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Molecular and Biochemical Evaluation of Genetic Effects of *Calotropis procera* (Ait.) Latex on *Aspergillus terreus* Thom

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Abstract. On treating dense conidial suspensions of *Aspergillus terreus* Thom with different concentrations of *Calotropis procera* latex, for investigating the genotoxicity of the latter, it was found that latex of *Calotropis procera* had potent lethal and mutagenic activities. Survival percentage decreased as concentration or time of exposure increased. Frequency of auxotrophic mutants increased with increase in concentration or exposure time. Most auxotrophic mutants were amino acid requiring mutants. DNA and total protein contents of each mutant was significantly lower than wild type of *Aspergillus terreus.* RAPD demonstrated polymorphic genetic bands of electrophoretic products of PCR for all mutants compared with the wild type strain. SDS-PAGE results expressed a polymorphism of protein bands as well. All these results indicated the mutagenicity of the latex of *Calotropis procera.*

Keywords: *Calotropis procera,* mutagenicity, *Aspergillus terreus,* genotoxicity

Introduction

Calotropis procera (Ait.) of the Asclepiadaceae family, commonly known as Sodom apple, is widely distributed in Saudi Arabia and is one of giant milk weeds (Calvin, 1979). The plant is popularly known because it produces large amount of latex, which is easily collected from its green parts when the plant is wounded. The aspect of this natural secretion resembles that of rubber tree, *Hevea brasiliensis*. The latex of *C. procera* extract is prescribed in folkloric medicine for the treatment of many diseases. Many recent studies proved its potential activities as wound healing, anti-inflammatory, anti-diarrhoeal and anti-rheumatic drug (Kumar *et al*., 2001; Rasik *et al*., 1999; Sharma and Sharma, 1999; Basu *et al*., 1997; Jain *et al*., 1996).

Some people use it successfully to combat some skin fungal infections and malaria. The abundance of latex in the green parts of the plant reinforces the idea that it is produced and accumulated as a defence strategy against organisms such as virus, fungi and insects, although wounded plants of *Calotropis* as well as insects visiting their leaves are also seen (Haque *et al*., 2000; Larhsini *et al*., 1997). When injured, leaves or the nearest other green parts of the tree exude the latex which has a clingy effect, capable of immobilizing insects. Additionally, the presence of plant defence-related proteins such as hevein, alpha-amylase inhibitor, among others, have been described to occur in the latex secretion of other plants (Pereira *et al*., 1999; Wititsuwannakul *et al*., 1998) and this seems to be also the case of *Calotropis* latex (Dubey and Jagannadham, 2003).

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Scientific literature mentions other relevant activities of the latex of *C. procera*, such as among others antibacterial, analgesic or *in vitro* schizonticidal activity, (Alencar *et al*., 2004; Dewan *et al*., 2000; Sharma and Sharma, 2000; Jain *et al*., 1996). A brief communication pointed out the whole latex of *C. procera* as a suitable source of active compounds exhibiting larvicidal activity (Ramos *et al*., 2006). The genotoxicity of *C. procera* latex extract was demonstrated for the first time by Baeshin and Al-Ahmadi (2004) employing root tip meristems of *Allium cepa* and *Vicia faba* chromosomal aberrations test. Cytotoxic and anti-tumour activities of the latex of *C. procera* were studied by Choedon *et al.* (2006). More investigations are required as a battery of tests to establish its genotoxic effect. The purpose of this study is to evaluate the genotoxic effects of the latex of *C. procera* by the *Aspergillus terreus* auxotrophic mutant test and DNA fingerprinting by RAPD as well as protein profile by SDS-PAGE test.

Materials and Methods

Latex preparation. The latex of *C. procera* was collected during the last week of August, 2006 and January, 2007 from plants growing in their natural habitats, located along the belt of *C. procera* in Al-Hajj Street of Makkah as described by Migahid (1988).

Fresh latex was collected from the healthy plants by making small incisions near the youngest leaves in sterile 250 ml conical flask. The latex samples were gently handled in ice bag under sterile conditions to maintain homogeneity and activity during transport to the laboratory and transferred to

a refrigerator for 2-3 h; afterwards, they were centrifuged (3000-4000 rpm) at room temperature (25 °C) in a non-refrigerated bench top centrifuge for 15 min to remove inter coagulum (Atal and Sethi, 1962). The supernatant was then filtered through a membrane filter and the filtrate (the pure latex) was either used directly in the experiment or kept in the refrigerator for no longer than 7 days for future use. Three concentrations of this stock extract were prepared with sterilized distilled water (1, 3, 9 and 27% of latex) for testing the genotoxic activities.

