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Study of Mutagenic Effect of *Coccinea cordifolia* (Linn.) Cogn. Leaf Extract on *Neurospora crassa* Fungus

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Abstract

Coccinea cordifolia (Linn.) Cogn. leaf extract showed significant mutagenic effect on *Neurospora crassa fungus.* Observation after 24 hours, control showed 2.9 cm linear vegetative growth of the mycelia where *C. cordifolia leaf* with *N. crassa* showed 0.25 cm growth and it is 12 times less than the control. Besides, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 ml extract showed gradual decrease of growth of mycelia. This result indicates that this plant was suitable for induction of mutation in *N. crassa*. After mutation of *N. crassa* Ema (5297) conidia with *C. cordifolia* leaf extract, 6 types of mutants were found namely- *dirty, ropy, albino, mat, cauliflower, conidial brand. Albino* mutant showed highest frequency (35%) where *ropy* showed lowest frequency (5%).

Key words: *Neurospora crassa, Coccinea cordifolia,* Mutants, *Dirty, Ropy, Albino, Mat,Cauliflower* and *Conidial brand.*

Introduction

Neurospora crassa is a type of red bread mold of the phylum Ascomycota. The genus name, meaning, "nerve spore" refers to the characteristic striations on the spores that resemble axons. *N. crassa* is used as a model organism because it is easy to grow and has a haploid life cycle that makes genetic analysis simple since recessive traits will show up in the offspring.

Neurospora was used by Beadle and Tatum (1941) in their experiments for which they won the Nobel Prize in Physiology or Medicine in 1958. Beadle and Tatum (1941) exposed *N. crassa* to x-rays, causing mutations. 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 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 Westergaard and Mitchel (1947) has made the most extensive and systemic surveys of the action of biochemical mutagens on fungi on *N. crassa* (Fincham *et al.*, 1979). Therefore, a plan was made to induce mutation morphological change by using leaf extracts of *C. cordifolia*. 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 *C. cordifolia* leaf aqueous extract 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 *C. cordifolia* present in the leaves.

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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 *C. cordifolia* were used in the experiments. The extraction procedure is given below:

Aqueous extract

Mature fresh green leaves of *C. cordifolia* 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 *C. cordifolia*

Experiments were made to find out the mutagenic effect of *C. cordifolia* 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 *C. cordifolia* leaves:

Different concentrations of the leaf extracts of *C. cordifolia* with sterilized distilled water were prepared.

Table I. Different doses of *C. cordifolia* Solution

Name of the solution	Amount of leaf	Amount of sterilized distilled water
N_P	20g	-
N_1	20g	10ml
N_2	10g	10ml
N_3	10g	18ml
N_4	10g	20ml
N_5	4 g	10ml

Effect of water extracts of *C. cordifolia* on the growth of *Neurospora crassa*

For testing the effect of water extracts of *C. cordifolia* 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 *C. cordifolia* 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°C under 15 Ib pressure for 20 minutes. The inoculation chamber, needle, centrifuge machine etc. were also sterilized with rectified spirit.

c) Centrifugation

10 ml *C. cordifolia* 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. 10 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 (Sorbose and Infanger, 1964) 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 (Vogel, 1956). 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.

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 *C. cordifolia* on *Neurospora crassa*

Mature fresh green leaves of *C. cordifolia* were washed with sterilized distilled water and then air-dried. 50 gm of clean leaves were ground with mortar and pestle. The paste was filtered through extracts. The filtered extract was centrifuged for 5 minutes. The supernatant was used for this experiment. 6 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 *C. cordifolia* 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

Table II. Characteristics of the mutants of *N. crassa* obtained by the induction with *C. cordifolia* (Different doses).

Name of the mutants	Percent of mutants	Characteristics of the mutants
Ropy	5	The mycelia look like beautiful ropes, conidia pinkish a orange in colour. Growth is less than wild type.
Albino	35	Less growth of mycelia, conidia are very scanty in number. Mycelia and conidia are completely colourless.
Mat	30	Mycelia have a characteristic frayed appearance like mat
Dirty	10	Small conidial lump scattered here and there in the tube.
Conidial brand.	12	Dense conidial growths form a band shaped structure at the top.
Cauliflower	8	It produces cauliflower-like buttons of growth.

isolates were examined and classified (Table II). Different concentrations of *C. cordifolia* extracts were used to test the mutagenic effect on *N. crassa*. Fixed concentrations had appropriate mutagenic effects on *N. crassa*.

Results and Discussion

C. cordifolia leaf extract effects of chemical mutagens on frequency and specificity of chemical mutations in *N. crassa*. Suspensions of *N. crassa* conidia were inactivated by *C. cordifolia* leaf extract. Chemical substances, which showed mutagenic activities, became longer in every year. The authors tested the mutagenic effect of leaf extracts of *C. cordifolia* on the fungus, *N. crassa*. The plant aerial parts contain protein, fat, vitamin C, sterols, ß-sitosterol, phenolic compounds, triterpenoids, bitter glycosidic constituents, alkaloids, cephalandrine A and B, alcohol, cephalandrol, tri-triacontane, heptacosane. (Ghani, 1998). To determine the efficacy of *C. cordifolia* 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 doses of *C. cordifolia* extracts used. By increasing the doses of leaf extracts of *C. cordifolia* radial growth of Ema was reduced in comparison to control. Np 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 between sterilized and non-sterilized doses on the growth of *N. crassa* {Table III (i and ii)}. Aqueous extracts of the leaves of *C. cordifolia* 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 2.9 cm linear vegetative growth of the mycelia was observed. 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 different concentrations of the aqueous leaf extracts. One hundred percent extracts produced seven types of mutants, namely- *dirty, ropy, albino, mat, cauliflower* and *conidial brand* (Table II and Fig.1) and *albino* showed highest frequency (35%)

