassa*, *Leucas aspera*, *Mutants*, *Dirty*, *Vigorous*, *Cauliflower* and *Conidial brand.*

## Introduction

*Neurospora crassa* is the most suitable material for genetic study. Genetic work with this fungus effectively began sixty-six years ago. The experimental material of *N. crassa* is a well-known pink bread mould. It is a filamentous fungus that belongs to the Class-Ascomycetes. Their extremely short life cycle and capacity to grow in minimal medium help the scientists of this branch to perform extensive work within a period of short time, which is one of the most essential factors of genitival study (Watson, 1970). Beadle and Tatum (1941) first used the *N. crassa* in genitival and at the same time for biochemical study. *N. crassa* was originally selected for studying biochemical genetics

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because it is an organism in which both formal genetic analysis and investigation of nutritional mutants appeared possible, (Beadle and Tatum, 1941). Plant extracts play an important role to check the growth of various fungi. Scientists are interested in evaluating the antifungal activities of plant extracts against plant pathogenic fungi (Ahmed and Sultana, 1984; Bashar and Rai, 1991; Anwar *et. al.*, 1994). Haque and Shamsi (1996) observed that the leaf extracts of Neem (*Azadirachta indica*) have antifungal properties and it decreased the radial growth of fungus but none of them studied the mutagenic effect of the plant extracts. By the application of artificial mutation many mutants can be obtained which fail to grow unless some specific substances, such as amino acid or vitamin are added to the culture medium. The actual mutation is supposed to take place in the level of DNA structure. Perhaps the most extensive and systemic surveys of the action of biochemical mutagens on fungi have been made by Westergaard on *N. crassa* (Fincham and Day, 1965). Therefore, a plan was made to induce mutation morphological by using leaf extracts of *L. aspera*. Few selected and interesting mutants were studied for there morphologically changes by studying the linear growth, mycelial weight, time required for germination, fertility, mating type, heterocaryosis in comparison with the wild type Ema. Chemical mutagens have the ability to penetrate the cells and to change the DNA. Presently, scientists are interested to evaluate the mutagenic properties of chemicals on *N. crassa.* So the present study was undertaken to treat swetadrone aqueous extracts by *N. crassa* to produce morphological mutants. If morphological mutants formed, then all of them were isolated and characterized separately to determine the mutagenic and growth inhibitory effects of *L. aspera* present in the leaves.

## Materials and Methods

*N. crassa* Ema (5297) was the experimental material. The wild type strain was received from Fungal Genetic Stock Centre, Department of Microbiology; University of Kansas Medical School, Kansas, U.S.A. Strain Ema (5297) was used. Vogel's minimal medium (VM) (Vogel, 1956) was used for maintaining the cultures. Solid VM was used for obtaining and measuring linear growth of conidia (Ryan *et. al.*, 1943). Different concentrations of aqueous extracts of the leaves of *L. aspera* were used in the experiments. The extraction procedure is given below:

### Aqueous extract

Mature fresh green leaves of *L. aspera* was washed with sterilized distilled water and then air dried and 100 g of clean leaves was ground with mortar and pestle. The paste was filtered to get extracts. The filtered extract was centrifuged for 5 minutes in 300 rpm at $25^{O}$C. The supernatant was used for this experiment.

**Preparation of solution at different concentration of swetadrone**

Experiments were made to find out the mutagenic effect of swetadrone extracts on *N. crassa.* Leaf extracts of different density were prepared with the help of sterilized distilled water. Leaves of different weights were taken separately, washed 3-4 times with tap water and 3 times with distilled water. After air-drying, the leaves were ground in an electric grinder with distilled water and without distilled water. The paste was filtered with a sterilized cotton cloth and finally filtered with filtre paper to get clean solution. Different extracts of known strengths were prepared using leaves and sterilized distilled water. 20 g leaves were pasted without distilled water and it was as $N_p$. The next solution of 20 g leaves and 10 ml distilled water ($N_1$), 10 g leaves and 10 ml distilled water ($N_2$), 10 g leaves and 18 ml distilled water ($N_3$), 10 g leaves and 20 ml distilled water ($N_4$), and 10 ml distilled water and 4 g leaves ($N_5$) were prepared which were denoted as $N_1$, $N_2$, $N_3$, $N_4$ and $N_5$ (Table I). All extracts namely $N_p$, $N_1$, $N_2$, $N_3$, $N_4$ and $N_5$ were preserved separately in sterilized test tubes in a refrigerator. Some of the extracts were also sterilized in autoclave.

