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ISOLATION AND CHARACTERIZATION OF ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER PRODUCED BY *ALTERNARIA ALTERNATA* OF GROUNDNUT

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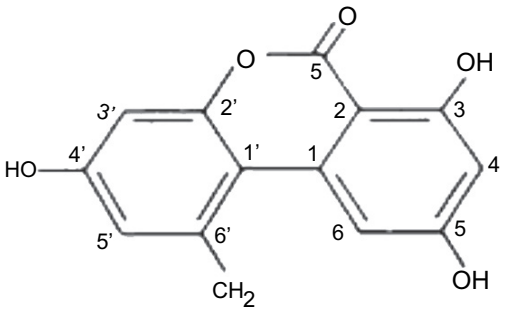
ABSTRACT
To study the patho-physiological role of Alternariol and Alternariol monomethyl ether in disease syndrome as well as their significance role in pathogenesis caused by *Alternaria alternata* (Fr.) keissler to Groundnut has been chemically characterized. The toxins Alternariol and Alternariol monomethyl ether identification was confirmed by Rf values on TLC in different solvent systems, UV and IR spectral analysis using authentic samples. The melting points 350ºC for AOH and 267ºC for AME were determined. It is believed that the present study would contribute not only to elucidate the role of toxins in plant parasite relationship but also to arrive an alternative concepts and strategies for plant protection.

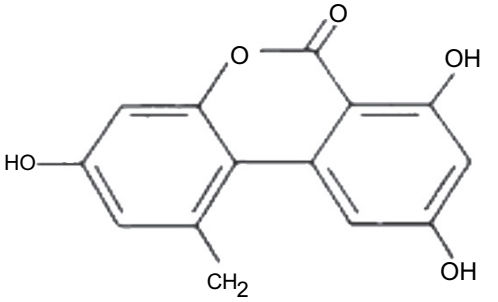
INTRODUCTION

Alternaria alternata (Fr.) Keissler, the causal agent of Alternariosis of Groundnut (*Arachis hypogaea* L.) and a week pathogen, was known to cause a large number of leaf spot and blight disease in plants. Several phytopathogenic species of *Alternaria* have been reported to produce phytotoxic metabolites, which play a significant role in pathogenesis and many of them have been chemically characterized. On Groundnut it has been reported to cause leaf spot and veinal necrosis or Alternariosis leading to considerable damage to the crop in the South Indian States. The symptoms of the disease suggest that the fungus may be producing some powerful toxic metabolite(s) during pathogenesis and preliminary studies indicated the same. The mycotoxins are Alternariol (AOH), Alternariol monomethyl ether and Tenuazonic acid (TA). Natural occurrences of AOH, AME and TA have been reported in various fruits, including Tomatoes, Olives, Mandarins, Melons, Peppers, Apples and Raspberries (Scott, 2001).

Alternariol and Alternariol monomethyl ether were first isolated and identified from the mycelium of *A.tenuis* by Raistrick *et al.,* (1953). The culture filtrates of each of four strains of *A.tenuis* auct., grown on Czapek-Dox medium, have been shown to contain one or more of five metabolic products (Rosett *et al*., 1957). Freeman (1965) reported the isolation of these



Alternariol



Alternariol methylether

compounds from *A.dauci* (Kuhn.). Staratt and White (1968), Seitz *et al.,* (1975), and Sauer *et al.,* (1978) reported the natural

occurrence of the toxins (Alternariol and Alternariol monomethyl ether) in weathered sorghum grain. Schroeder and Cole, (1977) reported Alternariols in discolored pecans.

Alternaria mycotoxins by *Alternaria alternata* isolated from Chinese weathered wheat kernels were first investigated on polished rice and durum wheat grains. These mycotoxins included Alternariol (AOH) and Alternariol monomethyl ether (AME), Altenuene (ALT), Altertoxin I (ATX-I), and Tenuazonic acid (TA) (Li F-Q.Toyazaki and Yoshizawa (2001)).

A limited survey of the natural occurrence of the major *Alternaria* mycotoxins has been carried out on olives (Visconti *et al*., 1986). A significant number of non-specific phytotoxins showing a wide range of disease symptoms and diverse chemical properties have been isolated from culture filtrates of several phytopathogenic *Alternaria* species (William *et al.,* 1987).

AME is cytotoxic and AOH and AME show synergetic effects (Motta and Soares, 2000).

The present study reports both the *in vitro* production of Alternariol and Alternariol monomethyl ether, by this pathogen, its isolation and characterization.

MATERIALS AND METHODS

Isolation and maintenance of the pathogen

Infected leaves of groundnut var. TMV-2 showing typical symptoms of the disease were obtained from the fields of Tirupati and Srikalahasti. Repeated isolations using conventional plant pathological techniques yielded Alternaria alternata. Monospore isolations of the pathogen were obtained and maintained on PDA for regular use. On the basis of morphological and cultural characters the causal organism was identified as *A.alternata* (Fr.) Keissler.

