Symbiosis, 2 (1986)
Balaban Publishers, Philadelphia/Rehovot

Evaluation of Mycotoxins Produced by *Alternaria Alternata* Isolated from Tomato in Iraq

JAWAD ABDUL-LATIF, M., M.I. HUSSAIN*, H. RASHID IMAD, A.A.W. RAZZAK and F. ALI KHULUD

Scientific Research Council, Biological Research Centre, Pharmacology and Pharmacognosy Department, Jadriyah, P.O. Box 2371, Baghdad, Iraq.

**Baghdad University, College of Agriculture, Abu-Ghreab, Iraq*

Abstract

Evaluation of the toxicity of 27 isolates of *Alternaria alternata* was carried out to determine their pathogenicity to chicken embryos and mice. These isolates were inoculated in yeast extract sucrose media and incubated for 14 days at 27±1°C. The culture fluid was extracted and the residue was re-dissolved in 2.5% ethanol to give a final concentration of 6 mg/ml. The results showed that some isolates were highly productive. The crude extract from the majority of the isolates was toxic. The highest toxic effect on chicken embryos was manifested by isolates, 4, 5, 6, 16, 20 and 26 which caused 100% embryonic mortality. The LD 50 for mice of the most potent isolate was 20 mg/kg d.wt. having upper and lower limits of 26.8 and 14.9 respectively.

Keywords: mycotoxins, *Alternaria alternata*

Abbreviations: SPF: Specific pathogen free, MF: morphological forms

1. Introduction

Previous reports indicated that *Alternaria alternata* (Fa.) Keissler, may be grouped with other *Alternaria* species as an important pathogen of tomato fruit (Ellis, 1971). Recently, there have been interesting studies on phytotoxins produced by *Alternaria* species and many toxic metabolites have been isolated and characterized (Janardhanan and Hussain, 1983; Wittkowski et

0334-5114/86/$03.00

al., 1983). Mycelial extracts proved to be toxic to brine shrimps, chicken embryo and rats (Sauer et al., 1978).

The object of this work was to study the tomato fruits contaminated with *A. alternata* and to determine the effect of the metabolites of this fungus on chicken embryo mortality and its acute toxicity in mice.

2. Materials and Methods

Isolation and cultural procedure

Samples of diseased tomato fruits superficially infected with black rot were collected in Nov. and Dec. 1983 and Jan. 1984 from various regions in Iraq. Isolation was made on petri dishes containing Potato Dextrose solidified with agar and 50 ppm of tetracycline was added as antibacterial agent. Inoculation was performed by transferring the surface-sterilized infected tissue into the center of the medium. The cultures were incubated at 30°C for 3–5 days. Conidial characteristics were used in the identification of the isolates (Ellis, 1971).

Suspension cultures were grown in yeast-extract sucrose as adapted by Davis et al. (1968). The cultures were maintained in 250 ml conical flasks (incubated for 14 days at 27±1°C in darkness). The cultures were then filtered, using Whatman (150 mm) filters attached to Buchner funnels with vacuum, and the dry weights of the mycelia were determined. The cultural fluids (150 ml) were extracted with chloroform. The chloroformic layer was separated over sodium sulfate and then filtered and evaporated in a rotary evaporating system (Heidolph, type WI) at 40°C. The residues were weighed and redissolved in 2.5% ethanol to give a final concentration of 6 mg/ml.

Bioassay methods

Chicken-embryo test

Fertilized specific pathogen free eggs (SPF) were incubated for 48–72 hr and the defective eggs were excluded. A special drill (1 mm in diameter) was used to make a hole in the center of the sterilized marked air sac. A sterile syringe (size 50μl) was used to inject the fungal metabolites into the air sac. The hole was then cemented with nail polish. Twenty-five fertilized eggs were used for each isolate.

Incubation was carried out in an automatically controlled commercial egg incubator at 37.8°C and 60% RH. The mortality and hatchability of embryos were determined daily up to 21 days post-incubation. Mortality percentage was estimated following the formula adapted by Abott (1925).

Acute toxicity test

Seven groups, each consisting of 6 randomly decided albino male mice (20–25 g) were used. The first, second, third, fourth, fifth and sixth group were injected i.p. with 10, 15, 20, 25, 30 and 35 mg/kg d.wt, respectively. The seventh group was injected i.p. with 2.5% ethyl alcohol as a control. The volume of injection was 0.1 ml/mouse. The groups were observed continuously for 2 hr and from time to time for 24 hr, to note toxic symptoms and to record mortality in each group. The lethal dose in 50% of the tested mice (LD50) was statistically calculated using the Logarithmic method of Litchfield and Wilcoxon (1949).

