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Characterization of Mutant *Aspergillus niger* and the Impact on Certain Plants

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Abstract: *Aspergillus niger* is a dangerous pathogen for many plants. It is a major cause of the destruction, rotting and decomposition of plant tissues. Toxicity caused by *A. niger* can be inhibited by mutation decreasing the destructive effect on plants. An 18S rDNA molecular tool was used to identify *A. niger* strains. Sodium azide (NaN_3) is a chemical mutagen that disturbs fungal enzymatic activity and causes microbial production of cellulose-degrading enzymes, decreasing mycotoxin production. Different concentrations of sodium azide were used to treat *A. niger* (30, 40 and 50 µM). The study was designed on two levels: the first level concerned the mutant *A. niger's* mode of action: the higher the mutagen concentration, the lower the growth diameter and spore counts. The mutant *A. niger's* total proteins and flavonoids were reduced when compared to control. RAPD-PCR showed genetic variation in the genetic content of mutant fungi compared to control resulting in a polymorphism percentage of 78.56%. The second level included the effect of these mutants on two plants (onion and maize). The greater the increase in mutant concentration, the greater the increase in the plants' morphological and physiological behavior. In conclusion, mutation reduced fungal activity and strengthened plant resistance.

Keywords: *Aspergillus niger*; *Allium cepa* L.; *Zea mays* L.; mutation; sodium azide; genotoxicity; RAPD-PCR; proteins; 18S rDNA

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1. Introduction

Aspergillus niger is used in industrial fermentation for production in several industrial fields, including the food industry. It is used to produce extracellular (food) enzymes, citric acid, for biotransformation and in waste treatment. However, *A. niger* is a serious plant pathogen and produces many mycotoxins (e.g., ochratoxins and aflatoxins) that contaminant foodstuffs (e.g., groundnut, maize, onion, etc.). Furthermore, *A. niger* causes rotting of vegetables and fruits and causes substantial losses to the economy and agriculture products [1–5]. The fungus reduces seed germination, seedling emergence, and root and shoot elongation, causing mortality at the point of plant emergence [6].

Fungal pathogenicity is related to the mechanism of mycotoxin effect; it allows the fungus to invade the tissue environment and boosts the host's defense lytic activity. Proteinases, lipases, and phospholipases are among the hydrolytic enzymes produced by fungi in culture conditions. These enzymes have a role in fungal pathogenesis and harm the host cells. Fungal nutritional absorption, tissue invasion, and nutrient delivery in a confined environment are all aided by these enzymes [7].

Induced chemical mutation is a technique for creating genetic variation in phytopathogens those results in new strains with varied traits. Induced mutations can be used to provide a new source of resistance to biotic and abiotic stress factors. This enables

the creation of new resistant varieties [8,9]. Treatment with chemical mutagens can enhance physiological behavior in many plants (e.g., wheat, broad bean) [10].

The chemical mutagen sodium azide (NaN_3) is one of the most potent mutagens in many phytopathogens. It is used because it is simple and cheap, and it produces mutations that enhance their characteristics. The efficiency of mutant production is influenced by several factors, including azide concentration and treatment time. It causes point mutations and chromosome damage, resulting in plant tolerance to a variety of adverse conditions [8]. At low doses, sodium azide is cytotoxic in a variety of animal and plant systems, inhibiting protein synthesis and replicative DNA synthesis. It has been utilized as a positive control in various systems since it is mutagenic in bacteria, fungi, higher plants, and human cells [9].

The initial goal of the study was to study how mutagens affect fungal growth and physiology (Figure 1). The second goal was to investigate how mutant and control *A. niger* strains affect specific plants (e.g., maize grains and onion seeds).

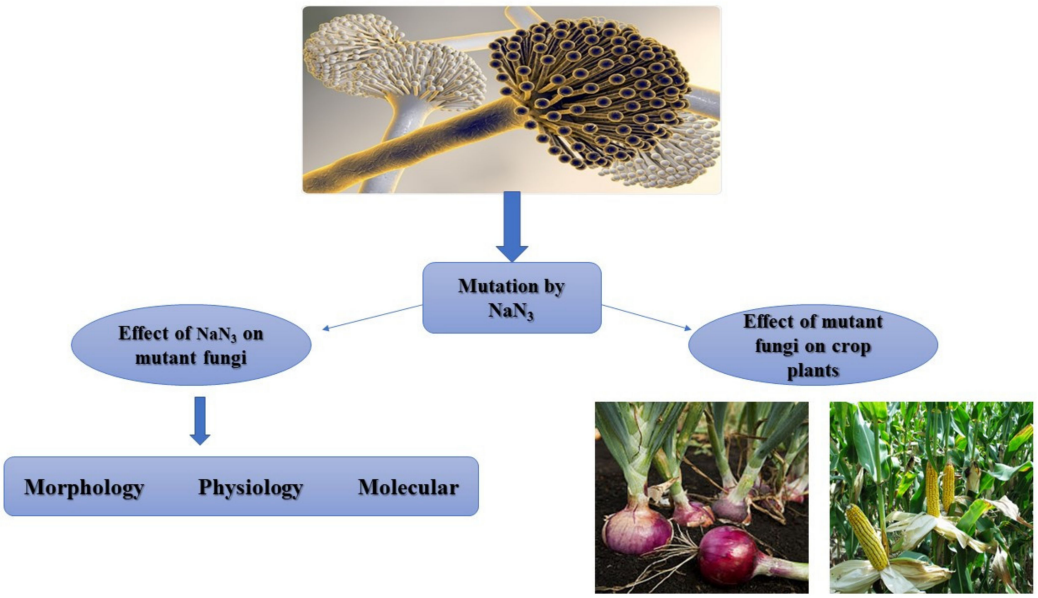

Figure 1. Graphical abstract for work design.