**Preparation of different extracts of swetadrone leaves**

Different concentrations of the leaf extracts of swetadrone with sterilized distilled water were prepared.

**Table I. Different doses of swetadrone solution**

| Name of the solution | Amount of leaf (g) | Amount of sterilized distilled water (ml) |
|---|---|---|
| $N_p$ | 20 | - |
| $N_1$ | 20 | 10 |
| $N_2$ | 10 | 10 |
| $N_3$ | 10 | 18 |
| $N_4$ | 10 | 20 |
| $N_5$ | 4 | 10 |

**Effect of water extracts of swetadrone on the growth of *Neurospora crassa***

For testing the effect of water extracts of swetadrone on the growth on *N. crassa,* different concentrations of it were prepared. Different solutions were taken separately on the petri dishes at the rate of 1 drop, 2 drops, 3 drops, 4 drops, 8 drops, 0.5 ml, 1ml, 1.5ml, 2ml, 3 ml and 4ml. Ten ml of molten SM medium was added in each Petri dish and rotated gently for uniform mixing of solution with the medium. When the medium became solid, the centre of the Petri dish was marked and fresh culture of Ema was inoculated at that point with a sterilized needle. All the Petri dishes were kept in an incubator at 25$^{O}$C after 16, 24, 40 and 48 hours, the radial growth of Ema of each petri dish was measured in cm.

**Treating of Ema with desired extracts of swetadrone leaves**

**a. Obtaining fresh culture**

To obtain fresh culture, Ema was cultured 4 times at 4 days intervals in each case. Five days old culture was used for treating conidia.

**b. Sterilization**

All the media, essential elements and instruments were sterilized in an autoclave at 120$^{O}$C under 15 Ib pressure for 20 minutes. The inoculation chamber, needle, centrifuge machine etc. were also sterilized with rectified spirit.

**c. Centrifugation**

Ten ml swetadrone leaf extracts of the concentration $N_p$, $N_1$, $N_2$, $N_5$ were taken into 4 centrifuge tubes. One loop of conidia (about 10,000 conidia) of Ema was taken into each tube and was shaken for homogenous solution. The solution was centrifuged with the help of a centrifuge machine for 20 minutes in 300 rpm at 25$^{O}$C.

**d. Filtration**

After centrifugation, the solution above conidia was poured out from the centrifuge tube. Ten ml of sterilized distilled water was added to the centrifuge tube and centrifuged for 3 minutes. Then the distilled water was poured out: The same procedure was repeated twice.

**e) Preparation of suspension with treated conidia**

10 ml of distilled water was added to the treated conidia remaining at the bottom of the centrifuge tube and the tube was shaken well.

**f. Plating of treated conidia**

The sterilized Petri dishes were marked as $N_p$, $N_1$, $N_2$, $N_5$ and 1 drop of the suspension of each was taken. Accordingly, 10 ml of molten SM medium was added to a Petri dish and were shaken gently to mix with the suspension and media. The plates were kept inside the incubator at 25$^{O}$C for maximum growth of conidia.

**g. Isolation of single conidial colony**

A number of well-separated colonies were isolated by cutting agar blocks from the conidial colony with an arrow shaped isolating needle and were inoculated into small tubes containing VM media. Precautions were taken so that separated growing conidial colonies can be isolated only.

**h. Classification**

After 5 days, all the cultures were observed and classified by comparing their characters with wild type Ema (Table II). The conidial cultures with any morphological variation were subcultured several times in small tubes and checked carefully whether any permanent morphological change occurs.

**Table II. Characteristics of the mutants of *N. crassa* obtained by the induction with swetadrone (100% doses).**

| Name of the mutants | Characteristics of the mutants |
|---|---|
| *Ropy* | The mycelia look like beautiful ropes, conidia pinkish a orange in colour. Growth is less than wild type. |
| *Albino* | Less growth of mycelia, conidia are very scanty in number. Mycelia and conidia are completely colourless. |
| *Mat plug* | Mycelia have a characteristic frayed appearance like mat<br>The mycelial growth reach the plug of the tube, deep pink conidia are formed outside the tube, conidia around the plug. |
| *Checked* | Growth lesser than wild, conidia light pink. |
| *Dirty* | Small conidial lump scattered here and there in the tube. |
| *Conidial brand.* | Dense conidial growths form a band shaped structure at the top. |
| *Vigorous* | Pink coloured profuse conidial growth, which reaches upto the plug. |
| *Ffluffy* | Mycelia colourless, conidia very few or totally absent, mycelia are cottony and growth is vigorous. |
| *Cauliflower* | It produces cauliflower-like buttons of growth. |

