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ASPOROGENIC MUTANTS OF *ALTERNARIA CASSIAE* GENERATED BY X-RAY IRRADIATION

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Abstract

The prevention of sporulation is an important factor in precluding the spread of transgenic biocontrol agents beyond desired limits; asporogenic deletion mutants could prevent such spread. Alternaria cassiae is a biological control agent for controlling sicklepod (Cassia obtusifolia) and coffee senna (Cassia occidentalis). The NEP1 transformed fungus is more virulent than the parent. A. cassiae forms microcolonies on 0.9 g/l sodium deoxycholate plates. The spores have a D_{10} value of 50,000 rads when subjected to X-ray beams. Five putative mutants were achieved and 3 proved to revert. Mutants xAlt541 and xAlt524 are stable with no spore-forming capability. The xAlt541 mutant grew more slowly (4.63 cm) on yeast starch agar than xAlt524 (5.94 cm) and the parent (5.77 cm). The mutant xAlt524 lacks aerial hyphae, pigmentation, and spore production. The mycelia of the parent fungus have rosy brown to peach puff pigment after 5 days of growth on medium plus 1 g/l tyrosine, feldspar pigment on medium plus 1 g/l phenylalanine and appear slate gray in the absence of an amino acid supplement. The xAlt524 and xAlt541 mutants had cream and dark salmon color, respectively. At 2.5×10^6 propagules/ml, mutants' xAlt541 (2.17a ± 0.31) and xAlt524 (2.0a ± 0.26) damage severity was not statistically different from that of the parent type AltNEP1 (2.5a ± 0.22) by 5 days post-inoculation.

Keywords: *Mycelia, NEP1, spore*

Introduction

The prevention of sporulation is the critical factor in preventing the spread of transgenic biocontrol agents beyond desired limits. Fungal mycelia have proven to be better inoculating agents than spores with fungi controlling *Orobanche* and *Abutilon* [2, 9], thus spores are not needed in these cases. Spores are dispensable structures, inessential for normal growth, and indeed, inessential for storage and propagation since the sexual cycle provides an alternative source of spores [8]. Therefore, the aim of these studies is to use irradiation to generate asporogenic mutants of *Alternaria cassiae*. A. *cassiae* spores are large, darkly pigmented, and surrounded by a thick, two-layered wall [24, 40]. Each spore is divided by transverse and longitudinal septa into multiple cells, with size ranging from 20 to 25 µm in width [24] and from 75 to 200 µm in length, with long filiform beaks [40], which do not exceed one-third of the spore length. *A. cassiae* spores have multiple nuclei each with prominent nucleolus [24]. In culture on YSA media, colonies of *A. cassiae* appear slate gray, and are cottony in texture. Different colors are produced on different media.

A. cassiae Jurair and Khan, an important filamentous fungal phytopathogen of economic importance in weed biological control, is used as a test fungus. *A. cassiae* is a biological control agent for sicklepod (*Cassia senna* L.) [14, 24, 29] and coffee senna (*Cassia occidentalis* L. # CASOC). It had also been reported on cowpea (*Vigna unguiculata*) [25]. Sicklepod is also a major biotic constraint to production of soybean (*Glycine max*) [41], peanut (*Arachis hypogaea*), and cotton (*Gossypium hirsutum*) [11].

Many of the early investigations were focused on the potential of mycoherbicides on target weeds. Now, there is a need to develop asporogenic mutant strains as researchers have identified that the mycoherbicides must be restricted to the target weed [9]. Safety concerns raised include the persistence of the fungi in the environment and the possibility of the transfer of a hypervirulent transgene from the biocontrol agent to a pathogen of a crop. Failsafe mechanisms proposed to prevent spread and mitigate introgression of transgenic hypervirulent biocontrol fungi include asporogenic deletion mutants of the transgenic hypervirulent fungi, which would prevent the spread of the biocontrol agent and environmental persistence [13, 32, 33].

The important characteristic of ionizing radiations is the localized release of large amounts of energy [15]. The energy dissipated per ionizing event is about 33 eV, which is more than enough to break a strong chemical bond; for example, the energy associated with a C=C bond is 4.9 eV [15]. Spores, like all living matter, are sensitive to the actions of radiation and though spores are more resistant to stress conditions than vegetative cells, no spore exists that cannot be killed by sufficient dosage. On the other hand, hyphae also are sensitive to the actions of ionizing radiation. Consequently, the idea is to make the fungi asporogenic with little damage to the hyphae. To this end, dose levels were identified which involve various levels of damage to *A. cassiae*. The intent of treatment is stable control of sporulation.

There is a dearth of information about the effect of radiation on fungi. Interest in this area is in connection with irradiation of fungi as it is necessary as a failsafe technique against off-target spread of transgenic fungi.

The objectives of the research reported in this study were: (1) to maximize spore production on chemically defined media in preparation for ionizing radiation of the fungal spores and for use in assessing the competence of irradiated spores to germinate and produce spores; (2) to determine the relationship between radiation and survival; and (3) to generate asporogenic fungi mutants which can tolerate normal growth conditions.

Materials and Methods

Cultivation media, fungi and culture maintenance. Media tested (see Table 1) were used alone and augmented with 1 g/l of either L-phenylalanine or L-tyrosine. The amino acids were added to media as melanin precursors in the hope that they would make it easy to identify asporogenic mutants. The spores of the test fungus, *NEP1* transformed *A. cassiae* (hereafter refer to as Alt*NEP1*), were cultivated at 28 ^{o}C under continuous cool white light conditions for 10-14 days on yeast-starch-agar (YSA). *A. cassiae* was grown on potato dextrose agar (PDA) slants at 28 ^{o}C for 14 days and maintained at 4 ^{o}C.