The stock culture was maintained at 10ºC in a refrigerator on PDA slants and successive sub-cultures were made at intervals of two months. Koch's postulates were followed in confirming the pathogenicity. All cultural characters exactly coincided with that isolate of the pathogen obtained from ICRISAT.

Collection of Fungal mycelium and culture filtrate

The cultures were harvested by filtration through a Whatman No.1 filter paper and fungal mat was blotted dry in folds of a filter paper. The cultures filtrates were collected separately. Both the mycelial mat and culture filtrates were analysed for various toxin constituents.

Isolation and characterization of Alternariol and Alternariol monomethyl ether

From mycelium

AOH and AME were isolated according to the method of Raistrick *et al*., (1953). The washed, dried and ground mycelium was extracted with light petroleum to remove fatty materials. It was then redried and reextracted with ether which removed completely the substances giving the purple ferric reaction. Again they were separated from the ether a colourless crystalline solid, which on fractionation from ethanol gave two pure crystalline substances *i.e.,* Alternariol and Alternariol monomethyl ether. Pero and Main (1970) and Schroeder and Cole (1977) used acetone water (7:3 v/v) as the primary extraction solvent of Alternaria cultures or discolored pecans. The filtrate of the acetone extract was evaporated to dryness and then extracted with hot tetrahydrofuran (THF). The THF extractives were dried and Alternariol and Alternariol monomethyl ether were isolated from the crude extract by chromatography on silica gel columns.

Quantitative assays for AOH and AME from different culture filtrates

From cultures grown in different culture media (Czapek-dox, Richard's, leaf extract and malt extract media) AOH and AME were isolated by above mentioned procedures. The isolated compounds were dissolved in 5 mL of ethanol.

1 ml of isolated extract (AOH and AME) was spotted on a TLC plate. The spot was eluted in 2 mL of ethanol after running in chloroform: methanol (90:10) solvent system and read at 250 nm for AOH and at 272 nm for AME in Hitachi spectrophotometer. The identification of AOH and AME was confirmed by UV and IR spectral analysis. AOH content and AME contents were determined by comparison with a standard curve prepared with the authentic sample.

RESULTS

Phytotoxicity of the culture filtrates

The inhibitory effects of culture filtrates on seed germination and radical length and development of necrotic spots on leaves of groundnut and rice were tested (Table 1). It seemed

Figure1: Loss of turgor cut shoot of groundnut in autoclaved media

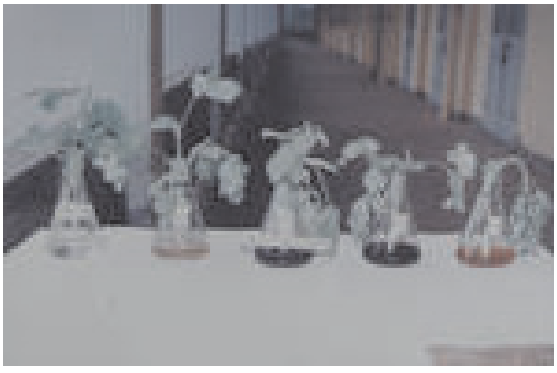


Figure 2: Loss of turgor of cut shoots of groundnut in uninoculated media

Table 1: Effect of different culture filtrates on seed germination of groundnut and rice

Nature of culture filtrate	Groundnut % of germination	% germination over control	Rice % of germination	% germination over control
I. Autoclaved media				
Czapek-dox	63.3	73.6	76.0	80.9
Richard's	00.0	00.0	65.0	69.1
Leaf extract	70.0	81.4	72.0	76.6
Malt extract	60.0	69.8	68.0	72.3
Control (uninoculated media)	86.0	-	94.0	-
II. Uninoculated media				
Czapek-dox	55.0	62.5	72.0	75.8
Richard's	00.0	00.0	68.0	71.6
Leaf extract	55.0	62.5	81.0	85.3
Malt extract	82.0	93.1	74.0	77.9
Control (uninoculated media)	88.0	-	95.0	-

Table 2: Effect of different culture filtrates on root elongation of groundnut and rice

Nature of culture filtrate	Groundnut Root elongation in mm	Rice % of elongation over control	Root elongation in mm	% of elongation over control
I.Autoclaved media				
Czapek-dox	6.4	51.2	17.5	38.7
Richard's	0.0	0.0	1.6	3.5
Leaf extract	8.4	67.2	15.0	33.2
Malt extract	9.2	73.6	37.1	82.0
Control (uninoculated media)	12.5	-	45.2	-
II.Uninoculated media				
Czapek-dox	7.8	60.9	17.5	38.9
Richard's	0.0	0.0	1.2	2.6
Leaf extract	8.4	65.6	22.6	49.8
Malt extract	7.9	61.7	39.4	86.8
Control (uninoculated media)	12.8	-	45.4	-

Figure 3: Loss of turgor cut shoot of rice in autoclaved media

Figure 4: Loss of turgor of cut shoots of rice in uninoculated media

necessary to undertake this study because several dominant colonizers, some of which are pathogenic, have not produced any toxins on microbial test organisms. Reduction in shoot length was also tested in rice (Table 3). The fungus (*A.alternata*) was cultured in Czapek-dox, Richard's, Leaf extract, Malt extract media and culture filtrates from all the media were tested for phytotoxicity and root elongation (Table 2). Phytotoxicity to cut shoots of groundnut and rice was also determined using the same culture filtrates (Table 4). Loss of turgor of cut shoots of groundnut in autoclaved media (Fig. 1) and in uninoculated media (Fig. 2). This is also tested in rice (Fig. 3 and Fig. 4).