2. Materials and Methods

2.1. Materials

Aspergillus niger ((Tiegh.): kindly supported by Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt. It was identified molecularly in this study.

Allium cepa L. (Giza-20 var.) seeds and *Zea mays* L. (Giza-10 var.) grains: purchased from Agricultural Research Center, Giza, Egypt.

2.2. Molecular Identification of Aspergillus niger

This was performed using 18S rDNA identification for *Aspergillus niger*. First DNA was extracted by Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research #D6007, CA, USA) according to the manufacturer's instructions. After that, PCR was applied using Maxima Hot Start PCR Master Mix (Thermo K1051, Waltham, MA, USA) as follows: 25 µL of Maxima® Hot Start PCR Master Mix (2X), 1 µL of each ITS1 primer, ITS-F:5′-TCCGTAGGTGAACCTGCGG-3′ and ITS1-R:5′- TTTCGCTGCGTTCTTCATCG-3′″ [11]. A 5 µL quantity of DNA template (50 ng/g) was made up to 50 µL by adding 18 µL of water, nuclease-free. PCR was performed using the recommended thermal cycling conditions outlined as follows: initial denaturation for 10 min at 95 °C, followed by 35 cycles of denaturation for 30 sec at 95 °C, annealing for 60 sec at 57 °C and extension for 90 sec at 72 °C. The final extension correction step was at 72 °C for 10 min. Then PCR cleanup was

performed to the PCR product using the GeneJET™ PCR Purification Kit (Thermo K0701, Waltham, MA, USA) according to the manufacturer's instructions.

Finally, sequencing of the PCR product was performed using the GATC Company ABI 3730xl DNA sequencer using forward and reverse primers according to the new 454 technology.

2.3. Preparation of Mutagen Solutions of Sodium Azide

Three different concentrations of sodium azide (Granular, Reagent, 39H015) were prepared (30, 40 and 50 µM) and a control solution (without mutagen) [12]. An amount of 50 µL of each concentration was poured into 50 mL PDA media plates.

2.4. Impact of Sodium azide

The main aim of this study was to estimate the effect of sodium azide mutagen on the morphological and physiological behavior of *A. niger*. After that, some crops were treated with the mutant fungi with others subject to a control condition, and the effects on the crops were evaluated. The impact of sodium azide mutagen was estimated at two levels:

Effect of mutagen on Aspergillus niger (direct evaluation).

Effect of these mutant and control A. niger on both Zea mays L. and Allium cepa L. (indirect evaluation).

2.4.1. Direct Evaluation

Fungal Isolates Preparation and Inoculation

The mycelium of *A. niger* isolates were inoculated in PDA plates (200 g potato extract, 20 g dextrose and 20 g agar/L) and incubated for 5 days at 28 °C for growth.

For the PDA plates, 50 µL of each mutagen concentration (30, 40, 50 µM) were inoculated in sterile Petri-dishes. Then sterile PDA medium was poured over them with rotation for homogenous distribution of the mutagen. After that, a fungal disc was inoculated in each plate compared to control. The plates were incubated for 5 days at 28 °C for growth.

For potato dextrose broth tubes, thirty microliters of each mutagen concentration were inoculated in falcon tubes containing potato dextrose broth media. These tubes were incubated for 5 days at 28 °C at 150 rpm for growth.

Morphological and Microscopic Examination of Mutant Fungal Spores

The growth of the inoculated fungal discs affected by different sodium azide concentrations compared to control was observed. A fungal disc (cut with 0.5 cm cork pourer) was removed into a test tube containing 10 mL of sterile dH_2O and thoroughly checked. The spores germinated from the mutant fungi were examined under a microscope.

Physiological Parameters of Mutant Fungi

(1) pH of Broth Media

The pH of the nutrient broth medium was measured using a JENWAY 3510 pH meter. This was measured to estimate the effect of the different NaN_3 concentrations on the growth media of *A. niger*.

(2) Total Protein of *A. niger*

The total protein was extracted from fungal mats according to Bradford [13] as follows: A half g was weighted and ground with liquid nitrogen. Then 500 µL of 2x protein buffer (1 M Tris -HCl (pH 8.8), 0.25 mM EDTA, 10% SDS, 50% glycerol) was added for each sample. Finally, the four samples were centrifuged at 21,952× *g* at 4 °C and the supernatants were transferred into new tubes. Protein concentration was estimated as follows: 0.1 mL of supernatant was transferred using a pipette into a test tube and 5 mL of protein reagent was added (100 mg Coomassie Brilliant Blue G-250 dissolved in 50 mL 95% ethanol, then 85% phosphoric acid was added and made up to 1 L with dH_2O). The samples were mixed with the reagent and measured by spectrophotometer at wavelength 595 nm. The concentration

of protein was determined according to the protein standard curve. The concentration was calculated according to the equation for standard curve calibration [14]:

$$X = \frac{Y - 0.030}{0.007}$$

X: concentration, Y: absorbance at 595 nm

(3) Total Flavonoids of *A. niger*

Total flavonoid content of the *A. niger* extract was determined using aluminum chloride colorimetric assay [15] as follows: Half mL of the extract was added to 150 µL of 5% $NaNO_3$ and allowed to stand for 6 min. Then 150 µL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, after which 200 µL solution of 1 M NaOH was added; then the mixture was made up to 5 mL with methanol and mixed well. After incubation for 15 min, the absorbance was measured spectrophotometrically against a blank at 510 nm. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram extract (mg QE/g). The standard curve of quercetin was used for calculation of total flavonoids.