Spore formation in different culture media and spore quantification. The agar medium was centrally inoculated with a small plug (4 mm) of mycelial mat and incubated at 28 ^{o}C for 10-14 days. There were at least two to three replica plates. On the basis of observations and spore quantification after 10-14 days incubation on agar media, a medium was selected for subsequent cultivations. Yeast-starch-agar (YSA) was prepared by mixing 15.0 g soluble starch, 4.0 g yeast extract (Difco), 0.5 g K_2HPO_4, 0.5 g $MgSO_47H_20$, and 15.0 g agar powder adjusted to 1000 ml with distilled water (pH 7.4).

Spores were dislodged from the mycelium of 10-14 day-old cultures in 2 ml sterile distilled water using a rubber policeman. All possible conidial residuals from the plates were rinsed three times with 2 ml sterile distilled water. The rinsing was added to the initial tube, and the final spore suspension was filtered through Nitex® Bolting Cloth (70 µm). Spores were counted on a grid from the surface of a hemocytometer mounted on a light microscope.

Spores per ml of original sample = spore count/dilution factor.

Irradiation of spores and dose determination. Five ml of the spore suspension (10^3 spores/ml of phosphate buffer, 0.02 M, pH 6.5) were placed into each 15 ml plastic tube and the lid was put in place. At the Weizmann irradiation unit, equal volumes of suspension were irradiated at room temperature with X-ray. The

available Andrex X-ray machine is capable of delivering approximately 580 rev./min at 70 keV, 5 mA with 0.5 mm aluminum filter and a target skin distance of 5 inches. Dosage was determined by time of exposure, because the emission of the X-ray tube was standardized. Next, 100 µl of one to two successive spore dilutions (from control and irradiated spore suspension) were plated in triplicate on media containing 50 mg/l chloramphenicol to restrict the growth and activity of bacteria. Every individual experiment included a control and irradiated series. The spores were incubated at 28 ^{o}C for 48 h. Single colonies were picked from plates containing about 50 colonies on media containing 0.9 mg/ml sodium deoxycholate and examined for nonsporulating mutants. The appropriate irradiation dose for 90 % kill was determined for *A. cassiae* from three replicate readings. The survival curve was plotted and the D_{10} value was calculated.

Assay for asporogenic mutants. Batches of fifty 24-well cluster dishes were prepared with YSA. Single colonies of putative mutants (visibly different from normal) were subcultured into each well of the cluster dishes from Petri plates. The single colonies were transferred to continuous cool white light at 28 ^{o}C for 10 days. The cultures were screened by checking under the inverted microscope with phase-contrast or under the light microscope for asporogenic mutants. Asporogenic mutants were picked and subcultured on 3.5 cm Petri dishes.

Observations for mutant strain stability and mutant characterization. Suspensions from parent strains and irradiated mutant strains were examined under the microscope. The cultures isolated as asporogenic were finally classified as asporogenic mutants after 5-7 successive ‘‘generations’’. Each transfer involved 10 days-culture on agar plates. The hyphae morphology was photographed 4 days after inoculation. Mutants were observed for morphology, mycelia biomass, pigment formation, and daily mycelia extension. Agar plates were inoculated centrally with one 5-mm diameter mycelial mat taken from the periphery of actively growing agar culture by means of a sterile cork borer. The dishes were incubated at 28 ^{o}C. For dry weight determination, five pieces of mycelial mat cultures 1.85 cm in diameter were grown for 3 days in triplicates in 50 ml PDB. The cultures were then filtered through a milipore filter and the excess moisture was absorbed on diapers. Mycelial pellets were dried to a constant weight at 37 ^{o}C and the dry weights determined.

Molecular analysis of mutants. The mycelia from asporogenic mutants were recovered from 48 h shake culture in potato glucose broth (PDB) (Pronadisa) (at 28 ^{o}C). The mycelia were collected and ground in liquid nitrogen with a mortar and pestle. Genomic DNA from mycelia was obtained as stated in Daboussi et al. [10]. To confirm that the mutants still carry NEP1, Polymerase Chain Reaction (PCR) detection of NEP1 was carried out. The primers designed to ampli-fy NEP1 were a forward primer 5’-CCGACGGTTGTCAGCCATACAC-3’ and a reverse primer 5’-CGGCAGCAGCGTAGAGGGTAG, which defined a 346-bp sequence spanning the NEP1. PCR reactions (25 µl) were incubated on a Biometra Thermocycler and contained 1 µl of 100 ng of genomic DNA, PCR mix and 1 µl of 10 µM of each primer. The amplification cycle consis-ted of denaturation at 94 ^{o}C for 5 min followed by 30 cycles consisting of 30 s at 95 ^{o}C (denaturation), 30 s at 55 ^{o}C (annealing), and 1.5 min at 72 ^{o}C (elongation). A positive control consis-ting of pure DNA from the source fungi and a negative control consisting of sterile water were used for each PCR run. PCR products were separated in a 1 % agarose gel, stained with ethidium bromide, and viewed on a Bio imaging system (Rhenium B.I.S. 202D) with Fuji film thermal imaging system (FTI-500). The PCR check was repeated three times. The forward and reverse primers were synthesized by the Weizmann Institute and were expected to amplify a 346-bp region of genes coding for NEP1. A 1 kb DNA ladder GeneRuler #smo321 (MBI Fermentas) was used as the size markers.