**Growth test of different morphological mutants on SM, VM (sucrose) and VM (glucose)**

Growth tests were made in sterilized Petri dishes containing sorbose minimal medium, Vogel's minimal medium (sucrose) and vogel's minimal medium (glucose was used in lieu of sucrose). The plates were divided into 24 compartments by glass marking pencil. Then conidia from a particular mutant were put in one compartment, 23 compartments contained conidia from different mutants and one contained wild type (Ema) as control.

**Study of mutagenic effect of extracts of swetadrone (*Leucas aspera*) on *Neurospora crassa***

Mature fresh green leaves of swetadrone were washed with sterilized distilled water and then air dried. 50g of clean leaves were ground with mortar and pestle. The paste

was filtered through the extracts. The filtered extract was centrifuged for 5 minutes. The supernatant was used for this experiment. Six sets of experiments were set taking 0.5 ml, 1 ml, 1.5 ml, 2 ml, 3 ml and 4 ml of extracts. Conidia of Ema (5297) of *N. crassa* were treated for 3-4 hours in 100% concentration of the extracts. It was centrifuged and the supernatant was discarded, treated conidia were washed twice with sterilized distilled water by pouring 1 ml sterilized distilled water in each centrifuge tube. Final suspension was made with sterilized distilled water. Then, 1 drop, 2 drops, 3 drops, 4 drops and 8 drops of conidial suspensions were taken in each of the 30 Petri dishes {Table III (i) and (ii)} and radial growths of Ema on VM containing swetadrone extracts were observed. Vogel's minimal medium (VM) was poured in each of the 30 Petri dishes. Plates were incubated for the formation of conidial colonies for 3 days. Observation was made daily for the appearance of the colony. The colonies were isolated in the test tubes containing Vogel's minimal medium (VM) and the isolates were incubated for growth at $25^{O}$C. After 4 days, the isolates were examined and classified (Table II). Different concentrations of swetadrone extracts were used to test the mutagenic effect on *N. crassa*. Fixed concentrations had appropriate mutagenic effects on *N. crassa.*

**Results and Discussion**

Chemical substances, which showed mutagenic activities, became longer in every year. The authors tested the mutagenic effect of leaf extracts of swetadrone (*Leucas aspera*) on the fungus, *N. crassa.* The plant leaves contain glucosides, tannins, saponins and sterols, a- and ß-sitosterol, oleic, linoleic, palmitic, stearic, oleanolic and ursolic acids have been isolated from this plant (Ghani, 1998). To determine the efficacy of swetadrone on the radial growth of *N. crassa* different doses of leaf extracts (sterilized and non-sterilized) were used. It was evident from Table III (i, ii) that the radial growth of *N. crassa* was proportional to the dosess of swetadrone extracts used. By increasing the doses of leaf extracts of swetadrone radial growth of Ema was reduced in comparison to control. $N_p$ doses were found to be more effective than $N_1$, $N_2$, $N_3$ and $N_4$ doses. An extensive study was conducted on different doses, so that *N. crassa* may tolerate little checked growth and to determine the extract amount to kill the fungus *N. crassa.*

From the Table III (i and ii) it was noted that 4 ml of doses decreased the radial growth extensively and the colony became very compressed and checked as compared to the control and 4 ml of dose killed the fungus *N. crassa.* There was no notable difference