3. Results and Discussion

A total of 27 isolates of *A. alternata* were identified. Two morphological forms (MF) were detected according to the sporulation density and aerial mycelium. The MF1 (Fig. 1) was highly sporulated with low density of aerial hyphae, while MF2 (Fig. 2) was less sporulated with higher density of aerial hyphae. The growth rate and the weight of the total extract for each isolate are shown in Table 1. The results indicate that the potential of the various isolates of *A. alternata* to produce extracellular extracts varied. Some isolates, for example 16, 17 and 19 were highly productive, yielding up to 182 mg extracellular products. However, the majority of isolates seem to be less productive.

The total growth rate (dry weight) was in the range of 1.12–4.21 g and the maximum growth rate was exhibited by isolate 15. Clearly, there is no correlation between the growth rate and the amount of extracellular products produced. Undoubtedly the production of certain secondary metabolites is under genetic control (Vanek et al., 1973). The bioassay test showed that the crude extracts of the majority of the isolates were toxic, although there was considerable variation amongst isolates. The maximum toxic effect on chicken embryos (100% mortality) was exerted by several isolates while other isolates, such as 2 and 12 were non-toxic (Table 2). The mortality percentage and lethal dose for mice are presented in Table 3 and Fig. 3. The results of the acute toxicity tests indicated that the LD 50 for the crude fungal metabolites was 20 mg/kg with 14.9 as lower limit and 26.8 as the upper limit. The symptoms of toxicity were manifested as nervous irritability, increase in rate of respiration followed by depression, then death. The post-mortem examination showed congestion of the whole internal organs, especially liver, heart, lungs and intestines. However, the severity of the symptoms and congestion

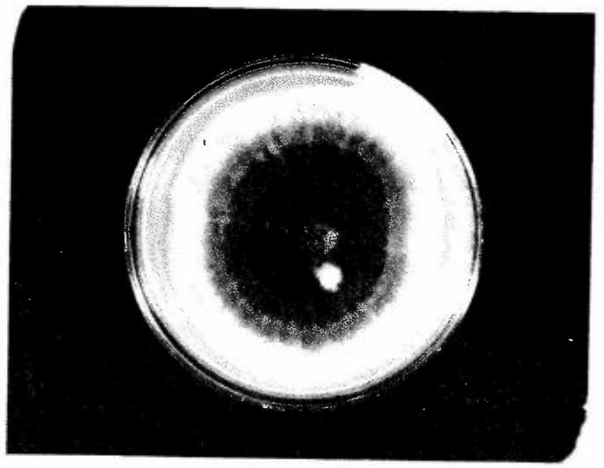

Figure 1. *Alternaria alternata*, highly sporulated with low density of aerial hyphae (MF1).

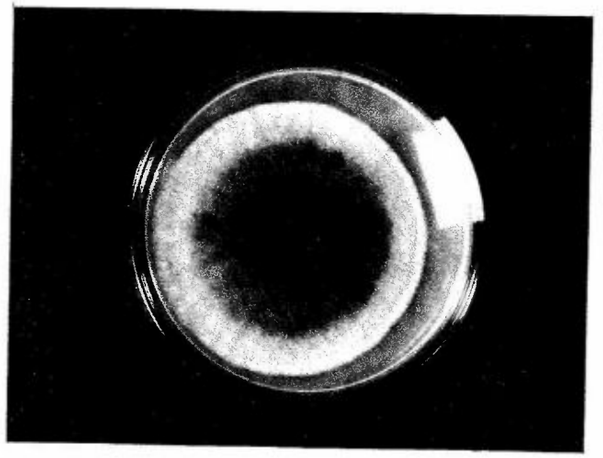

Figure 2. *Alternaria alternata*, less sporulated with higher density of aerial hyphae (MF2).

Table 1. Growth rate and crude extracellular products weight of *Alternaria alternata* grown at 27°C for 14 days on Yeast-extract sucrose medium.

Isolate No.	Dry weight (g)	Dry weight crude extract (g)
1	2.63	0.046
2	3.09	0.030
3	2.63	0.032
4	2.53	0.034
5	2.27	0.026
6	2.45	0.034
7	1.12	0.044
*8	2.11	0.034
9	1.75	0.028
10	2.71	0.064
11	2.57	0.064
*12	2.99	0.052
*13	2.01	0.036
14	2.77	0.074
15	4.21	0.026
16	3.52	0.134
17	3.72	0.182
18	3.03	0.084
19	3.77	0.140
*20	1.76	0.044
21	3.11	0.036
22	3.86	0.044
23	3.99	0.056
24	3.66	0.054
25	3.00	0.045
26	2.76	0.040
27	2.82	0.058

* Morphological form MF1 (see Fig. 1)

varied according to doses. As previously shown by various workers, chicken embryo test is a reliable and sensitive system for testing toxic mold metabolites. Thus, it appears that infected tomato fruit could be contaminated with highly toxic metabolites indistinguishable from those recovered from diseased tissue (Silver and Gilchrist, 1983). These results were confirmed by the acute toxicity test in this study. The toxicity might be due to the substances mentioned by Janardhanan and Hussain (1983) or other unidentified compounds produced by this fungus. Since large amounts of tomato fruit infected with black rot are offered to the consumer particularly between November to January, *Alternaria* contamination may seriously affect public health.