Pathogenicity assay. Pure mycelial mats of *A. cassiae* (3 g) were suspended in 100 ml distilled water, chopped for 1 min at 6,000 rpm using an Ultra-Turrax® homogenizer (IKA T18), and collected in vacuo on Miracloth. The chopped *A. cassiae* mycelia were resuspended in 33 ml of sterile distilled water per gram of filtered mycelia. Aliquots were removed for later determination of the propagules concentration by serial dilution and plating on PDA, following the procedure of Amsellem et al. [2].

Greenhouse grown *Cassia senna* L. plants in 8.5 X 8.5 X 6 cm plastic containers were inoculated with chopped mycelial suspensions (10^6 propagules/ml) in different experiments, as follows: (i) sterile distilled water as control, (ii) AltNEP1 as control, (iii) each of the mutants. A surfactant (0.01 % polyoxyethylene-sorbitan monolaurate - Tween 20, Sigma) was added to each spray suspension to spread the solution. Seedlings at the cotyledonary (8-10 days after planting [dap]) and two-leaf stages (15-20 dap)

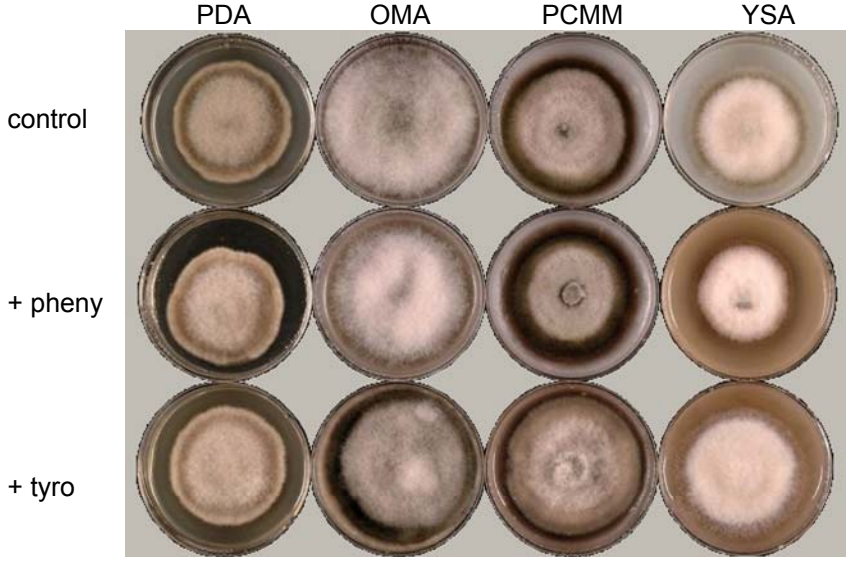
were sprayed until runoff, ensuring uniform coverage of the plants. Six replicate plants were inoculated in each treatment. Two experiments were done with cotyledonary stage plants and one experiment was done with plants at the two-leaf stage. The plants were thinned to the same number per container in each experiment. Inoculated plants were placed in boxes on water-soaked paper toweling and enclosed in plastic bags to maintain 100 % relative humidity in the dark for about 24 h in the growth room. Thereafter, all plants were grown in a growth room at 28 ^{o}C with 14 h daylight and a maximum photosynthetically active light intensity of 52 µE/m^2/s (LI-COR, Inc., Model LI-188B photometer) at a plant height provided by six 40 W cool white fluorescent tubes suspended 65 cm above the benches. Plants were watered as needed with deionized water.

For statistical analyses, the damage was rated visually for disease progression at 2 and 5 d post-inoculation using a 0-3 scale [2]: 0 = no infection or a hypersensitive reaction; 1 = moderate infection, plants still alive; 2 = severe infection, plants hardly alive, but one leaf may be alive and the plants can recover; 3 = complete desiccation and abscission of all leaves, dead plants. The results are shown as means with standard errors, and were further statistically analyzed as described below. To retain pathogenicity, the fungi were reisolated from the sprayed plants 2 days after inoculation.

Statistical analysis. Fungal characterizations were carried out twice under identical conditions, each with three replica platings. Data generated are reproducible. Means ± standard error are indicated in the text. Analysis of variance (ANOVA) for the ratios obtained and post-hoc tests were performed by using SAS version 9.1 [34]. Fisher's protected least significance test (LSD) test was used to determine the significance of pairwise comparisons between treatments. Statistical significances were judged at the 5 % level. Damage severity from mutants was compared with the parent type by Kruskall-Wallis ANOVA test.

Results and discussion

Cultivation media, spore germination, and quantification. The influence of medium composition was tested on fungal sporulation (Table 1). The aim was to optimize spore production on chemically defined media in preparation for ionizing radiation of the fungal spores. The sporulation ability of Alt*NEP1* was studied and compared on agar media. The results showed that the highest number of spores were formed (1.7 to 4.7 x 10^{-3} spores/ml) on YSA (Fig. 1, Table 1). However, in PDA, a predominantly vegetative mycelium was formed (Fig. 1). *A. cassiae* thrived on PDA. PDA, however, encourages the growth of mycelium rather than spore formation. The fungi were maintained by serial transfer from stale to fresh, solid media.



NEP1 *A. cassiae*

Fig. 1. Effect of media composition on sporulation. Mycelia pieces were placed on agar with or without an amino acid supplement (phenylalanine or tyrosine, g/l) and grown at 28 ^{o}C for 10-14 days. PDA = potato dextrose agar; OMA = oatmeal agar; PCMM = potato corn meal agar; and YSA = yeast starch agar.