**Table III (i) Effect of swetadrone extracts on the radial growth of *N. crassa* Ema.**

| Doses of the solution | Solution type | Treatment time (hr.) | Amount of solution taken in a Petri dish and growth obtained in cm | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 drop (Control) | 1 drop | 2 drops | 3 drops | 4 drops | 8 drops | 0.5 ml. | 0.1 ml. | 1.5 ml. | 2 ml. | 3 ml. | 4 ml. |
| 10 g leaves + 20 ml distilled water ($N_4$) | Sterilized | 16 | 4.70 | 3.60 | 2.70 | 1.80 | 1.40 | 1.30 | 5.20 | 4.10 | 3.20 | 2.80 | 2.20 | 0 |
| | | 24 | 4.70 | 3.80 | 2.90 | 2.60 | 2.30 | 1.80 | 3.90 | 3.20 | 2.50 | 1.60 | 0.90 | 0 |
| | | 40 | Over | Over | Over | 4.90 | 3.80 | 3.90 | 3.70 | 2.60 | 1.40 | 0.90 | 0.20 | 0 |
| | | 48 | Over | Over | Over | Over | 4.10 | 4.50 | 3.90 | 3.80 | 3.50 | 3.50 | 0.50 | 0 |
| | Unsterilized | 16 | Over | 1.90 | 1.80 | 1.70 | 1.60 | 1.40 | 4.45 | 3.20 | 2.10 | 1.90 | 0.30 | 0 |
| | | 24 | Over | Over | 5.20 | 4.50 | 3.10 | 2.80 | 3.90 | 3.20 | 1.50 | 1.20 | 0.60 | 0 |
| | | 40 | Over | Over | Over | Over | 3.80 | 3.70 | 3.60 | 3.20 | 3.00 | 2.60 | 1.40 | 0 |
| | | 48 | Over | Over | Over | Over | Over | 4.10 | 3.10 | 2.90 | 1.70 | 1.60 | 1.50 | 0 |
| 10 g leaves + 18 ml distilled water ($N_3$) | Sterilized | 16 | 3.90 | 3.70 | 2.80 | 2.30 | 1.80 | 1.20 | 5.10 | 4.90 | 4.10 | 3.90 | 0.20 | 0 |
| | | 24 | 4.80 | 4.50 | 3.90 | 3.10 | 2.50 | 1.80 | 3.90 | 3.20 | 2.20 | 1.10 | 0.90 | 0 |
| | | 40 | Over | Over | 5.50 | 5.00 | 4.90 | 4.60 | 3.40 | 3.30 | 3.20 | 2.90 | 0.50 | 0 |
| | | 48 | Over | Over | over | 5.30 | 5.20 | 5.10 | 4.90 | 3.80 | 3.50 | 3.10 | 0.60 | 0 |
| | Unsterilized | 16 | 3.70 | 3.60 | 2.50 | 2.40 | 2.10 | 1.90 | 4.90 | 4.70 | 3.60 | 2.40 | 0.80 | 0 |
| | | 24 | 4.50 | 3.90 | 2.60 | 2.60 | 2.20 | 1.80 | 3.90 | 3.20 | 2.10 | 2.00 | 0.90 | 0 |
| | | 40 | over | Over | Over | 5.30 | 4.80 | 3.70 | 3.40 | 3.30 | 3.20 | 2.90 | 1.70 | 0 |
| | | 48 | Over | Over | over | Over | 5.20 | 4.90 | 4.70 | 4.60 | 3.90 | 2.50 | 1.90 | 0 |

**Table III (ii)**

| Doses of the solution | Treatme-nt time (hr.) | Amount of solution taken in a Petri dish and growth obtained in cm | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0drop (Control) | 1 drop | 2 drops | 3 drops | 4 drops | 8 drops | 0.5 ml. | 0.1 ml. | 1.5 ml. | 2 ml. | 3 ml. | 4 ml. |
| 10g leaves + 10 ml distilled water ($N_2$) | 16 | 2.20 | 1.80 | 1.70 | 1.50 | 1.30 | 1.20 | 3.90 | 3.70 | 2.50 | 1.30 | 0.10 | 0 |
| | 24 | 2.50 | 2.40 | 2.35 | 2.30 | 2.20 | 1.90 | 3.90 | 3.20 | 1.40 | 1.30 | 0.20 | 0 |
| | 40 | over | 3.50 | 3.40 | 3.30 | 3.20 | 3.00 | 2.90 | 2.70 | 2.20 | 1.50 | 0.30 | 0 |
| | 48 | over | 4.10 | 3.90 | 3.80 | 3.70 | 3.50 | 3.40 | 3.10 | 2.80 | 1.70 | 0.40 | 0 |
| 20g leaves + 10 ml distilled water ($N_1$) | 16 | 3.60 | 2.20 | 1.90 | 1.80 | 1.60 | 1.10 | 3.20 | 3.00 | 2.80 | 1.00 | 0.40 | 0 |
| | 24 | 4.50 | 4.10 | 3.40 | 2.60 | 2.20 | 1.90 | 3.90 | 3.20 | 2.10 | 1.10 | 0.50 | 0 |
| | 40 | over | 4.70 | 3.90 | 3.80 | 3.70 | 3.10 | 2.90 | 2.60 | 2.30 | 1.20 | 1.00 | 0 |
| | 48 | over | 5.20 | 4.90 | 3.70 | 3.60 | 3.50 | 3.10 | 2.80 | 2.30 | 1.30 | 1.10 | 0 |
| 20g leaves ($N_p$) | 16 | 4.90 | 4.70 | 4.20 | 3.50 | 3.30 | 3.20 | 3.40 | 3.10 | 2.60 | 1.30 | 1.20 | 0 |
| | 24 | 4.80 | 4.50 | 4.30 | 3.70 | 3.50 | 3.30 | 3.90 | 3.20 | 2.10 | 1.40 | 1.30 | 0 |
| | 40 | over | over | over | 4.20 | 4.10 | 3.90 | 3.80 | 3.50 | 2.40 | 1.60 | 1.40 | 0 |
| | 48 | over | over | over | 4.70 | 4.40 | 3.30 | 4.10 | 3.90 | 3.80 | 1.90 | 1.60 | 0 |

