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Full Length Research Paper

Identification and characterization of tenuazonic acid as the causative agent of *Alternaria alternata* toxicity towards groundnut

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***Alternaria alternata* (Fr.) Keissler, the causal agent of Alternariosis of groundnut seeds (*Arachis hypogaea* L.) was toxigenic when cultured on various laboratory media. The cell free extract or culture filtrate induced severe chlorosis and necrosis on leaves, inhibition of root and shoot growth of germinating seeds and wilting of seedlings. A phytotoxin was isolated both from cell free extract/culture filtrate and infected tissues, purified and identified as tenuazonic acid, by using thin layer chromatography, ultra violet and infra red spectral analysis. Toxicity was monitored with standard bioassay techniques. The results show patho-physiological significance of tenuazonic acid in disease syndrome.**

Key words: *Alternaria alternata*, groundnut, alternariosis, tenuazonic acid.

INTRODUCTION

Several phytopathogenic species of *Alternaria* have been reported to produce phytotoxic metabolites, many of which have been chemically characterized and play a significant role in pathogenesis. *Alternaria alternata*, an important pathogen of many plants, produces tenuazonic acid (TA) with bioactivity to microbes, plants and animals. TA is one of the main mycotoxin to humans and other organisms (Zhou and Qiang, 2008).

A. alternata (Fr.) Keissler, though a weak pathogen, is known to cause a large number of leaf spot and blight diseases in plants. The phytotoxic metabolites isolated from this fungus have been implicated in the disease syndrome.

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, where the crop is grown intensively as post-rainy season crop (Subramanyam et al., 1981). *A. alternata* species produce several mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME) or 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).

Tenuazonic acid, iso-tenuazonic acid, and their salts exhibit herbicidal activity with broad spectrum, quick killing, and high efficiency. The addition of adjuvants improves the herbicidal activity of these compounds

The symptoms of the disease indicate that the fungus may be producing some powerful toxic metabolite(s) during pathogenesis and preliminary studies indicated the same. The present study, reports both the *in vitro* and *in vivo* production of tenuazonic acid, by this pathogen, its isolation and characterization.

MATERIALS AND METHODS

In vitro production of toxic extract and assay for phytoxicity

A virulent strain of *A. alternata* isolated from infected groundnut leaves was used in the present study. The production of tenuazonic acid was carried out in the medium consisting following chemical

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composition (g/L) (Glucose-40; KH_2PO_4-1,$(NH_4)_2HPO_4$- 2; $MgSo_4$-$7H_20$, 0.5; KCl, 0.5; yeast extract-1, were dissolved in 1000 ml distilled water, and the medium pH was adjused to 5.5. 200 ml medium aliquotes was transferred in 1 L Roux bottles. The fungus was grown for 3 weeks at 29 ± 2°C as stationary culture and filtered through several layers of cheese cloth.

Half litre portion of the culture filtrate was autoclaved at 15 lbs pressure for 10 min, to inactivate any possible enzymatic activity before being used for phytotoxicity testing on the leaves of groundnut by leaf spot assay tests. Growth inhibiting activity of the culture filtrate was assayed on germinating groundnut seeds. One hundred germinating seeds were placed in Petri dishes containing filter papers moistened with culture filtrate and incubated at 30°C; root and shoot elongation was measured after the incubation period of 6 days in order to evaluate the activity. The phytotoxic activity was also assayed on one month old seedlings of groundnut by dipping the cut shoots in 100 ml of culture filtrate in 250 ml Erlenmeyer flasks. Uninoculated medium served as control in all the above experiments.

Isolation of toxin from culture filtrate

The toxin isolation was carried out by the method of Janardhanan and Hussain (1975). The fungal filtrate was collected by filtering through cotton wool. An equal volume of methanol was added to the fungal filtrate to precipitate proteins and kept in a refrigerator for 24h. The precipitate was filtered off and methanol recovered at 40 - 50°C *in vaccuo*. The filtrate was then extracted with ethyl acetate 3 times and the extract concentrated with 40 - 50°C *in vaccuo*. An orange red viscous substance obtained was dissolved in acetone, absorbed on activated charcoal and then eluted with the same solvent. The acetone eluate was concentrated *in vaccuo* for further purification and identification.