Table 1. Effect of various cultivation media (broth and agar) and the influence of two amino acids on *A. cassiae* culture pigmentation and sporulation.

Amino acid	Medium	Mycelia color		Quantity (spores/ml, x 10^{-3})
		Broth medium	Agar medium	
control	Potato dextrose	pink	sandy brown	0.0 ± 0.0
	Glucose yeast extract	pink	white	ND
	Oat flake	dark olive green	grey black	ND
	Oat meal	dark olive green	saddle brown	1.0 ± 0.4
	Peptone-corn meal	wheat	saddle brown	1.7 ± 0.2
	Potato carrot	pink	misty rose	2.3 ± 0.4
	Yeast starch	army green	slate gray	0.0 ± 0.0
L-phenylalanine (g/l)	Potato dextrose	pink	Navajo white	0.0 ± 0.0
	Glucose yeast extract	pink	misty rose	ND
	Oat flake	dark olive green	slate gray	ND
	Oat meal	dark olive green	rosy brown	3.5 ± 0.5
	Peptone-corn meal	dark red	saddle brown	1.8 ± 0.2
	Potato carrot	pink	misty rose	4.3 ± 0.8
	Yeast starch	rosy brown, feldspar, dim gray	feldspar	1.7 ± 0.3
L-tyrosine (g/l)	Potato dextrose	pink	rosy brown	0.0 ± 0.0
	Glucose yeast extract	pink	dark salmon	ND
	Oat flake	dark olive green	Navajo white	ND
	Oat meal	dark olive green	rosy brown	0.6 ± 0.01
	Peptone-corn meal	rosy brown	saddle brown	1.7 ± 0.6
	Potato carrot	pink	sienna	2.4 ± 0.6
	Yeast starch	pink	peach puff	4.7 ± 0.6

The aim of screening different media was to identify the media which could induce the highest spore production. YSA showed the presence of spores for *A. cassiae*. That was highly desired in the process to check for asporogenic mutants and, when possible, the likely revertants. YSA is a nutrient media, yet it was found effective for the study purpose. A previous study [39] observed a slight increase in sporulation of some fungi strains with yeast extract which was attributed to the vitamins in yeast extract. Yeast dextrose agar is recommended for fungal sporulation [22].

It is known that cultural conditions determine the type of pigment formed [39]. Tyrosine was added to the agar medium. Tyrosinase is the rate-limiting enzyme in melanin synthesis [12] and melanin is formed from tyrosine.

Formation of microcolonies. To permit the use of higher plating densities (about 50 colonies/plate) than is normally possible when single colonies are desired, 0.9 g/l sodium deoxycholate was added to the agar medium before plating. A. *cassiae* formed microcolonies on 0.9 g/l sodium deoxycholate plates. The colony forming unit per plate was adjusted to get a countable number of colonies.

Dose-response curve. To assess the optimum dose of X-ray irradiation for total asporogenesis of *A. cassiae,* X-ray doses were sequentially increased and many irradiation doses were investigated (25,000 to 100,000 rads). *A. cassiae* curve demonstrates clearly that the X-ray survival curve has an initial ‘‘shoulder’’. The initial shoulder region may be attributed to the multinuclei nature of the *A. cassiae* spores. The existence of a shoulder on the survival curve of cells has been interpreted to imply a multi-target, multi-hit effect of radiation [7]. According to target theory, the mathematical approach to the interpretation of irradiation survival curves [16], survival curves with ‘‘shoul-

ders'', as shown by *A. cassiae* (Fig. 2), indicate that the killing of certain cells requires one or more hits on each of several critical targets. Such cells are capable of accumulating a certain ''threshold dose'' before an effect is observed [19]. This was referred to as an ''induction'' dose (D_i), which is defined as ''the point of intersection between lines parallel with the x-axis and the extrapolation line of the linear portion of the survival curve to the y-axis'' [26].

Some plates with X-ray irradiated spores gave higher propagule counts than the controls. The dose-response curve of *A. cassiae* to X- rays varies critically with a changing dose rate and it is hard to say conclusively at which dose fungal cultures remain viable but with no spore-forming capability. Energy from X-rays is deposited unevenly in ''spurs'' and ''blobs''. This may lead to sites with multiple damages, that is, a combination of a double-stranded break and a base damage.

The purpose of these findings is to determine the D_{10} radiation dose of *A. cassiae* spores. The spores were irradiated with 25,000 to 100,000 rads units and the irradiated cultures were plated. The log of the number of survivors against the dose for getting the survivor curve was plotted. The spore has a D_{10} value of 50,000 rads. The sensitivity of *A. cassiae* to radiation ranges from ≥ 25,000 to 100,000 rads (Fig. 2). It seems clear that these spores are quite resistant to X-radiation, since more than half of them survived a dose of 60,000 rads (Fig. 2). The present data show the potential of *A. cassiae*'s resistance to irradiation, the destruction of which follows a statistical pattern. *Alternaria* has been reported to show resistance to irradiation [31]. The resistance of *A. cassiae* to irradiation is a result of its pigmentation. *A. cassiae* is dematiaceous - a fungus having brown or black melanotic pigment in the cell wall. The decrease in the sensitivity of the *A. nidulans* mutant to UV correlates with the accumulation of a red pigment by the strain, that is, the pigment makes the spores less sensitive to irradiation [18].