Table 2. Mortality by 27 isolates of *Alternaria alternata* crude extract injected into the aircell of fertile SPF eggs. Eggs were incubated for 21 days in an automatically controlled incubator; mortality is expressed as per cent killed embryos.

Isolate No.	Mortality
1	21.8
2	4.4
3	30.4
4	100.0
5	100.0
6	100.00
7	69.6
8	13.1
9	17.4
10	74.0
11	95.7
12	0.0
13	21.8
14	78.3
15	95.7
16	100.0
17	65.3
18	56.6
19	78.3
20	100.0
21	100.0
22	95.7
23	78.3
24	74.0
25	95.7
26	100.0
27	82.6
NT	0.0
MC	8.7
EA	8.7
PO	4.4

NT = Not treated (control); MC = Medium crude extract;
EA = Ethyl alcohol only; PO = Pore only,
SPF = Specific pathogen free eggs

Table 3. The mortality 24 hour after i.p. injection of the the most potent fungal extracellular extracts in groups of 6 mice.

Groups	Dose mg/kg B.Wt.	Mortality no. over 24 hr	Mortality % over 24 hr
1	10	0	0
2	15	1	16.33
3	20	3	50.00
4	25	4	66.66
5	30	5	83.33
6	35	6	100.00

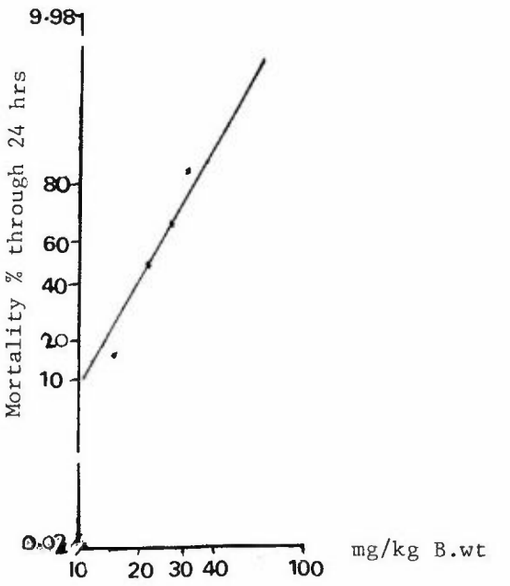

Figure 3. Determination of lethal dose of 50% animals (LD 50) for crude fungal metabolites injected i/p in the mice (mg/kg B.Wt.)

The purification and identification of the active metabolites were made by several workers. Analogous studies seem to be of prime importance of some highly toxic metabolites in various fruit and vegetable products. Public health service should consider the problem of the contaminated fruits and vegetables seriously and simple procedures must be developed to estimate the extent of contamination of fruit and vegetables with various toxins.

REFERENCES

Abott, W. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Enthomology* **18**, 265–267.

Davis, N.D., Diener, U.L., and Eldridge, S.W. 1968. Production of aflatoxin B1 and G1 by *Aspergillus flavus* in semisynthetic medium. *Appl. Microbiol.* **14**, 387.

Ellis, M.B. 1971. *Dematoaceous Hyphomycetes.* The Eastern Press Ltd., London and Reading.

Janardhanan, K.K. and Hussain, A. 1983. Isolation, purification and identification of Tenuazonic acid a phytotoxin produced by *Alternaria alternata* causing leaf blight of *Datura inxia. Mycopathologia* **83**, 135–140.

Litchfield, J.T. and Wilcoxon, F. 1949. A simplified method of evaluation dose-effect experiments. *J. Pharm. Exp. and Therap.* **96**, 99.

Sauer, D.B., Seitz, L.M., Rosemary, B., Harol, E.M., John, L.W., Roy, J.M., and Harry, D.A. 1978. Toxicity of *Alternaria* metabolites found in weathered sorghum grain at harvest. *J. Agric. Food Chem.* **26**, 1380.

Siler, D.J. and Gilchrist, D.G. 1983. Properties of specific toxins produced by *Alternaria alternata* f.sp. *cycopersici* in culture and in Tomato plants. *Physiol. Plant. Pathol.* **23**, 265–274.

Vanek, Z., Hostalek, Z., and Cudlin, J. 1973. *Genetics of Industrial Microorganisms.* Prague, Academic.

Wittkowski, M., Bales, W., Kroenert, W., and Weber, R. 1983. Determination of *Alternaria* toxins in fruit and vegetable products. *Z. Lebensm.-Unter. Forsch.* **177**, 447–453.