Identification of toxin

The concentrated eluate containing the toxic metabolite(s) isolated from the culture filtrate was chromatographed by thin layer chromatography (TLC) on silica gel G using chloroform : methanol (99:1), chloroform : methanol (80:20) and ethyl acetate and benzene (99:1) as solvent systems. Some of the known *Alternaria* toxins namely AOH, AME and TA were used as reference samples in order to identify the compound by comparison with its Rf value. The spots were detected by treatment with iodine vapours or spraying with ethanolic ferric chloride. The tenuazonic acid was confirmed by UV and IR spectral analyses. UV absorbance was measured in Spectrophotometer (Hitach) and IR spectrum was determined. UV and IR spectra of the reference sample taken under identical conditions were used for comparison.

Isolation of the toxin from infected leaves

The method followed was that of Janardhanan and Hussain (1983). Infected leaves (5 gms) showing early and advanced stages of infection were collected from inoculated plants and processed by homogenizing with 100 ml 80% aqueous acetone. The homogenate was filtered through Whatman No.1 filter paper and concentrated at low temperature. The aqueous extract thus obtained was acidified with dilute HCL to pH 2 and extracted thrice with 10 ml 5% $NaHCO_3$. The aqueous layer was separated, pH adjusted to 2 with dilute HCl and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and the solvent evaporated at low temperature *in vaccuo* and the residue was chromatographed by TLC on silica gel G using chloroform: methanol (90:10) solvent system using a standard sample of tenuazonic acid as reference. The spots were developed by exposing the TLC plates to iodine vapours/ethanolic ferric chloride. Samples of leaves from uninoculated plants and processed in a similar manner were used as control.

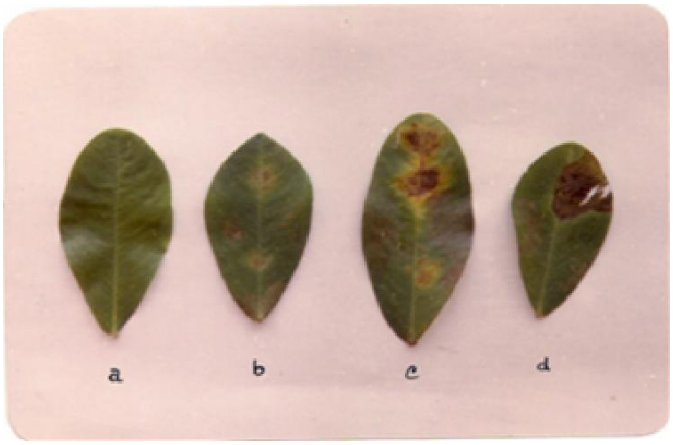

Figure 1a. Effect of phytotoxin of crude culture filtrate on groundnut leaves applying 50 ul at 12, 24 and 36 h of incubation. (a) control (b) 12 h (c) 24 h (d) 36 h of incubation.

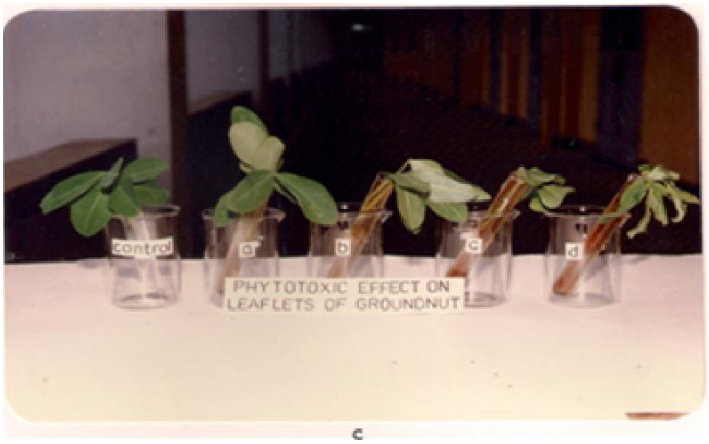

Figure 1b. Effect of phytotoxin on groundnut leaflets at different dilutions. Control, (a) 4:1 (b) 1:1 (c) 1:4 (d) phytotoxin (water/phytotoxin)