Strains. ***Wild type strain.*** The haploid phase of the wild type strain of *Aspergillus terreus* Thom was the test organism in the present study. It was obtained from the Biology Department, Faculty of Sciences, King Abdul-Aziz University, Jeddah where it had been maintained for several years. It had been isolated from Makkah Road by El-Sharkawi *et al.* (1981) and was identified by the Common Wealth Mycological Institute, England.

Mutant strains. Induced mutant strains by *C. procera* latex in this experiment were isolated and maintained on Potato Dextrose Agar (PDA) and used later in this study for determining their nutritional requirements and to run RAPD and SDS-PAGE tests on them. Growth medium supplies were purchased from Difco Laboratories.

Chemicals. Standard concentrations of hydrolysed casein (CAS), mixed vitamin solutions (VITS), hydrolysed yeast ribonucleic acid (RNA), individual amino acids, individual vitamins and individual purines and pyrimidines were used as supplements to the minimal media (Table 1). All the chemicals used in the study were provided by Sigma Company, except DNA polymerase (from Perkin-Elmer Cetus) and the dNTPs (from Boehringer Mannheim). Difco Czapek Dox agar (DOX) was used as the minimal medium and Potato Dextrose Agar (PDA) was employed as the complete medium.

Test of genotoxicity. ***Auxotrophic mutation.*** Latex of *C. procera* was tested for biochemical genetic activities following the method described by Sabir and Baeshin (1999) for the induction of auxotrophic mutants. A dense conidial suspension was made and number of 1,000,000 conidia/ml was estimated using a hemocytometer. Five ml of this suspension was immediately added to 5 ml of the latex extract and one ml sample of this mixture was immediately diluted in 9 ml sterile distilled water to serve as untreated (positive) control. Subsequent samples were taken at regular intervals (15 min) for a period of 1h after exposure and serially diluted in sterile distilled water to halt the mutagenic treatment. Samples of the final dilutions containing about 100 conidia/ml were spreaded on PDA plates and incubated at 28 °C for 4 days. This was repeated for each of the four different concentrations of *Calotropis* latex (1, 3, 9 and 27% of latex).

Table 1. Concentrations of medium supplements

Supplement	Concentration of stock solution (mg/ml)	Concentration used in media (mg/ml)
Hydrolysed casein	60	3
Individual amino acids	10	10^{-1}
Hydrolysed yeast		
Ribonucleic acid	10	5×10^{-1}
Individual purines and pyrimidines	4	2×10^{-2}
*Vitamins**		
Aneurin	5	5×10^{-4}
Biotin	0.02	2×10^{-6}
Choline chloride	20	2×10^{-3}
Inositole	40	4×10^{-3}
Nicotinic acid	10	10^{-3}
Pyridoxine	5	5×10^{-4}
P-aminobenzoic acid	1	10^{-4}
Pantothenic acid	20	2×10^{-3}
Riboflavine	10	10^{-3}

* = mixed vitamins: 1 ml of each of the individual vitamin solutions was mixed and 0.2 ml of this mixture was added to 200 ml of media (0.2 ml of 1/10 dilution/plate)

Mutants were isolated according to the method described by Fincham *et al.* (1979). At each time interval, a monoconidial inoculum was inoculated in each of 26 loci/plate containing PDA and served as template. The template was in turn replicated on the minimal medium (DOX) to detect auxotrophic mutants. All replicates were incubated for 5 days at 28 °C. Auxotrophic mutants were those which failed to grow on DOX after incubation for 5 days at 28 °C. All mutant colonies were isolated on PDA templates and replicated on the following supplemented media for determination of their nutritional requirements:

1. DOX (minimal media).
2. DOX (minimal media) + CAS (add casein) + VITS (add vitamins) – RNA (without ribonucleic acid)
3. DOX (minimal media) + CAS (add casein) - VITS (without vitamins) + RNA (add ribonucleic acid)
4. DOX (minimal media) – CAS (without casein) + VITS (add vitamins) + RNA (add ribonucleic acid)
5. DOX (minimal media) + CAS (add casein) + VITS (add vitamins) + RNA (add ribonucleic acid)

A colony which failed to grow on any of number 2, 3 or 4 media requires the chemical, missing from that medium. The auxanographic technique by Pontecorvo (1949) was used to

specify the particular nutritional requirement of each mutant. One ml of a dense conidial suspension of the mutant was mixed with cooled molten DOX (45 °C) in dishes and left to solidify. A few crystals of the nutrients to be tested were placed at marked positions around the periphery of the agar plate. Each mutant grew after 5 days incubation in the immediate vicinity of the nutrient required.