The total flavonoid content was calculated according to the following equation:

$$X = \frac{(R - B) * dilution\ factor * factor}{1000}$$

*X: concentration (mg/g), R: samples absorbance, B blank absorbance

(4) DNA Isolation and Molecular Marker (RAPD-PCR)

The total genomic DNA of mutant *A. niger* was isolated using Doyle and Doyle's [16] CTAB method as follows: Half g of fungal mat was ground in 700 µL of 2% CTAB buffer and incubated for 30 min at 65 °C with vertexing every 10 min. The supernatant was transferred into new Eppendorf tubes after centrifugation at 16,128× g for 10 min. Each tube was filled with an equal volume of chloroform: isoamyl alcohol (24:1) and allowed to sit for 2 min at room temperature before being centrifuged at 16,128× g for 10 min at 4 °C. The upper aqueous layer was transferred to new tubes, which were then filled with 800 µL of ice-cold absolute ethanol and stored at −20 °C for approximately 2 h. The solution was centrifuged to obtain DNA pellets, which were then washed in 70% ice-cold ethanol. Finally, the pellets were resuspended in 50 µL TE buffer and stored at −20 °C until the RAPD-PCR reaction was performed.

For RAPD-PCR bioassay, seven RAPD decamers were used, and the results were consistent and clear. In the tables, a list of primers is shown (in Results section). The RAPD-PCR reaction was carried out in a Biometra thermocycler. The reaction mixture contained 12.5 µL Taq master mix (COSMO PCR RED M. Mix, W1020300x), 2 µL genomic DNA, and 1 µL for each primer in a total volume of 25 µL (Willowfort) and 9.5 µL ddH_2O. The reaction program consisted of 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at different degrees for each primer, as shown in tables, and extension for 1 min at 72 °C. After that, one step of final extension at 72 °C for 10 min was followed by cooling at 4 °C. On a 1.4% agarose gel, the amplified PCR product was evaluated using a Willofort, BERUS 100 bp DNA ladder.

2.4.2. Indirect Evaluation

Plant Germination and Infection

Seeds and grains of both plants (*A. cepa* and *Z. mays*) were geminated in Petri-dishes with filter papers. There were ten seeds in each plate with 5 replicates. In addition, plant seeds were germinated in pots. Ten seeds were sown in each pot with five pots selected for infection with mutant fungi (−ve control, +ve control, 30, 40 and 50 µM). One milliliter (1100 spores/mL) of each mutant fungus was used for the inoculation of different plant seeds. One germination test was applied as a negative control, in which the seed germinated without

any fungal infection. The positive control involved seeds infected with non-mutant *A. niger*. The germinated seeds and grains were incubated at room temperature for two weeks.

Morphological Measurements

After ten days of germination, the fresh weight and shoot length were measured and the percentage loss in seedling parameters were estimated compared to controls. The general effect on plant growth behavior was estimated from seedlings germinated in pots and infected with different mutant *A. niger* compared to positive and negative control as previously mentioned.

Physiological Measurements of plants infected with mutant fungi

(1) Total proteins

The total protein was extracted according to [12] as follows: An amount of 0.5 g of leaves was weighed and ground thoroughly with 0.5 mL of [2×] buffer. Then the mixture was vortexed for 10 min and centrifuged for 15 min at 21,952× *g* at 4° C. The supernatant contained the total protein content of the plant species. Finally, the protein concentration was estimated according to [12] as follows: 0.1 mL of supernatant was pipetted into a test tube and 5 mL of protein reagent was added, mixed, and measured by spectrophotometer at wavelength 595 nm. The concentration of protein was determined from the protein standard curve. The concentration was calculated according to the following equation [13]:

$$X = \frac{Y - 0.030}{0.007}$$

(2) Total flavonoids

Total flavonoid content of both *A. cepa* and *Z. mays* extract was determined using aluminum chloride colorimetric assay [15] as follows: Half ml of the extract was added to 150 µL of 5% $NaNO_3$ and allowed to stand for 6 min. Then 150 µL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, after which 200 µL solution of 1 M NaOH was added; then the mixture was completed to 5 mL with methanol and mixed well. After incubation for 15 min, the absorbance was measured spectrophotometrically against a blank at 510 nm. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram extract (mg QE/g). The standard curve of quercetin was used for calculation of total flavonoids.

The total flavonoid content was estimated according to the following equation:

$$X = \frac{(R - B) * dilution\ factor * factor}{1000}$$

*X: concentration (mg/g), R: samples absorbance, B: blank absorbance

2.5. Statistical Analysis

The presence of a band was coded as 1, while the absence of a band was coded as 0. The images obtained from gel electrophoresis were analyzed, and the presence of a band was coded as 1. After generating a pairwise similarity matrix using Jaccard's similarity coefficient, the unweighted pair group method with the arithmetic averaging algorithm (UPGMA) was used to develop a dendrogram. Bio-Rad Quantity (4.6.2) (Dubai Biotechnology and Research Park (DUBIOTECH)) was used to perform these calculations [17].