RESULTS

Phytotoxic activity of culture filtrates

Culture filtrate applied on leaves of groundnut produced chlorotic spots after 24 h of treatment, which enlarged and turned necrotic after 48 h. The necrotic spots were surrounded by chlorotic margin at later stages. The toxic symptoms elaborated were almost similar to natural infection. Leaflets of groundnut treated with culture filtrate exhibited irreversible wilting and dehydration within 24 h (Figure 1)

Figure 2a. Isolation of toxin from culture treating with iodine vapours, B. Authentic sample of TA, B. Isolated toxic metabolite from culture filtrate

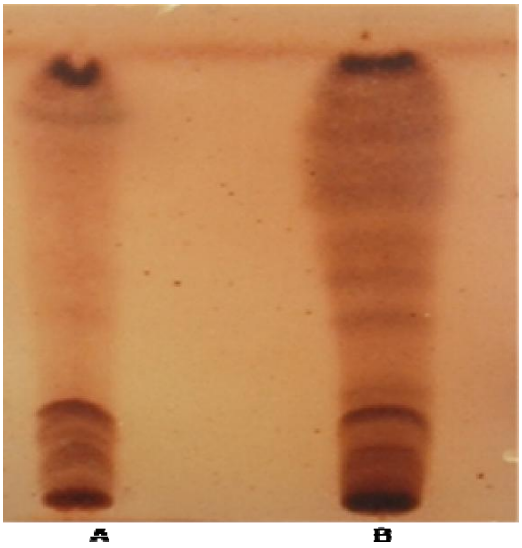

Figure 2b. Isolation of toxin from filtrate treating with Iodine vapours, A. Authentic sample of TA, B. Isolated toxic metabolite from culture filtrate

Isolation of tenuazonic acid from the culture filtrate

The analysis of the crude toxin by TLC, using chloroform: methanol (90:10), showed 6 spots on exposing the TLC plates to iodine vapours. Toxicity test of the spots eluted from unexposed TLC plates showed that the spot having Rf value 0.40 was the major phytotoxic principle. Other spots were either non toxic or weakly toxic (Figures 2a and b).

Identification of tenuazonic acid (TA)

Tenuazonic acid was identified based on the comparison of the Rf value of the toxic metabolite isolate from the culture filtrate with that of AOH, AME and TA showed agreement with that of tenuazonic acid. The toxin and the reference sample of tenuazonic acid produced brilliant orange colour spots on TLC plates by ethanolic $FeCl_3$ spray. Rf values of the toxic metabolite as compared to tenuazonic acid on TLC using various solvent systems was presented in Table 1.

The compound showed UV absorption peaks at 226 and 288 nm in spectrophotometry (Figure 3a). These were found to be identical with UV spectra of standard tenuazonic acid (Figure 3b). IR spectral analysis of tenuazonic acid isolated from culture filtrate showed absorption peaks at 3300, 2990, 2140, 1930, 1650, 1460, 1390, 1275, 1090, 1050 and 800 cm^{-1} (Figure 4a). Comparison of IR spectrum with that of standard TA revealed striking similarity (Figure 4b). Strong absorption was noticed in the region of 2990, 1650 cm^{-1}. Based on these observations the phytotoxin was identified as tenuazonic acid.

Detection of tenuazonic acid in infected plants

Tenuazonic acid in infected plants dectected by TLC analysis of toxin isolated from infected leaves indicated the presence of ethanolic $FeCl_3$ positive orange colored spot with identical Rf value and similar UV (Figure 3a) and IR (Figures 4a and b) spectral peaks as of standard tenuazonic acid. The spots were developed by exposing the plates to iodine vapours.

DISCUSSION

Experimental results indicate that *A. alternata* culture filtrate induced Alternariosis on leaves, as well as growth inhibition of germinating seeds. The observations of Fulton et al. (1965) support these conclusions, demonstrating that the pathogen produces several metabolites into culture filtrate, one of which was tenuazonic acid. Identification of the compound was done by TLC, besides UV and IR spectral analysis support this conclusion.

Tenuazonic acid was first isolated by Rosett et al. (1957), from *Alternaria tenuis* and its structure was established by Stickings (1959) as 3-acetyl-5-sec butyl-4-hydroxy-3pyrolin-one. Later, it was isolated from *Alternaria longiceps, Alternaria.kikuchiana, Alternaria mali* and *Pyricularia oryzae* as a phytotoxin and from

Table 1. Comparison of Rf value of the toxin isolated from *A. alternata* with tenuazonic acid by TLC.