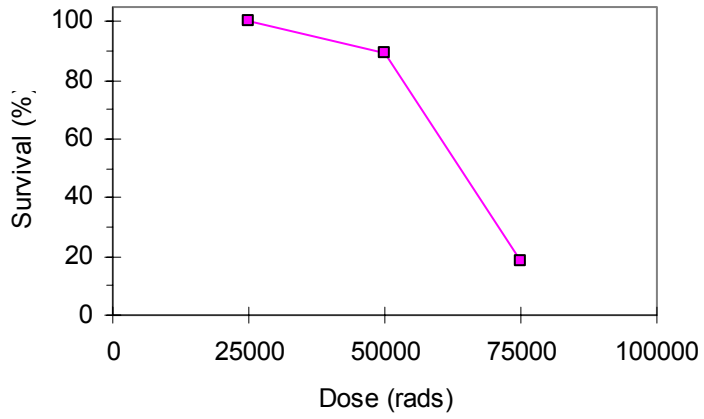


Fig. 2. Radiation sensitivity of spores of *A. cassiae* in suspensions initially containing 3 x 10^3 spores/ml.

Assay of asporogenic mutants. A number of colonies were examined and about 4,000 colonies have been screened. The five mutants isolated have been given the prefix xAlt, for X-ray derived asporogenic *A. cassiae*. The mutants are xAlt539, xAlt541, xAlt525, xAlt543, and xAlt524.

Given that an average of one asporogenic mutant is expected per ten thousand colonies without irradiation, a mutation rate of one in a million is expected. With irradiation at D_{10} dose, one mutation is expected from 100,000. If there are 10 possible mutation sites for sporulation one in 10,000 is expected. The current study has concentrated on the mutant derived from *A. cassiae*. Earlier work on sporulation mutation showed that many genes control various steps in the sporulation pathways [37, 38]. The latter could be one of the reasons why the mutants are relatively difficult to get. Mutation rates per locus per rad obtained from experimental organisms extend over an enormous range of three orders of magnitude, being 1 x 10^{-9} in *Escherichia coli* B/r, and 2.7 x 10^{-9} in *Neurospora* [1].

Observations for mutant strain stability. Three of the cultures, xAlt539, xAlt525, and xAlt543, obtained by X-ray experiments and recorded as mutants, reverted to normal after the third transfer. About half the mutants obtained by X-ray experiments reverted to normal after one or several subcultures [21]. Many sin-

gle-strand breaks are produced in DNA by radiation but are readily repaired using the opposite DNA strand as a template. Spontaneous morphological changes in filamentous fungi have been reported in phytopathogenic filamentous fungi [17], with a similar correlation between morphological changes. The ability to sporulate is nuclearly controlled but not cytoplasmic. In addition, sporulation is conditioned by at least several nuclearly inherited functions [42]. Several unlinked sporulation gene clusters seem likely to exist [35], so if one of the sporulation genes is unavailable, the presence of other sporulation genes may take over the function of the lost gene.

When DNA is irradiated with ionizing radiations, irreversible damage occurs only when a very large number of hydrogen bonds are all broken at the same time [4]. This means there is likely to have been a main-chain scission (a break in each of the adjacent chains less than about 5 nucleotide units apart) caused by the radiation in xAlt541 and xAlt524. The mutants that reverted (xAlt539, xAlt525 and xAlt543) might have had only crosslinks in the DNA molecule.

Molecular analysis. Molecular analysis was performed to check whether the NEP1 fragment was not disrupted by radiation treatment. The NEP1 fragment (346-bp) in each of the mutants was intact (Fig. 3). The results suggested that the mutation does not occur in loci involving the NEP1 fragment or there was radiation-inducible DNA repair in the fragment. This means that the three- to four-fold reduction of transformation frequency below the spontaneous level caused by low dose irradiation [36] is overcome as the added NEP1 fragment could still be expressed.

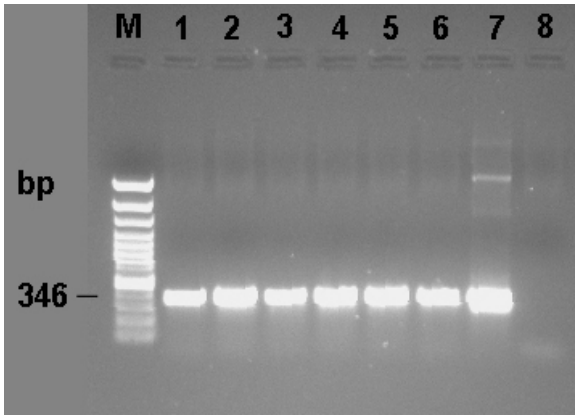


Fig. 3. PCR amplification of *NEP1* gene fragment in the parent strain and asporogenic mutants. Lanes: molecular weight standards (M), Alt*NEP1*, the parent strain (1), xAlt539 (2), xAlt541 (3), xAlt525 (4), xAlt543 (5), xAlt524 (6), *NEP1* plasmid (7), water control (8).

Mutant characterization. To reveal possible variants in morphology due to radiation treatments, the mutants were characterized. The asporogenic mutants xAlt543, xAlt524, xAlt525, xAlt539, and xAlt541, were isolated from survivors of *NEP1 A. cassiae* after X-ray irradiation. The colony morphology of *NEP1 A. cassiae* and those of the mutants are shown in Fig. 4. The irradiation leads to some different outgrowth patterns observed in visible colony diameter, mycelia biomass, pigment formation, growth morphology, and the daily mycelia extension compared with the *NEP1 A. cassiae* control. Mutant xAlt524 seems to assume the form of a dimorphic fungus as a result of the irradiation treatment.

Resulting mutants xAlt539, xAlt541, xAlt525, xAlt543, and xAlt524, which produce no spores, were checked for morphological variants (Fig. 4). Diversity of colonial forms, ranging from tiny to vigorous forms, had been reported in *Neurospora crassa* [23].