The statistical analysis for the morphological and physiological data were performed using SPSS 21 (IBM, USA). The data was subjected to analysis of variance. Standard deviations and means were calculated.

3. Results

Different sodium azide mutagen concentrations were prepared to affect the bioactivity of *Aspergillus niger*. This included different morphological, physiological, and molecular

parameters of *A. niger*. In addition, the effect of these mutant fungi on the activity of both *A. cepa* and *Z. may* was assessed.

3.1. Molecular Identification of Aspergillus Niger

The molecular identification results obtained from 18S rDNA and sequencing showed that the tested fungus was *Aspergillus niger* (lcl|Query_20417) (Figure 2). The tree obtained from the alignment results, obtained from the NCBI data base, showed that the provided specimen was *A. niger* which had 100% identity with *Aspergillus niger* strain URM7014 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

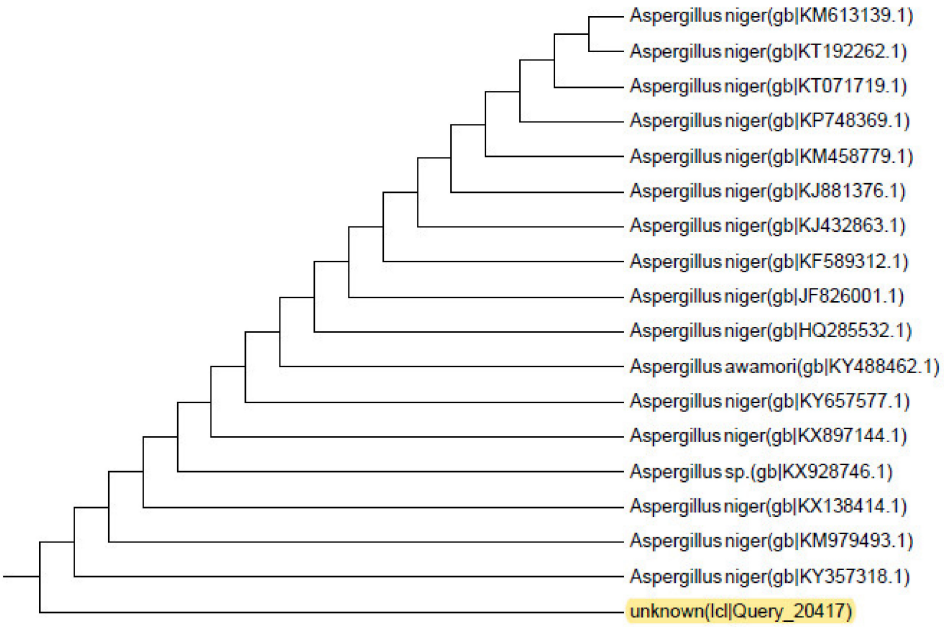

Figure 2. Phylogenetic tree based on 18S rDNA identification with ITS1 primers. showing the relation of the target sample to other *Aspergillus niger* strains.

3.2. Direct Evaluation for Mutant Fungi

3.2.1. Morphology of Fungi and Their Spores

The mutant *A. niger* growth diameter was estimate manually. The results (Table 1) indicated that the growth diameter of *A. niger* decreased with increase in sodium azide concentration compared to control. For spore suspension, the examined spores of mutant *A. niger* showed spore inhibition in comparison to control.

Table 1. Growth diameter and spore inhibition of mutant *A. niger* treated with sodium azide.

	Control	**30 μM**	**40 μM**	**50 μM**
Growth diameter, cm	7.76 ± 0.25	1.73 ± 0.15	1.10 ± 0.10	0.78 ± 0.27
Spores count, spore/mL	93.66 ± 5.68	47.00 ± 11.00	30.33 ± 3.05	22.66 ± 4.04

3.2.2. Physiological Parameters of Mutant Fungi

The measured physiological parameters of mutant *A. niger* compared to control included pH of culture media, total protein of fungi and total flavonoids (from both fungal extract and media). The variation of these physiological parameters was estimated for mutant fungi compared to control (Table 2). All the physiological parameters showed significant differences between the different strains.

Table 2. Some physiological parameters of *A. niger* in response to sodium azide.

	Control	30 µM	40 µM	50 µM	*p*-Value
pH of media	5.21 ± 0.34	5.65 ± 0.02	5.48 ± 0.26	5.74 ± 0.01	0.002 **
Total protein "mg/g"	50.52 ± 1.95	64.42 ± 0.14	64.90 ± 0.08	32.57 ± 0.37	0.028 *
Total flavonoids "mg/g" (fungi)	20.61 ± 0.47	10.45 ± 0.43	15.49 ± 0.47	12.38 ± 0.58	0.008 **
Total flavonoids "mg/g" (media)	61.04 ± 0.08	42.70 ± 0.93	47.40 ± 0.98	43.48 ± 0.74	0.009 **

* Significant; ** highly significant.

For pH of medium, the original pH of the negative control medium (medium only without fungal inoculum nor mutagen) was 6.23. When the medium was inoculated with mutant fungi, the pH decreased because of different secretions of the mutant fungi. However, compared to control, pH increased when the mutagen concentration increasesd. The highest pH value was for the sodium azide treatment of 50 µM. The total fungal proteins increased in mutants compared to control. Otherwise, in 50 µM, the proteins were inhibited to be less than the control. The total flavonoid exosecretion in the medium was higher than the endosecretion within fungal cells. However, generally they decreased in mutants compared to control. Except in 40 µM treatments in comparison to other treatments, it was higher for 30 and 50 µM. Generally, there was no definite characterization for the physiological parameters of fungi as they sometimes increased, and at other times decreased in response to mutagen treatment.