Isolation and characterization of Alternariol and Alternariol monomethyl ether

Table 3: Effect of different culture filtrates on shoot elongation of rice

Nature of culture filtrate	Rice Shoot elongation in mm	% of elongation over control
I. Autoclaved media		
Czapek-dox	19.0	49.7
Richard's	11.2	29.3
Leaf extract	19.0	49.7
Malt extract	26.0	68.1
Control (uninoculated media)	38.2	-
II. Uninoculated media		
Czapek-dox	28.8	64.0
Richard's	20.0	44.4
Leaf extract	27.6	61.3
Malt extract	36.0	80.0
Control (uninoculated media)	45.0	-

Figure 5: A. Isolated alternariol (AOH); B. Authentic sample

Figure 6: A. Isolated alternariol monomethyl ether (AME); B. Authentic sample

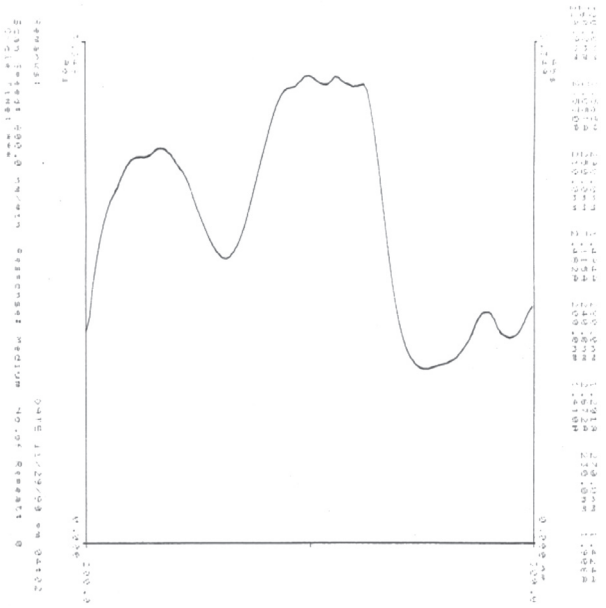


Figure 7: UV spectrum of isolated AOH

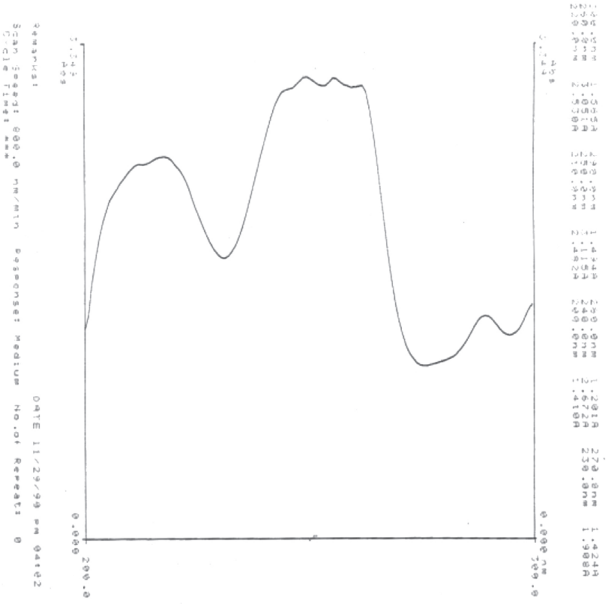


Figure 8: UV spectrum of authentic AOH

From mycelium

Two methods were used for isolation of metabolic products (AOH and AME) from the dried mycelium of *A.alternata*. In the first experiment, two pure crystalline substances were obtained. In the second, the two compounds were separated by TLC.