Mutant xAlt524 lacks aerial hyphae, pigmentation, and spore production. When xAlt524 was subcultured, it grew no further and could not give rise to a colony. In liquid culture, the mutant was completely autolyzed by 48 h. Mutant xAlt524 displays morphological abnormalities and cell lysis. Accordingly, colonial growth of xAlt524 was scanty. The xAlt524 mutant is assumed to be producing lytic enzymes for self-degradation. The autolysis of filamentous fungi was observed in [27, 30]. The irradiation leads to some different outgrowth patterns because of mutations of the fungal colonies surviving the irradiation treatment. X-ray treatment induces both gene mutations and chromosomal aberrations, including inversions [23].

The *NEP1* type produced a rosy brown to peach puff pigment after 5 days of growth on YSA

plus tyrosine (Fig. 4). The xAlt524 mutant produced a cream and xAlt541 produced dark salmon color. Pigmentations of xAlt543 and xAlt339 were the same, having a Navajo white, dark golden rod, or light salmon mycelial color (Fig. 4). A distinctive feature of a *Magnaporthe grisea* sporulation mutant (abnormal conidium morphology and a reduction in sporulation) was delayed and reduced pigment formation [38]. The xAlt524 mutant lost its pigmentation and so can be readily lysed. Melanized appressoria are resistant to lysis by antagonistic bacteria [20].

Except for xAlt524, all the other mutants retained fluffy mycelia. Alt*NEP1* has abundant mycelia which were still retained by xAlt539 and xAlt541. The cultures of xAlt543 had moderate but non abundant mycelial density. Mutants xAlt524 and xAlt525 had scanty mycelia (Fig. 4).

Comparison of phenotypes of the asporogenic mutants xAlt539, xAlt541, xAlt525, xAlt543, xAlt524, and the parent Alt*NEP1* revealed that xAlt541 grew significantly more slowly (4.63 cm) on YSA than all the other mutants (5.84 to 7.32) as well as compared to the parent Alt*NEP1* (5.77 cm) (Fig. 5). No statistically significant difference in radial growth was detected among Alt*NEP1*, xAlt525, xAlt543, and xAlt524. There was no substantial difference in the radial growth of all the isolates until day 5 after inoculation.

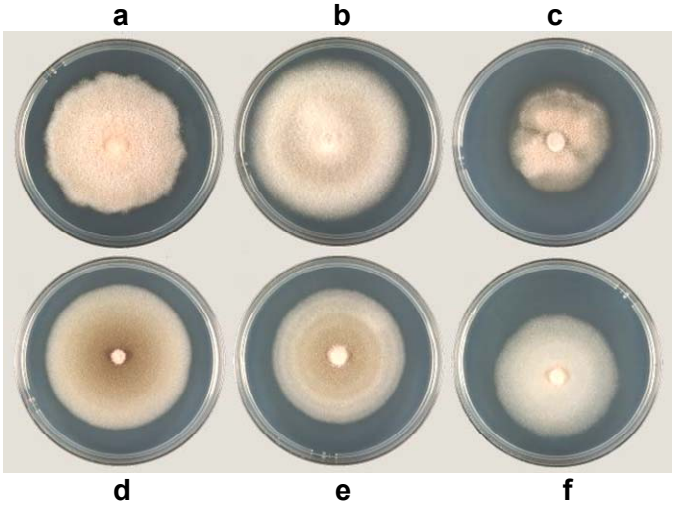


Fig. 4. Growth characteristics of the parent type and the mutants grown on YSA plates. Morphology of *NEP1* fungus (a), xAlt539 (b), xAlt541 (c), xAlt525(d), xAlt543(e), xAlt524(f). Plates (b) to (f) are irradiation mutants. Photographs were taken 7 days after inoculation.

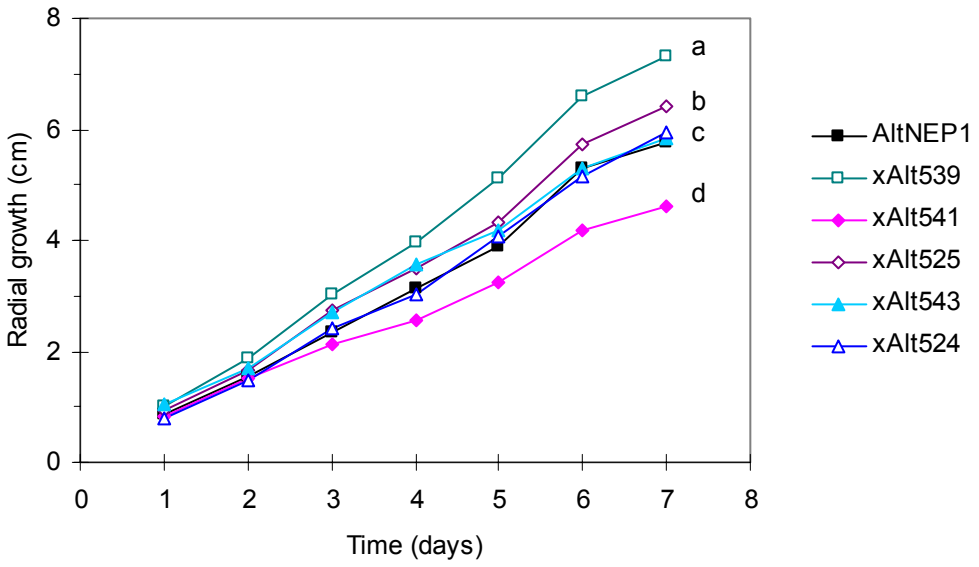


Fig. 5. Radial growth of parent transgenic type and irradiation mutants; 7 days growth on yeast starch agar under continuous cool white light (48 µE m^{-2} s^{-1}) at 28 ^{o}C. The data were analyzed by ANOVA