3.2.3. Molecular Variation

The genomic DNA of control and different mutant *A. niger* strains was isolated from fungal mats in broth media to be used in RAPD-PCR bioassay which was performed using 5 oligonucleotide RAPD primers. Only 3 of them gave reproducible bands. These primers were OPA-12, OPB-17 and OPZ-07 (Table 3), with P4 and RFU-25 not producing clear bands. A total of 19 different bands were obtained from these primers. Their data are listed and illustrated in Figure 3.

Table 3. RAPD primer data and polymorphism percentage.

Primer Code	Primer Sequence	GC%	Tm	Polymorphism %
OPB-17	5′-AGGGAACGAG-3′	60	33.1	66.66
OPA-12	5′-TCGGCGATAG-3′	60	34	85.7
OPZ-07	5′-CCAGGAGGAC-3′	70	34.6	83.33
	Total polymorphism percentage (%)			78.56

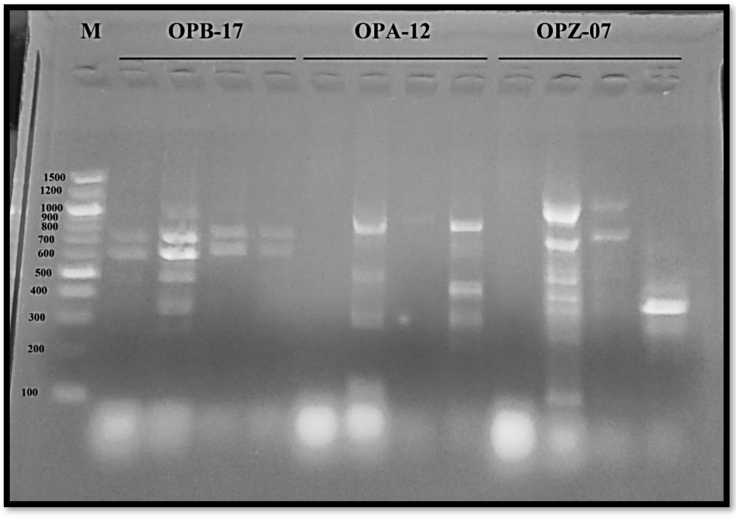

Figure 3. Agarose gel electrophoresis for RAPD-PCR of mutant *A. niger* with control. For each primer, the samples were arranged as follows: control: non-mutant *A. niger*; 30 µM: mutant *A. niger* with 30 µM sodium azide; 40 mM: mutant *A. niger* with 40 mM sodium azide; 50 µM: mutant *A. niger* with 50 µM sodium azide.

The total similarity matrix resulting from these mutants is shown in Table 4. The matrix shows that the mutant *A. niger* (50 mM) was genetically close to the control. The 40 mM mutant was very distant and occupied a separate clade.

Table 4. Total similarity matrix based on all RAPD-PCR primers using BioRad Quantity One software.

	Control	30 µM	40 µM	50 µM
Control	100	24.23	25.87	36.87
30 µM	24.23	100	16.5	31.27
40 µM	25.87	16.5	100	17.8
50 µM	36.87	31.27	17.8	100

Figure 4 represents a total dendrogram based on morphological, physiological, and molecular data. It illustrates that both the control and the 30 mM mutant were relatively close to each other. However, the dendrogram also confirms the separation of the 40 mM mutant in a separate clade.

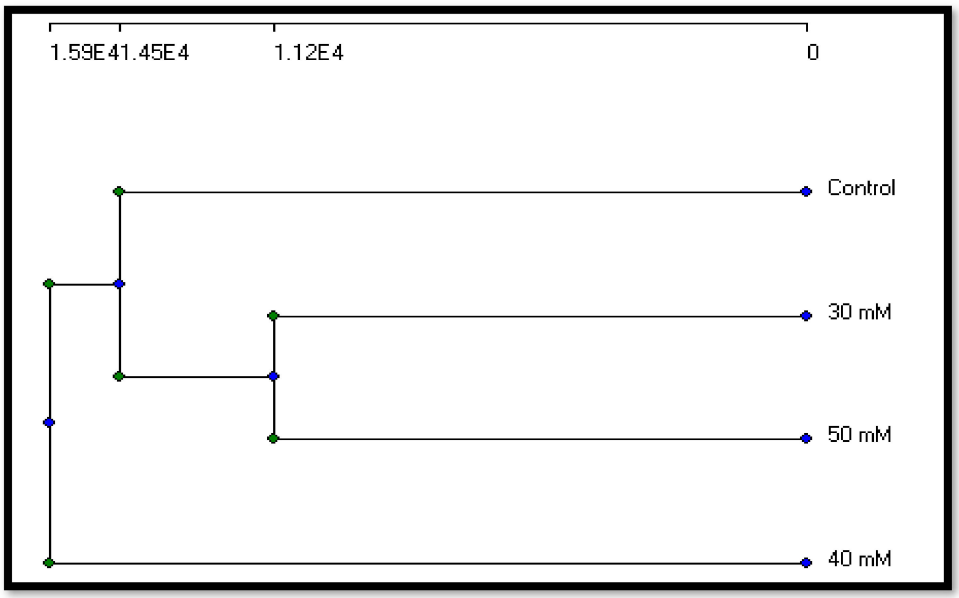

Figure 4. Dendrogram based on total parameters (morphology, physiology and molecular) for *A. niger* mutants using Community Analysis Package (CAP software).