RAPD profile. Auxotrophic mutants were incubated for 5 days at 28 °C in broth PDA media, then, they were frozen in liquid nitrogen, ground with mortar and pestle and incubated in 1.5 ml of extraction buffer (100 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM EDTA pH 8, 2% SDS and 0.1 mg/ml proteinase K) for 1.5 h at 37 °C. DNA was extracted according to Scott *et al.*, (1991) with minor modifications.

PCR reactions were conducted using three arbitrary 10-mer primers (Operon Tech. Inc.). The names and sequences of these oligoprimers are listed in Table 2. The reaction conditions were optimized and mixtures (25 µl total volume) consisted of: P10X PCR buffer (pH 8.3), KCl (50 mM), $MgCl_2$ (3 mM), dNTPs (2.5 mM), primer (20 µM), template DNA (30 ng/µl), Taq DNA polymerase (1 unit), all under a drop of mineral oil. DNA amplification was carried out in a Perkin Elemar G thermalcycler. Samples were preheated at 94 ºC for 1 min. Amplification reactions were run for 40 cycles consisting of: 1 min at 94 ºC, 2 min at 36 ºC, 1 min at 72 ºC with a final extraction time of 7 min at 72 ºC. Amplification products were size-separated by electrophoresis in 2.5% agarose gel and visualized by ultraviolet illumination after staining with ethidium bromide.

Protein profile. Protein was extracted by crushing frozen fungi which were incubated for 5 days at 28 °C in broth PDA media from each mutant and wild type in 500 µl sample buffer. The mixture was denatured for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on total protein using the slab gel apparatus (Hoffer SE 600 vertical slab unit) according to the method of Laemmli (1970). The protein contents of both wild *A. terreus* and mutants were estimated by the colorimetric method of Coomassie Brilliant Blue according to Bradford (1976).

Statistical Analysis. Data of survival percentage and frequency of auxotrophic mutants were analyzed by analysis of variance (ANOVA), with the calculations of the F-statistic and respective P values; P values were compared with the value of the significant difference for P=0.05% or P=0.01%.

Table 2. Primers and their sequences

Primer	Sequence	G+C (%)
OPB-16	5′ TTT GCC CGG A′3	60
OPC-20	5′ ACT TCG CCA C′3	60
OPE-03	5′ CCA GAT GCA C′3	60

Results and Discussion

Auxotrophic mutation. The survival percentage and recovery of auxotrophic mutants among survivors of *A. terreus* cultured cells, as a function of treatment with different concentrations of *C. procera* latex, are presented in Table 3 and 4.

Table 3. Effect of concentrations of *C. procera* latex and exposure time on survival percentage (SP) of conidia of *Aspergillus terreus*

Extract conc. (%)	Exposure time (min)					Mean of SP (%) within conc.
	0	15	30	45	60	
1	100	96.74	87.91	78.37	76.74	87.95*
3	100	94.96	87.65	82.62	83.62	89.77*
9	100	79.48	70.51	65.64	64.87	76.10**
27	100	60.87	15.11	46.17	45.18	60.66**

* = significant from wild type (control at 0 time) at P>0.05; ** = significant from wild type (control at 0 time) at P>0.01

Table 4. Effect of concentrations of *C. procera* latex and exposure time on auxotrophic mutation percentage (AMP) for 208 isolated colonies of *A. terreus*

Extract conc. (%)	Exposure time (min)					Mean of AMP (%) within conc.
	0	15	30	45	60	
1	0.00	0.00	0.00	0.48	0.48	0.192*
3	0.00	0.00	0.48	0.00	0.96	0.288**
9	0.00	0.00	0.48	0.48	0.96	0.384**
27	0.00	0.00	0.00	0.48	0.00	0.096**

* = significant from wild type (control at 0 time) at P>0.05; ** = significant from wild type (control at 0 time) at P>0.01

The highest possible percentage of mutation (1.92%) is achieved with the dose of 9% at exposure time of 60 min which is the optimal dose for the induction of auxotrophic

mutants with *C. procera* latex. All of the auxotrophic mutants recovered from *C. procera* latex-extract-treated conidia of *A. terreus* were amino acid-requiring mutants (Table 5). Some of the mutants restored growth with any one of different alternative nutritional requirements (e.g., tyrosine or proline).