For mycelia dry weight determination, data from six experiments showed that the parent Alt*NEP1* developed more mycelium than all the mutants (Fig. 6). There was a striking significant difference between dry weight of xAlt524 (6.46 mg) and the parent type fungus (16.13 mg). This reduction led to approximately only 40% growth. The other mutants also showed reduced dry weights, respectively xAlt539 (86.29%), xAlt541 (63.55%), xAlt525 (94.48%), and xAlt543 (71.6%). There was no significant difference between Alt*NEP1* and two of the mutants (xAlt525 and xAlt539).

The hyphal growth and branching of the parent fungus Alt*NEP1* and the stable mutants xAlt541and xAlt524 are depicted in Fig. 7 (a, b and c, respectively). If the fungal colonization ability of the fungus (hyphal growth and branching) is reduced, then the pathogenicity may be reduced.

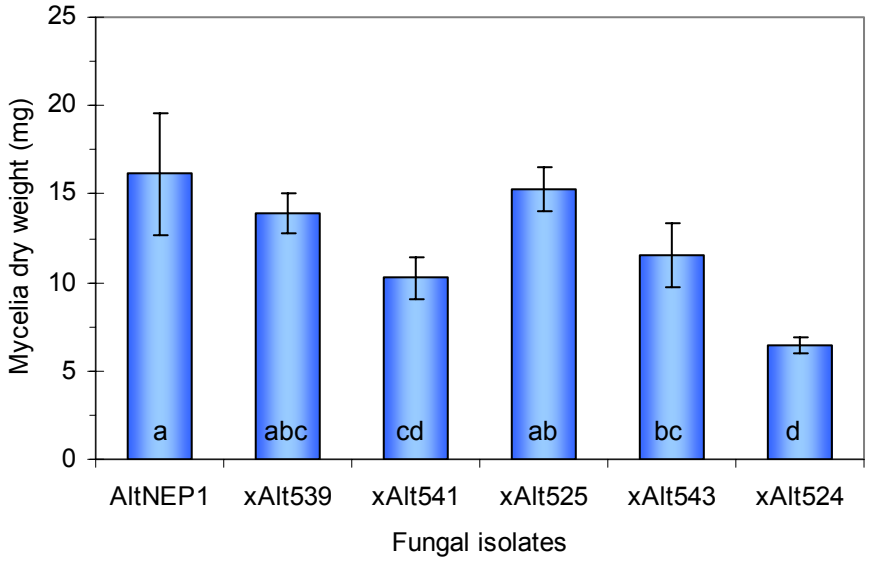


Fig. 6. Mycelia dry weight of Alt*NEP1* and five asporogenic mutants of Alt*NEP1*. Bars represent the means ± standard error of six replicas. The data were analyzed by ANOVA.

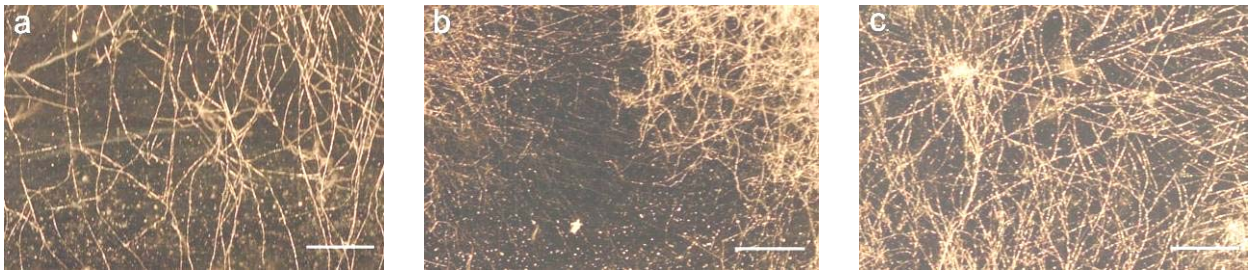


Fig. 7. Light microscopic observation of hyphal morphology of Alt*NEP1* (a) and mutants xAlt541 (b) and xAlt524 (c). They were grown for 4 days on glass slides covered with half strength PDA. Slides were placed on a dark field background, the growing mycelium was directly observed and photographs were taken of the upper surfaces of the colonies. The bar represents 1000 µm.

Virulence test. Pathogenicity of the mutants was examined to determine whether the mutants were still virulent despite the mutation effect of irradiation on their sporulation capability. All the mutants caused wilting and necrosis with xAlt525 being statistically (2.67ab ± 0.1) insignificantly different in vigor than the parent *NEP1* culture (Fig. 8, top panel).

To see clearly the infection vigor of the mutants on older plants, xAlt541 and xAlt524 (stable mutants) were sprayed on 2-leaf stage cassia. Alt*NEP1* (1.5a ± 0.22), xAlt541 (1.33ab ± 0.21), and xAlt524 (1.0b ± 0.00) damage severity was statistically different by two days post-inoculation. However, damage severity from Alt*NEP1* (2.5a ± 0.22), xAlt541 (2.17a ± 0.31), and xAlt524 (2.0a ± 0.26) was not statistically different by 5 days post-inoculation (Fig. 8, bottom panel).