3.3. Indirect Evaluation of Plants Infected with Mutant Fungi

This section explains the effect of different mutants and control *A. niger* on two plants. Morphological and physiological parameters were estimated for two plants: *A. cepa* and *Z. mays*.

3.3.1. Morphological Parameters and Seed Germination of Infected Plants

Two different plants were tested for resistance of *A. niger*. Both *A. cepa* and *Z. mays* showed gradient resistant to mutant treatments of *A. niger* compared to the positive control. The results are illustrated in Table 5 and Figure 5a,b for *A. cepa* and *Z. mays*, respectively. The results showed that the pot experiment of germinated plant seeds infected with different mutations and controls of *A. niger* showed a loss in the total measure % when compared to controls (for before and after measurements). All these results indicated and confirmed the following: when the mutant concentration increased in *A. niger*, this had a negative effect on the fungi but positive effects on the infected plants. This means that plants were more resistant to the mutant fungi compared to the +ve control. The infection symptoms were more obvious in plants infected with the +ve control *A. niger* rather than mutant fungi. All this is a reflection of the inhibitory effect of sodium azide on *A. niger*.

Table 5. Plant morphological and seed germination responses to infection with the sodium azide mutant *A. niger.*

Plant	Mutants	Fresh Weight Loss %	F-Value	Shoot Length Loss %	F-Value	Seed Germination Loss %	F-Value
Zea mays	−Ve Control	0.00 ± 0.00	136.32 ***	0.00 ± 0.00	93.56 ***	0.00 ± 0.00	306.67 ***
	+Ve Control	80.3 ± 2.34		54.2 ± 2.14		93.5 ± 9.2	
	30 µM	50.5 ± 2.17		37.3 ± 1.24		47.5 ± 4.2	
	40 µM	26.4 ± 2.11		30.2 ± 1.11		33.3 ± 1.4	
	50 µM	12.5 ± 0.89		15.7 ± 0.52		20.2 ± 1.1	
Allium cepa	−Ve Control	0.00 ± 0.00	122.67 ***	0.00 ± 0.00	110.33 ***	0.00 ± 0.00	197.33 ***
	+Ve Control	86.3 ± 5.67		79.7 ± 5.47		91.7 ± 9.25	
	30 µM	59.7 ± 2.45		47.5 ± 3.33		67.7 ± 8.33	
	40 µM	42.5 ± 2.11		36.3 ± 1.27		29.3 ± 3.29	
	50 µM	23.7 ± 1.57		22.9 ± 1.15		11.6 ± 0.77	

*** highly significant. −Ve control: plants without fungal treatment, +Ve control: plants infected with non-mutant A. niger, 30 µM: plants infected with mutant A. niger with 30 µM sodium azide, 40 mM: plants infected with mutant A. niger with 40 µM sodium azide, 50 µM: plants infected with mutant *A. niger* with 50 µM sodium azide.