Identification of AOH

The general properties and colour reaction of first fraction from both the procedure agreed with those of standard Alternariol. The isolated toxin fraction showed similar Rf values as standard AOH in various solvent systems (Table 5). The toxin fraction and AOH reference sample produced purple

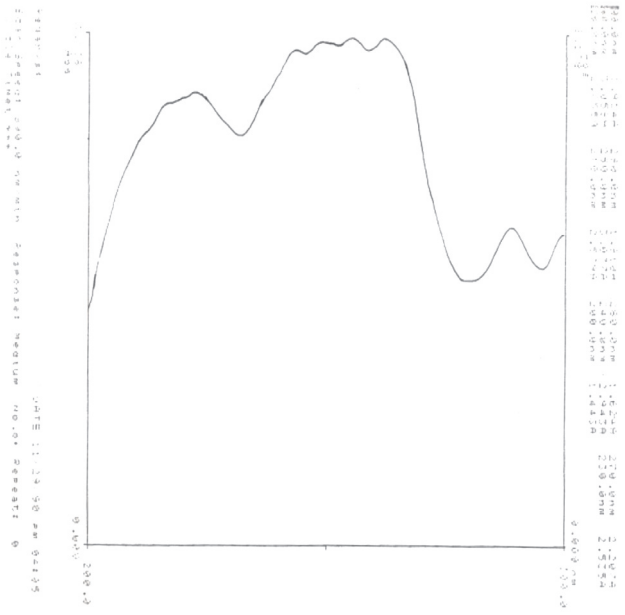


Figure 9: UV spectrum of isolated AME

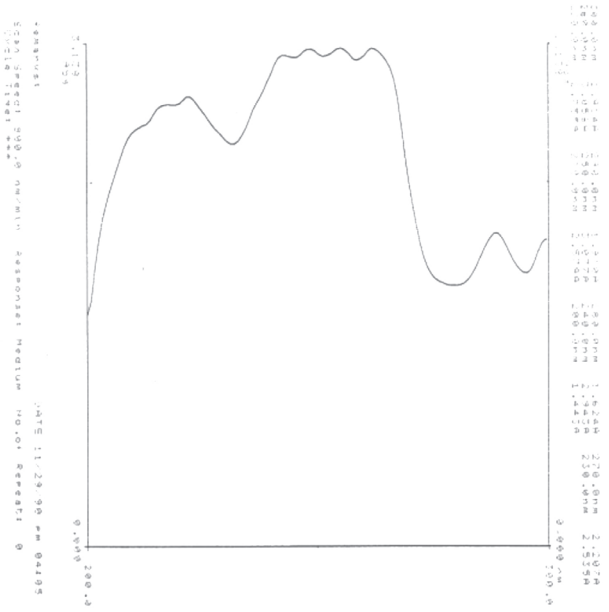


Figure 10: UV spectrum of authentic AME

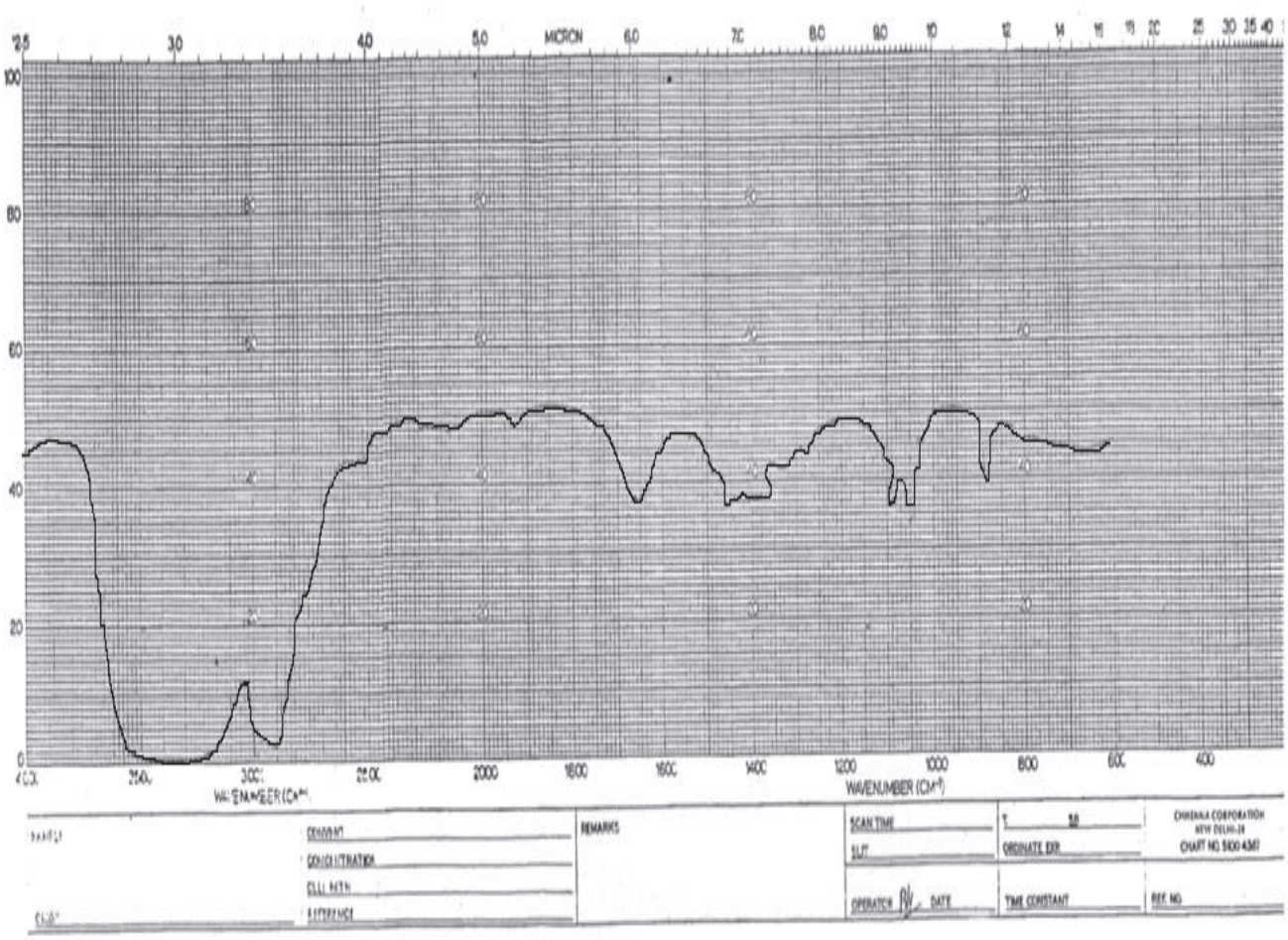


Figure11: I R spectrum of isolated AOH

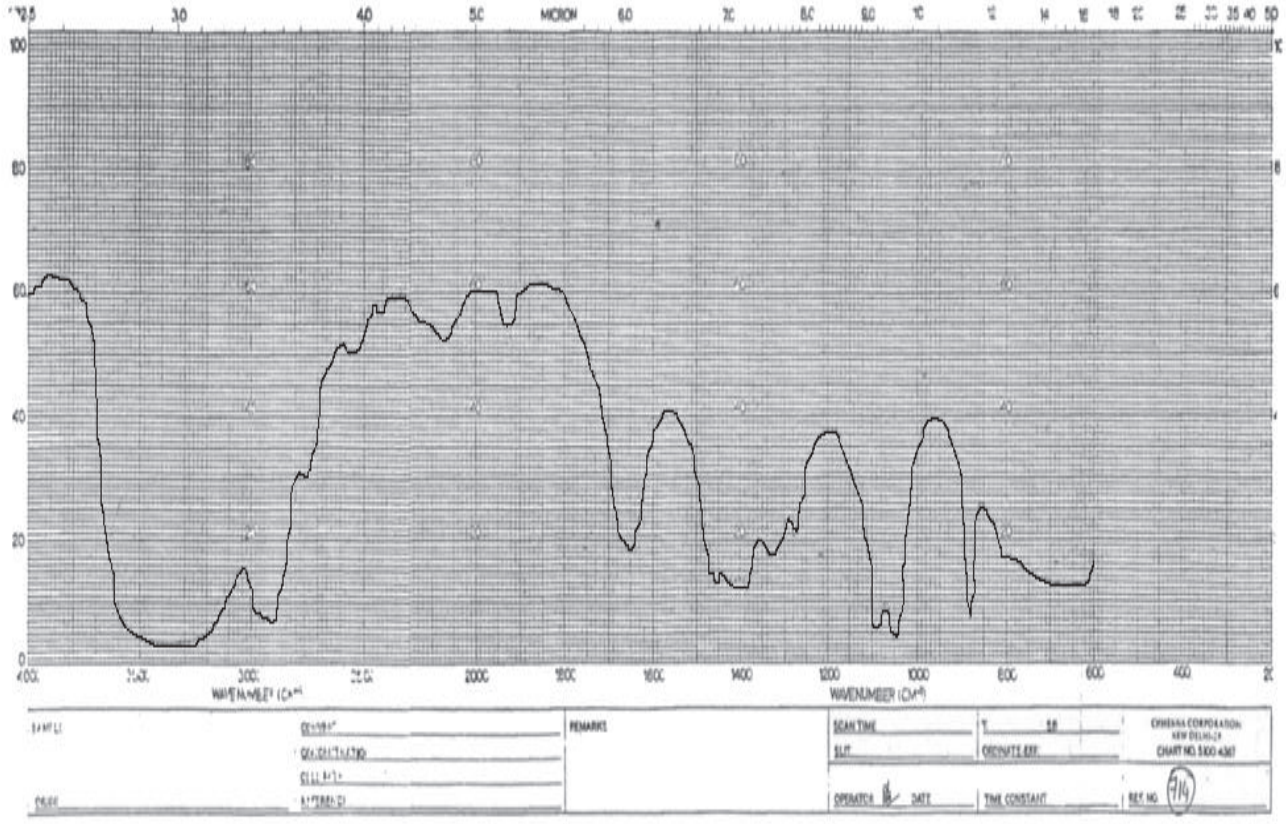


Figure12: I R spectrum of authentic AOH

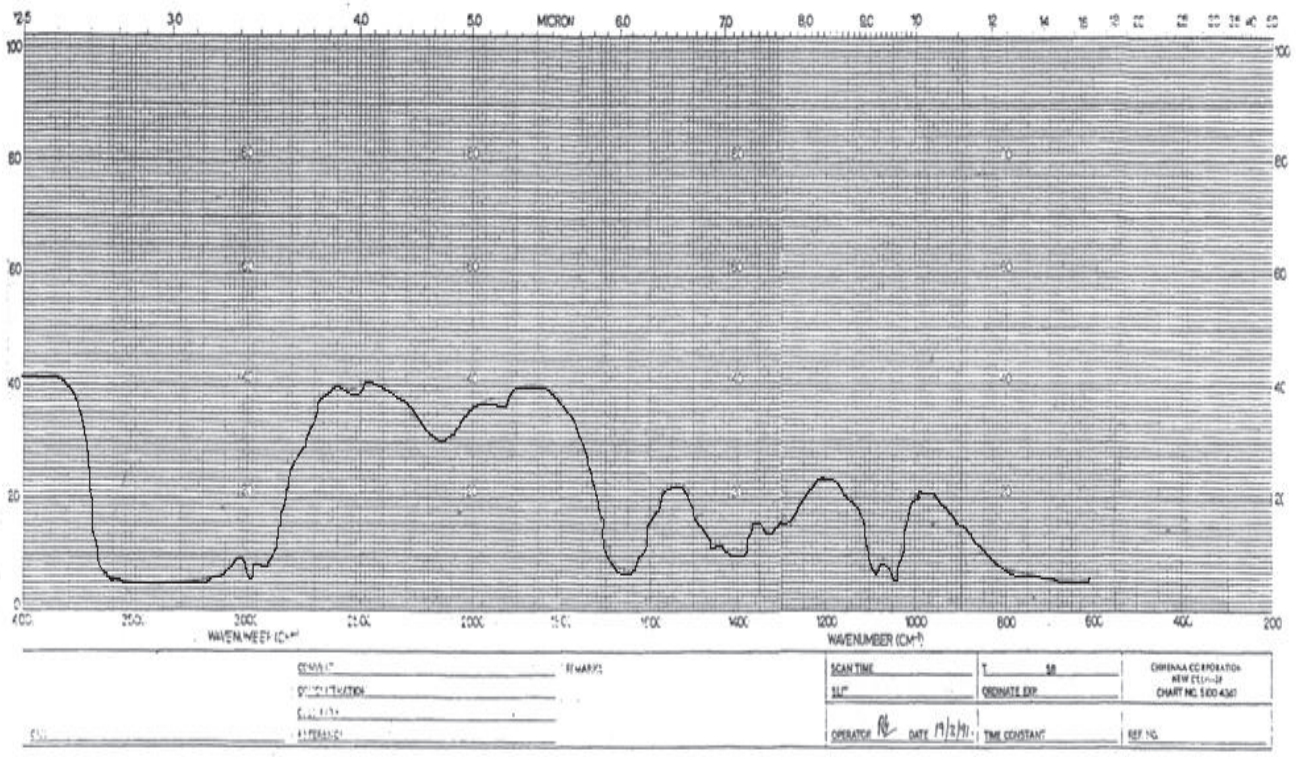


Figure13: I R spectrum of isolated AME

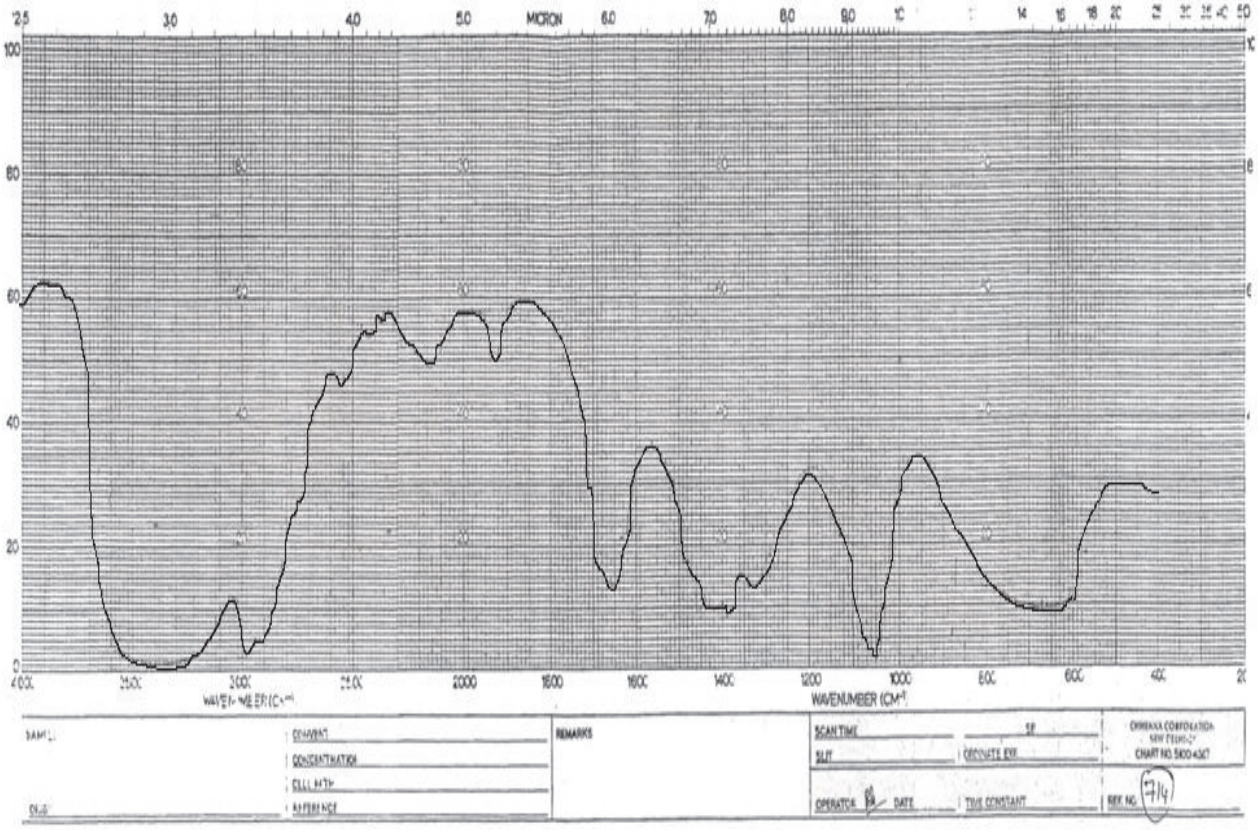


Figure14: I R spectrum of authentic AME

Table 4: Phytotoxicity of different culture filtrates to cut shoots of groundnut and rice