DNA and total protein quantity of mutants (Table 6) decreased significantly (P> 0.01) as compared to the wild type which is a consequence of molecular changes in the genetic material of *A. terreus*.

Table 5. List of auxotrophic mutants recovered from *C. procera* latex treated conidia of *A. terreus*

Mutants code no.	Latex conc. (%)	Exposure time (min)	Requirement
AMC1T45	1	45	glutamine
AMC1T60	1	60	lysine
AMC3T30	3	30	proline (or) tyrosine
AMC3T45-a	3	45	tyrosine
AMC3T45-b	3	45	glutamine
AMC9T30	9	30	lysine
AMC9T45	9	45	tyrosine (or) proline
AMC9T60-a	9	60	glutamine
AMC9T60-b	9	60	lysine
AMC27T45	27	45	lysine

Table 6. Quantities of DNA and total protein isolated from wild type and auxotrophic mutants of *A. terreus*

Aspergillus terreus strains	Exposure time (min)	Treatment conc. (%)	DNA quantity (µg/ml)	Total protein quantity (µg/ml)	% of decrease total protein
Wild Type	-	-	600	3190	0
AMC1T45	45	1	543*	2820	11.59**
AMC1T60	60	1	445**	2930	8.15**
AMC3T30	30	3	340**	3009	5.67**
AMC3T45-a	45	3	310**	2706	15.17**
AMC3T45-b	45	3	315**	2742	14.04**
AMC9T30	30	9	300**	2685	15.83**
AMC9T45	45	9	221**	2689	15.70**
AMC9T60-a	60	9	260**	2627	17.64**
AMC9T60-b	60	9	247**	2349	26.36**
AMC27T45	45	27	117**	2476	22.38**

* = significant from wild type of *A. terreus* at P>0.05; ** = significant from wild type of *A. terreus* at P>0.01

RAPD profile. The RAPD results illustrated polymorphic numbers of the genetic bands (Fig. 1), which were the electrophoretic products of PCR for all mutants compared with the wild type. Table 7 illustrates that the highest number of polymorphic bands among mutants was generated in reactions with the primers OPB-16 which was 78 genetic bands and the lowest number of polymorphic bands obtained with primer OPE-03 which was 46, whereas the number of polymorphic bands of primers OPC-20 was 58. The percentage of polymorphic bands to source of polymorphism are 42.86, 25.27 and 31.86 for OPB-16, OPE-03 and OPC-20, respectively.

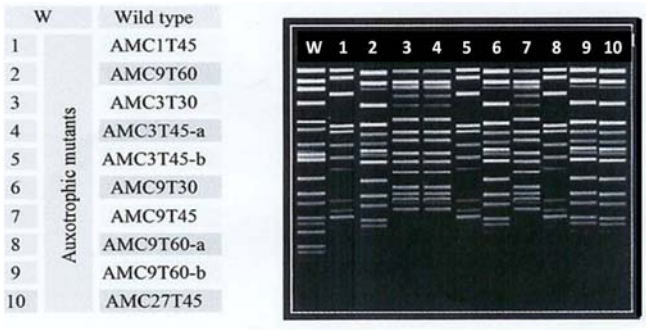

Fig. 1. RAPD-DNA genetic bands of auxotrophic mutants in *Aspergillus terreus* caused by C. *procera* latex (OPB-16).

Protein profile. The results of total protein profile and applied SDS-PAGE technique are expressed as a decrease in the total protein in all mutants compared to wild type (Table 7); a polymorphism of protein bands are clear in Fig. 2. All these results strongly point out the mutagenicity of the latex extract of *C. procera*.

It has been clearly shown that the increase in concentration and exposure time to *C. procera* latex lead to a decrease in survival percentage and an increase in auxotrophic mutation percentage of *A. terreus*. These results are generally in agreement with the rule mentioned by Fincham *et al*. (1979) who stated that by using chemical mutagens, there was a constant relation between the dose and the mutation percentage which increases to a certain limit with the increase in the dose. This provides additional evidence for the genotoxicity of this latex extract earlier demonstrated by Baeshin and Al-Ahmadi (2004) for the first time.