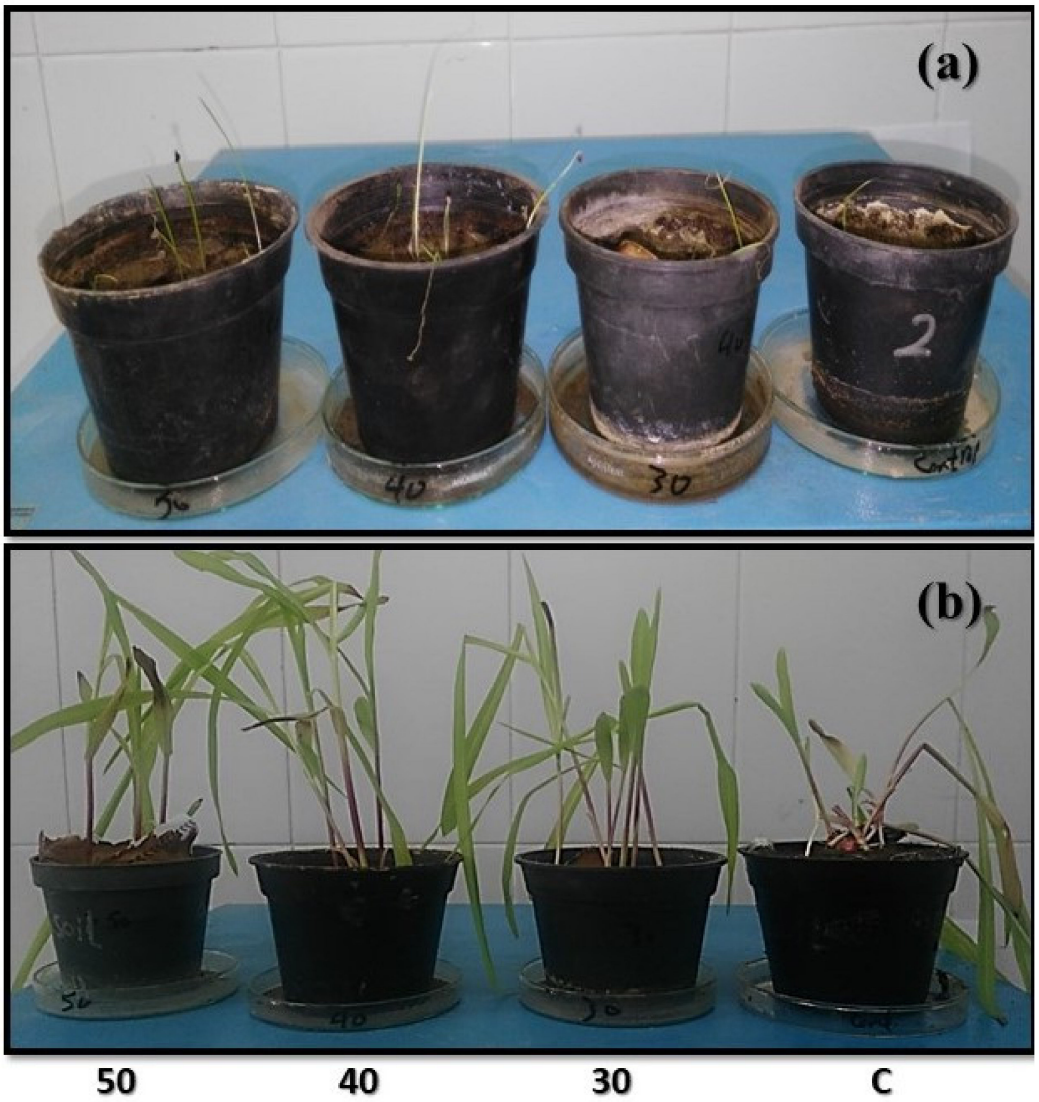

Figure 5. Pot experiment of plants. (**a**) *Allium cepa* infected with mutants and control *A. niger*. (**b**) *Zea mays* infected with mutants and control *A. niger*. The concentrations were: C: control treatment (non-mutant), 30 µM, 40 µM, 50 µM sodium azide mutagen.

3.3.2. Physiological Parameters of Infected Plants

Total proteins and total flavonoids of mutant *A. niger*-infected *A. cepa* and *Z. mays* were quantified and compared to control. In terms of total proteins, the higher the mutant concentration in *A. niger*, the higher the total protein content of these plants. This suggests that sodium azide reduces *A. niger's* impact while increasing plant resilience. Table 6 depicts these findings.

Table 6. The total proteins of plants infected with mutant *A. niger.*

Plant	Mutants	Proteins		Flavonoids	
		Mean	F-Value	Mean	F-Value
Zea mays	−Ve Control	49.54 ± 0.37	298.799 ***	0.073 ± 0.004	246.315 ***
	+Ve Control	14.33 ± 0.35		0.065 ± 0.005	
	30 µM	23.28 ± 2.10		0.144 ± 0.006	
	40 µM	36.09 ± 0.81		0.158 ± 0.003	
	50 µM	38.90 ± 0.217			
Allium cepa	−Ve Control	28.26 ± 0.39	77.138 ***	0.057 ± 0.002	101.025 ***
	+Ve Control	2.38 ± 0.21		0.064 ± 0.002	
	30 µM	5.19 ± 0.21		0.089 ± 0.005	
	40 µM	6.85 ± 0.37		0.097 ± 0.002	
	50 µM	15.85 ± 2.24			

*** highly significant. −Ve control: plants without fungal treatment, +Ve control: plants infected with non-mutant *A. niger*, 30 µM: plants infected with mutant *A. niger* with 30 µM sodium azide, 40 µM: plants infected with mutant *A. niger* with 40 µM sodium azide, 50 µM: plants infected with mutant *A. niger* with 50 µM sodium azide.

For total flavonoids, the greater the increase in mutant concentration in *A. niger*, the greater the decrease in total flavonoids in these plants. This means that sodium azide had a negative effect on *A. niger*, while positively affecting the plants and enhancing resistance in the plants. This may be explained as follows: the plants secret more secondary metabolites (e.g, flavonoids) to resist the different mycotoxins secreted by fungi. All these results are illustrated in Table 6. Flavonoids are structurally varied secondary metabolites that serve a variety of roles in plants. These include roles in plant development, pigmentation, and UV protection, as well as a variety of defense and signaling functions between plants and microbes.

4. Discussion

The main purpose of choosing *A. niger* for this study is that it produces organic acids, enzymes, plant growth regulators, mycotoxins, and antibiotics. Under normal conditions, *A. niger* may create certain enzymes and is a prolific exporter of homologous protein species.

Pathogenic fungi secrete enzymes which are integral to their pathogenesis. The *A. niger* group is composed of black-spored *Aspergillus* species. The spores are responsible for secretion of several secondary metabolites, known as mycotoxins. *A. niger* is widely distributed in many habitats; it has not only been isolated from host cells but was recognized and isolated from space [18]. Molecular identification was used to define *A. niger* isolates collected from U.S. segment surfaces of the International Space Station. According to Shankar [3] and Zohri et al. [7], the fungal secretions are virulent factors of fungi to facilitate the adherence and hydrolysis of the components of the cells of the host. Schuster et al. [1] examined the idea that *A. niger* strains produce mycotoxins (e.g., aflatoxins, ephrotoxic and carcinogenic mycotoxin ochratoxin A). They suggested that these toxins were produced under uncontrolled storage conditions for *A. niger*. In addition, [2] demonstrated that *A. niger* isolates are responsible for production of mycotoxins that affect food grade enzymes. Soares et al. [19] reported the infection of maize with *A. niger* which produced fumonisin B2 and ochratoxin A, while [20] found that mycotoxins, such as ochratoxins, could be isolated from grapes infected by *A. niger* and *A. carbonarius* strains.