Nature of culture filtrate	Test plant Groundnut	Rice
I.Autoclaved media		
Czapek-dox	+	+
Richard's	+	+
Leaf extract	+	+
Malt extract	+	+
Control (uninoculated media)	-	-
II.Uninoculated media		
Czapek-dox	+	+
Richard's	+	+
Leaf extract	+	+
Malt extract	+	+
Control (uninoculated media)	-	-

coloured spots on TLC plates by ethanolic $FeCl_3$ spray. AOH exposed to iodine vapours appeared as dark brown spot (Fig. 5). The melting point of the fraction was determined as 350ºC.

The following peaks were observed in UV spectrum of an ethanolic solution: 218, 256, 288 nm, maximum extinction was at 256 nm (Fig. 7 and Fig. 8). These absorption peaks were found to be identical with UV spectrum of standard AOH. The IR spectrum determined on a Nujol mull, (RSIC, IIT, Madras), showed absorption bands at 3390, 2980, 1930, 1650, 1090, 1050, 880 cm^{-1}. Comparison of IR spectrum with

Table 5: comparison of Rf value of the toxin isolated from A.alternata with standard Alternariol by TLC

Solvent systems	Rf values Isolated toxin	Standard AOH
Chloroform: Methanol(90:10)	0.69	0.69
Chloroform: Methanol(80:20)	0.83	0.82
Ethylacetate: Benzene(99:1)	0.29	0.29

Table 6: Comparison of Rf value of the toxin isolated from A.alternata with standard Alternariol monomethyl ether by TLC

Solvent systems	Rf values Isolated toxin	Standard AME
Chloroform: Methanol(90:10)	0.73	0.73
Chloroform: Methanol(80:20)	0.87	0.82
Ethylacetate: Benzene(99:1)	0.39	0.38

that of standard AOH revealed striking similarity (Fig. 11 and Fig. 12). Based on these observations the toxin fraction was identified as AOH.

Identification of AME

The general properties and colour reaction of second fraction from both procedures agreed with those of standard AME. This toxin fraction showed similar Rf values as standard AME in various solvent systems (Table 6). The toxin fraction and AME reference sample produced purple coloured spots on TLC plates by ethanolic $FeCl_3$ spray. AME exposed to iodine vapour appeared as dark brown spot on TLC (Fig. 6). The melting point of the substance was determined as 267ºC. The following peaks were observed in UV spectrum of an ethanolic

solution: 223, 263, 287 nm (Fig. 9). These absorption peaks were found to be identical with UV spectrum of standard AME (Fig. 10). The IR spectrum determined on a Nujol mull, (RSIC, IIT, Madras), showed absorption bands at 3390, 2980, 1930, 1650, 1090, 1050, 880 cm^{-1}. Comparison of IR spectrum with that of standard AME revealed striking similarity (Fig. 13 and Fig. 14). Based on these observations the toxin fraction was identified as AME.