The present study confirms that *C. procera* latex extract exhibits strong mutagenic activities as compared to the potent chemical mutagenic agent N.T.G. It was found by Baeshin and Sabir (1987) that N.T.G. yielded 3.8% of auxotrophs in *A. terreus* with the optimal dose of 0.0075 g/10 ml on 70 min. exposure, whereas *C. procera* latex extract resulted in

Table 7. Polymorphic bands of each genetic primer and percentage of polymorphism in auxotrophic mutants of *A. terreus*

Primers	Total bands	Polymorphic bands to control bands (no.)	Polymorphic bands to total bands of primers (%)	Polymorphic bands to source of polymorphism (%)	Polymorphic bands to total bands (%)
OPB-16	161	78	48.45	42.86	19.84
OPE-03	109	46	42.20	25.27	11.70
OPC-20	123	58	47.15	31.86	14.75
Total	393	182	-	-	-

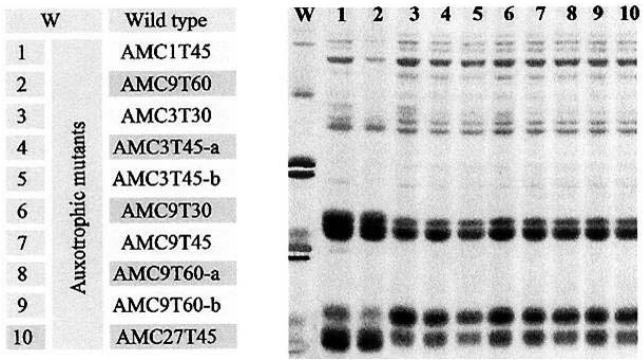

Fig. 2. Protein bands of auxotrophic mutants in *A. terreus* caused by *C. procera* latex.

1.92% auxotrophic mutants with a dose of 9% at 60 min exposure, in the present study.

All of the auxotrophic mutants recovered from *C. procera* latex extract treated-conidia of *A. terreus* were amino acid-requiring mutants. Some of the mutants restored growth with any one of different alternative nutritional requirements. These are probably leaky auxotrophic mutants i.e., they fail to completely prevent the action of a gene and permit some residual functions, specifically of glutamine, proline, tyrosine and lysine. According to the selected target, locus of this extract at the molecular level is suggested. Similar results were obtained by Sabir (2005), Baeshin and Sabir (1987) and Tayl (1975). This observation led to a molecular study of DNA and protein of the auxotrophic mutants with the wild type as control.

The DNA and protein study revealed a significant decrease in all auxotrophic mutants as compared with the wild type, suggesting a molecular change causing a deletion at one or more loci; this probably affected gene expression and consequent interruption in biochemical pathways of DNA and protein synthesis; alkaloids, glycosides and functional enzymes in *C. procera* latex extract often exhibit similar activity. These results are consistent with the results obtained by Choedon *et al.* (2006).

The auxotrophic mutation resulted due to a change (defect in the metabolism pathway) on genomic DNA; the results of RAPD genetic bands have given strong evidence that auxotrophic mutants induced genomic DNA changes. So the observation of the RAPD results have provided good evidence of the ability of *C. procera* latex to induce point mutation as a result of deletion of at least one nucleotide; this is revealed by the disappearance of many genetic bands as compared with the wild type. Some of the components of *C. procera* may act as intercalation agent or generate free radicals which interact with DNA to account for the observed deletions; similar results were obtained by Ansah *et al.* (2005) in their study of *Cryptolepis sangvinolehta*. Also a polymorphism of protein bands, obtained as a result of PAGE protein profile, confirms the ability of *C. procera* to cause frame shift mutation in *A. terreus*. All these results strongly point out the mutagenicity of the latex extract of *C. procera* in *A. terreus*.

Generally, latex of *C. procera* showed mutagenic effect on the conidian cells of *A. terreus* in increasing manner along with concentration and treatment time. Further studies need to be made to separate the active ingredient of that latex which produces the mutagenic effect and to explore the toxicity and other pharmacological assay in order to make herbal safety products as mentioned in our local folkloric medicine.

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