Table III. (i) Effect of *C. cordifolia* 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
10 g leaves + 20 ml distilled water (N_4)	Sterilized	16	4.80	3.80	2.90	1.90	1.70	1.60	4.20	4.00	3.50	2.90	2.30	0
		24	4.70	3.80	2.90	2.60	2.30	1.80	2.90	2.50	2.10	1.90	0.80	0
		40	over	5.10	5.00	4.70	3.80	3.90	3.70	2.60	1.40	0.90	0.30	0
		48	over	over	5.20	5.00	4.90	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	0.60	0
		24	over	over	4.20	4.10	3.10	2.80	2.90	2.20	1.50	1.20	0.70	0
		40	over	5.00	4.90	4.00	3.80	3.70	3.60	3.20	3.00	2.60	0.40	0.10
		48	over	over	5.10	5.00	4.50	4.10	3.10	2.90	1.70	1.60	0.50	0.20
10 g leaves + 20 ml distilled water (N_3)	Sterilized	16	4.90	4.70	3.80	3.30	2.80	2.20	5.00	4.90	4.10	3.90	0.20	0
		24	4.70	4.50	3.90	3.10	2.50	1.80	2.90	2.60	2.20	1.10	0.90	0
		40	over	5.20	5.10	5.00	4.90	4.60	3.40	3.30	3.20	2.90	0.50	0
		48	over	over	5.20	5.30	5.20	5.00	4.90	3.80	3.50	3.10	0.60	0
	Unsterilized	16	3.70	3.60	3.50	2.40	2.10	1.90	4.90	4.70	3.60	2.40	0.80	0
		24	4.50	3.90	3.60	2.60	2.20	1.80	2.90	2.20	2.10	2.00	0.90	0
		40	over	over	5.10	5.00	4.80	3.70	3.40	3.30	3.20	2.90	1.70	0.10
		48	over	over	over	5.10	5.20	4.90	4.70	4.60	3.90	2.50	1.90	0.20

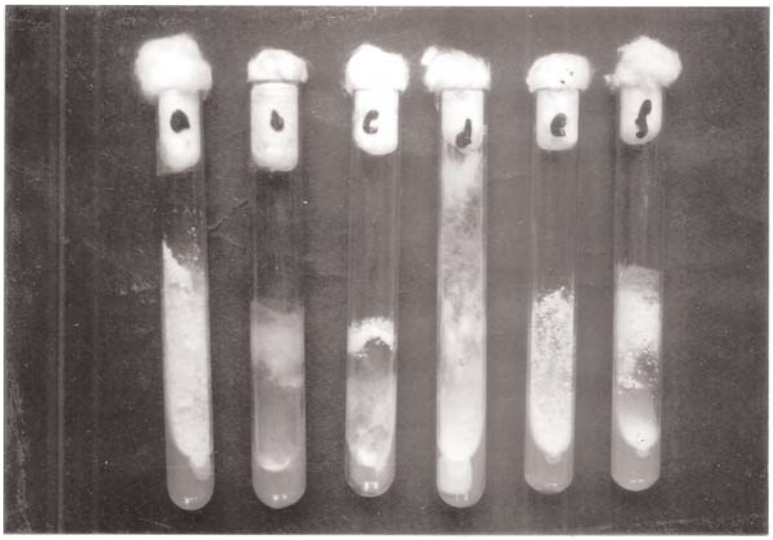

Fig. 1. Growth pattern of different mutants of *N. crass*a with wild type Ema.

Table III. (ii) Contd *C. cordifolia* extracts on the radial growth of *N. crassa* Ema.

Doses of the solution	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
10 g leaves + 10 ml distilled water (N_2)	16	2.30	1.90	1.80	1.40	1.30	1.20	2.90	2.70	2.50	1.30	0.10	0
	24	2.50	2.40	2.35	2.30	2.20	1.90	2.90	2.80	2.40	2.30	1.20	0
	40	4.00	3.50	3.40	3.30	3.20	3.00	2.90	2.70	2.20	1.50	0.30	0
	48	over	4.20	3.90	3.80	3.70	3.50	3.40	3.10	2.80	1.70	0.40	0
20 g 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	2.90	2.70	2.50	2.10	1.50	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	over	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	2.90	2.60	2.40	2.10	1.20	0
	40	over	5.10	4.80	4.20	4.10	3.90	3.80	3.50	2.40	1.60	1.40	0.20
	48	over	over	5.00	4.70	4.40	3.50	4.10	3.60	3.10	1.90	1.60	0.30

where *ropy* showed lowest frequency (5%). Others showed *dirty* (10%), *mat* (30%), *cauliflower* (8%) and *conidial brand* (12%). The types and frequency of mutants obtained with the leaf extracts of *C. cordifolia* were slightly different from that recorded earlier with leaf extracts of *Averrhoa carambola* (Bhuiyan *et al.*, 2007), *Azadirichta indica* (Keya, 1998), bulb extract of *Allium sativum* (Yesmin, 1998), leaf extracts of *Zingiber officinale* and *Andrographis paniculata* (Bhuiyan, 2003). So, we concluded that from leaf extracts of *C. cordifolia* we found total six different types of mutants (*dirty, ropy, albino, mat, cauliflower* and *conidial brand*).

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