Quantitative assays of AOH and AME from different culture filtrates

The amount of AOH and AME were determined in different culture filtrates (Table and Fig). The amount of AOH was more in Czapek-Dox medium-culture filtrate and low in leaf extract medium-culture filtrate. The amount of AME was also more in Czapek-Dox medium-culture filtrate andless in leaf extract medium-culture filtrate.

DISCUSSION

Experimental results indicate that A.alternata causing alternariosis of groundnut produces a heat stable phytotoxic metabolite in vitro. Necrosis and Chlorosis induced by the culture filtrate on leaves, besides the growth inhibition of germinating seeds Fulton *et al.,* (1965) support this conclusion. Studies on the isolation of the phytotoxin reveal that the pathogen produces several metabolites into culture filtrate, as evident from TLC analysis. However, identification of the compound by TLC, besides UV and IR spectral analysis support this conclusion.

Alternariol and Alternariol monomethyl ether were first isolated from the mycelium of A.tenuis by Raistrick *et al*., (1953). Freeman reported the isolation of these compounds from A. dauci (Kuhn). Quantitative assays of Alternariol and Alternariol monomethyl ether, showed more amount of these compounds in Czapek-dox medium when compared to the other media (Richard's, Leaf extract and Malt extract media). *Alternaria* toxins exhibit both acute and chronic effects. The LD_{50} values for Alternariol monomethyl ether, Alternariol, Altenuene, and Altertoxin I in mice is reported as 400, 400, 50 and 200 mg/kg b. w. respectively. Those for Tenuazonic acid are 162 and 115 mg/kg b.w. (i.v.) for male and female mice respectively.

The toxins Alternariol and Alternariol monomethyl ether identification was confirmed by Rf values on TLC in different solvent systems, UV and IR spectral analysis using authentic samples. The melting points 350ºC for AOH and 267ºC for AME were determined.

The aim of the present study was to study the toxins in vitro and in vivo and their role in disease initiation and production. It is believed that the present study would contribute not only to elucidate the role of toxins in plant parasite relationship but also to arrive at alternative concepts and strategies for plant protection.

REFERENCES

Freeman, G. G., 1965. Isolation of Alternariol and Alternariol monomethyl ether from Alternaria dauci (kuhn) groves and skolko. *Phytochemistry*. **5**: 719-725.

Fulton, N. D., Katharina Bollenbacher and Templeton, G. E. 1965. A metabolite from Alternaria tenuis that inhibits chlorophyll production. *Phytopathology*. **55**: 49.

Li, F-Q., Toyazaki, N. and Yoshizawa, T. 2001. Production of *Alternaria* Mycotoxins by *Alternaria alternata* Isolated from Weather-Damaged Wheat. *J. Food Protection®*. **64(4):** 567-571(5).

Motta, S. D. and Soares, L. M. V. 2000. Simultaneous determination of Tenuazonic and Cyclopiazonic acids in tomato products. *Food Chemistry*. **71**: 111-116.

Pero, R. W. and Main, C. E. 1970. Chlorosis of tobacco induced by alternariol monomethyl ether produced by *Alternaria dauci*. *Phytopathology*. **60**: 1570-1573.

Raistrick, H., Stickings, C. E. and Thomas, R. 1953. Studies in the Biochemistry of microorganisms. 90. Alternariol and Alternariol monomethyl ether, metabolic products of *A. tenuis. Biochem. J.* **55**: 421.

Rosett, T., Sankhala, R. H., Stickings, C. E., Taylor, M. E. U. and Thomas, R. 1957. Studies in the Biochemistry of microorganisms 103. Metabolites of *A. tenuis Auct.: Biochem. J.* **67**: 390-400.

Sauer, D. B., Larry, M., Seitz, R., Burroughs, H. E., Mohr, J. L., West, R. J. Milleret and Anthony, H. D. 1978. Toxicity of Alternaria metabolites found in weathered Sorghum grain at harvest. *J. Agric. Food. Chem.* **26**: 1380-1383.

Schroeder, H. W. and Cole, R. J. 1977. Natural occurrence of alternariols in discolored pecans. *J. Agric. Food Chem.* **25**: 204-206.

Scott, P. M. 2001. Analysis of agricultural commodities of foods for Alternaria mycotoxins. *J. AOAC International*. **6:** 1809-1817.

Seitz, L. M., Sauer, D. B., Mohr, H. E. and Burroughs. 1975. weathered grain Sorghum: natural occurrence of alternariols and storability of the grain. *Phytopathology*. **65:** 1259-1263.

Staratt, A. N., and White, G. A.1968. Identification of some metabolites of Alternaria cucumerina (E.and E.) E11. *Phytochemistry*. **7**: 1883-1884.

Visconti, Angelo, Antonio Logrieco and Bottalico, A. 1986. Natural occurrence of Alternaria mycotoxins in olives: Their production and possible transfer into the oil.

William, A. Ayer and Luis, M. Pena-Rodriguez. 1987. Metabolites produced by A.brassicae, the blackspot pathogen of canola. Part I, the phytotoxic components. *J. Natural products*. **50**: 400-407.