In this study, spore count, and fungal growth were negatively affected as mutagen concentration increased. Fungal spores are important as causes of disease, pollution, and

as biological warfare agents. Dijksterhuis and Samson [21] demonstrated that spores have several advantages over their vegetative counterparts. Spores have unique characteristics, such as tolerance to extreme temperatures, poisonous chemicals, desiccation, and radiation, among others. So, in this study it was essential to estimate the spore count to evaluate any changes due to exposure to mutation. The germination and count of spores varied in response to mutation according to this study.

Kumar and Parikh [22] suggested that change in fungal growth diameter could be explained as a result of chemical mutagens causing the production of cellulose-degrading enzymes and inhibiting extracellular protein production in fungi which inhibits fungal cell wall formation.

To prevent spore germination, sodium azide was added to the bioconversion medium. Spores could be used as a catalyst in the bioconversion of glucose to gluconic acid because of these treatments [23]. Sodium azide, either as the free acid HN_3, or as an ionic compound, binds metal sites in enzymes. The azide anion acts as a reversible or irreversible inhibitor of catalytic hemoproteins, such as catalase and horseradish peroxidase, in various situations. In previous studies, sodium azide was used as the bioconversion medium. Its effect on the enzyme was compared to that of commercial glucose oxidase because it was found to be toxic. When sodium azide was added to the bioconversion medium containing commercial glucose oxidase, 50% of the activity was lost when compared to the control [23]. As a result, sodium azide was discovered to be a glucose oxidase enzyme inhibitor. There have been reports that sodium azide inhibits peroxidase and laccase [24,25]. With *A. niger*, a sodium azide mutagen was used; [26] worked on *A. niger* mutants to increase glucose oxidase enzyme production.

For the physiological parameters, this study showed that mutation causes variation in responses and indicated that a concentration of 40 µM sodium azide has a better response rather than the other two concentrations. These findings could be explained by [27–29] who concluded that many enzymes, including invertase and galactosidase, are found in *Aspergillus spp*. Aflatoxigenic and probable aflatoxin makers, *Aspergillus* spp., infect maize, peanuts, and tree nuts. Chemical (sodium azide, ethyl methane sulphonates, and nitrous acid) and physical (ultraviolet radiation) mutagens have a significant impact on them. Aflatoxigenic fungi contaminate food, hence mutation can be utilized to eliminate this impact.

The present study found that mutation caused genotoxicity and instability in DNA content. Other studies have also found this, using molecular markers to estimate the variation caused by mutations. Many researchers [30–32] have shown that molecular markers, such as inter-simple sequence repeats (ISSRs), random-amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLPs) are three DNA profiling approaches that can be effectively utilized to assess genetic diversity. They are dominant markers that use anonymous regions enabling study of genomic diversity using universal primers. All these approaches were related to this investigation which used RAPD-PCR as a molecular technique to estimate the genetic diversity in *A. niger* resulting from exposure to the sodium azide mutagen. Similar results from chemical mutagenic treatments of the strain could predict the activation of specific genes because of mutant mutations. According to [33] a mutation caused *aflR* overexpression in *A. flavus*, which increased aflatoxin pathway gene transcription and aflatoxin accumulation.

The evolutionary history of a pathogen population determines the genetic makeup of that population. Genetic makeup data is thought to provide insight into the evolutionary potential of disease populations in the future. The distribution and amount of genetic variability among and within populations is referred to as genetic structure. The genetic makeup includes both genotype and gene diversity. Gene diversity refers to the number and frequencies of alleles at particular loci in a population, whereas genotype diversity refers to the number and frequencies of multi-locus genotypes in a population [34,35].

In this study, both *Z. maize* and *A. cepa* showed high resistance, in morphological and physiological parameters, in response to mutant *A. niger* strains compared to control. This agrees with [36] who identified three fungal species that infect maize, *Aspergillus flavus*,

Aspergillus niger and *Fusarium* spp. These species have inhibitory effects on maize with percentage reductions of 42, 96 and 13%. Moreover, [37] found that ten fungal endophytes of maize cause severe diseases in maize.

5. Conclusions

Aspergillus niger is a common phytopathogen which infects many fruits and vegetables, such as onion, maize and others. *A. niger* was molecularly identified using 18S rDNA. Sodium azide is a chemical mutagen that inhibits fungal enzymatic activity and as a result decreases mycotoxin production. Different concentrations of sodium azide were used to treat and inhibit the vital activities of *A. niger*. The study was divided into two parts: the manner of action of mutagen concentrations on *A. niger*, and the infection by these mutant fungi of two different plants (onion and maize). Morphologically, the greater the increase in concentration, the greater the decrease in growth diameter and spore counts of *A. niger* compared to control was found. Physiologically, the total proteins and flavonoids of mutant *A. niger* decreased compared to control. The pH of the medium was not significantly affected by the growth of mutant fungi. At the molecular level, there was genetic variation in the genetic content of mutant fungi compared to control. RAPD-PCR molecular marker analysis resulted in a polymorphism percentage of 78.56%. The second part of the study explained that the greater the increase in mutant concentration, the greater was the increase in the physiological activity of the plants which were treated with these fungi. The innovative aspect of using *A. niger* in this study, was to apply the chemical mutation to infect the fungus, then to compare the effect of mutant fungi on essential crops, compared to control infection. To sum up, sodium azide inhibits fungal activity and enhances plant resistance.

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