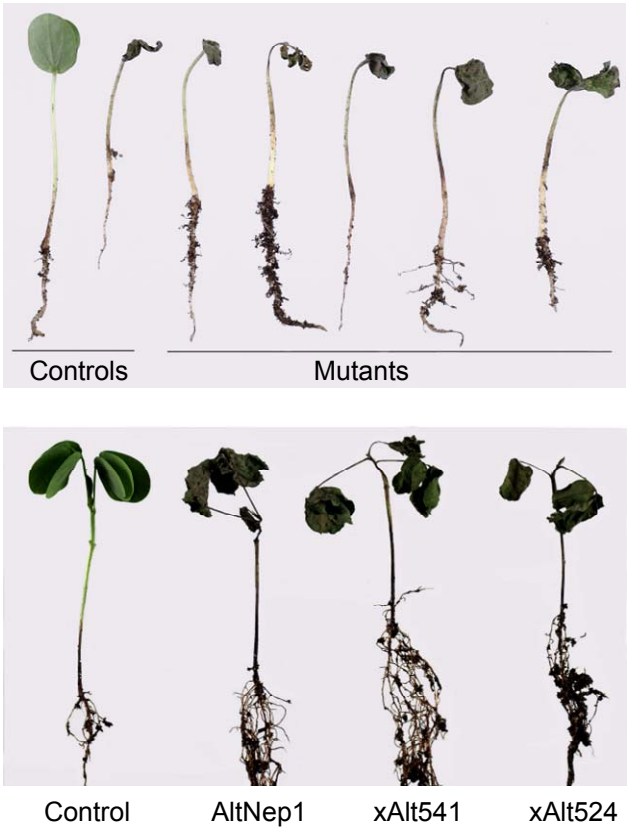


Fig. 8. Evaluation of virulence of asporogenic mutants applied as a foliar spray on seedlings at cotyledonnary (top panel) and 2-leaf stages (bottom panel). Ten ml of chopped mycelia (10^6 propagules/ml) of *A. cassiae NEP1* type or mutant strains sprayed to runoff and controls inoculated with water. They were placed in a dew chamber (100 % RH) for 24 h, then on growth room benches. The photograph represents typical shoots 4 days after spraying. Top panel: the controls are water and Alt*NEP1*. The mutants are xAlt539, xAlt541, xAlt525, xAlt543, and xAlt524. The bottom panel is as labeled.

The mutants had the capacity to cause disease on cassia plants and the levels of damage severity equaled that of the parent strain over time. Either the sporulation of *A. cassiae* is not related solely to fungal virulence or the irradiation treatment had not affected some of the components vital for colonization and hence virulence. The present results are in agreement with that of [5] who reported that X-ray sensitive mutants are similar, if not identical, to their wild-type counterparts in other biological functions. Asporogenic fungi could mitigate risks of transgene introgression into related fungi. The mutants have the *NEP1* gene and could also still express the gene on target plants.

Melanin biosynthesis is an important determinant of pathogenicity and virulence [6, 28]. However, not all pathogenic fungi are melanotic, as seen with xAlt524. Even on YSA plus tyrosine xAlt524 does not form a pigment.

Although X-ray irradiation has an effect on fungi mutagenesis, some of the mutants revert and could not be differentiated from the parent isolate. By 5 days post-inoculation, the fungal virulence of the mutants had no statistically significant difference from that of the parent isolate. It is possible that there could be stabilization of the mutated gene in the revertants if they were again irradiated, but this hypothesis requires further research. Results from this study have made it clear that asporogenic mutants could reduce the ecological constraint of the use of mycoherbicides. The mutants could be mass-produced and stored for reasonable periods e.g. > 9 months, [3] prior to field application.

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АСПОРОГЕННИ МУТАНТИ НА *ALTERNARIA CASSIAE,* СЪЗДАДЕНИ ЧРЕЗ ОБЛЪЧВАНЕ С РЕНТГЕНОВИ ЛЪЧИ

Олубукола Бабалола

Резюме

Предотвратяването на спорулацията е важен фактор, за да се попречи на разпространението на трансгенни агенти за биоконтрол над желаните граници. Това разпространение би могло да се избегне с аспорогенни делеционни мутанти. Alternaria cassiae е агент за биологичен контрол на тропическите билкови растения Cassia obtusifolia и Cassia occidentalis. Трансформираната с NEP1 гъба е по-вирулентна от родителската. A. cassiae образува микроколонии на среда с 0.9 г/л натриев дезоксихолат. При облъчване на спорите с рентгенови лъчи, стойността D_{10} е 50 000 рада. Получени са пет предполагаеми мутанта, като е доказано, че три реевертират. Мутантите xAlt541 и xAlt524 са стабилни и не могат да образуват спори. Мутантът xAlt541 расте по-бавно (4,63 см) на дрождево-скорбелен агар от xAlt524 (5,94 см) и изходния щам (5,77 см). Мутантът xAlt524 няма въздушен мицел и пигментация. Мицелът на родителската гъба е розово кафяв до прасковен след 5 дни растеж на среда с 1 г/л тирозин, с цвят на минерала фелдшпат на среда с 1 г/л фенилаланин и изглежда сив при отсъствие на добавена аминокиселина. Мутантите xAlt524 и xAlt541 имат съответно кремав и тъмно розово-оранжев цвят. При концентрация 2.5×10^6 пропагули/мл, тежестта на увреждания на растенията от мутантите xAlt541 (2,17a ± 0,31) и xAlt524 (2,0a ± 0,26) 5 дни след инокулация не се различава статистически от тази при родителския тип AltNEP1 (2,5a ± 0,22).