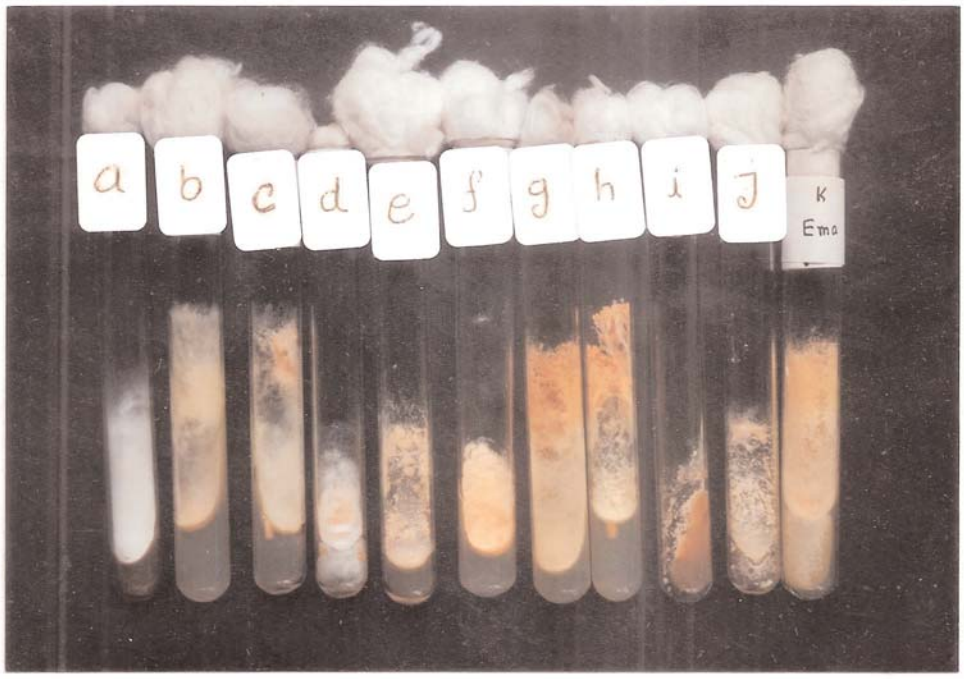


**Fig 1: Growth pattern of different mutants of N. crassa with wild type Ema.**

between sterilized and non-sterilized doses on the growth of *N. crassa* {Table III (i and ii)}. Aqueous extracts of the leaves of *L. aspera* showed significant inhibition of growth and mutagenesis on *N. crassa*. Result showed that 1 ml extract reacted with the test organism of *N. crassa* effectively. During the period of 24 hours, it produced only 2.6 cm linear vegetative growth of the mycelia was observed. Whereas, 1ml and 0.50 ml extracts produced less reactions (3.20 and 3.90 cm. respectively). The linear growth decreased with the increase of the doses of the extracts. Conidia of Ema (5297) of *N. crassa* were treated for 3-4 hours in 100% concentrations of the aqueous leaf extracts. One hundred percent extracts produced ten types of mutants, namely- *dirty, vigorous, ropy, albino, mat, plug, checked, fluffy, cauliflower* and *conidial brand* (Table. II and Fig.1). The types and frequency of mutants obtained with the leaf extracts of *L. aspera* were slightly different from that recorded earlier with leaf extracts of *Azadirichta indica* bulb extract of *Allium sativum* (Yesmin, 1998), leaf extracts of *Zingiber officinale* and *Andrographis paniculata* (Nazrul, 2003).

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