Solvent	Rf value	
	Isolated phytotoxin	Standard tenuazonic acid
Chloroform : Methanol (90:10)	0.40	0.40
Chloroform : Methanol (80:10)	0.60	0.63
Ethyl acetate : Benzene (99:1)	0.21	0.21

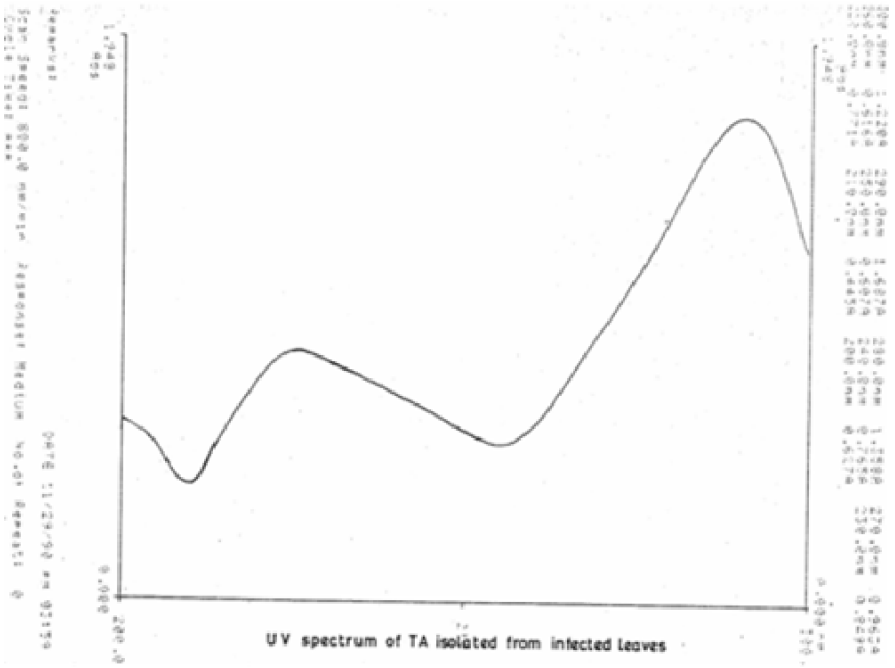

Figure 3a. UV absorption peaks of authentic TA at 226 nm.

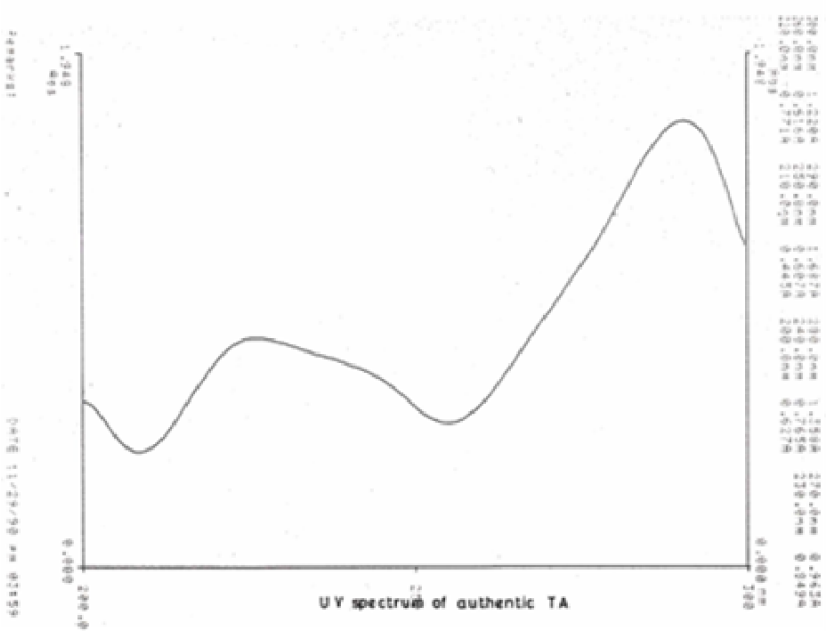

Figure 3b. UV absorption peaks of TA isolated from infected leaves at 226 nm.

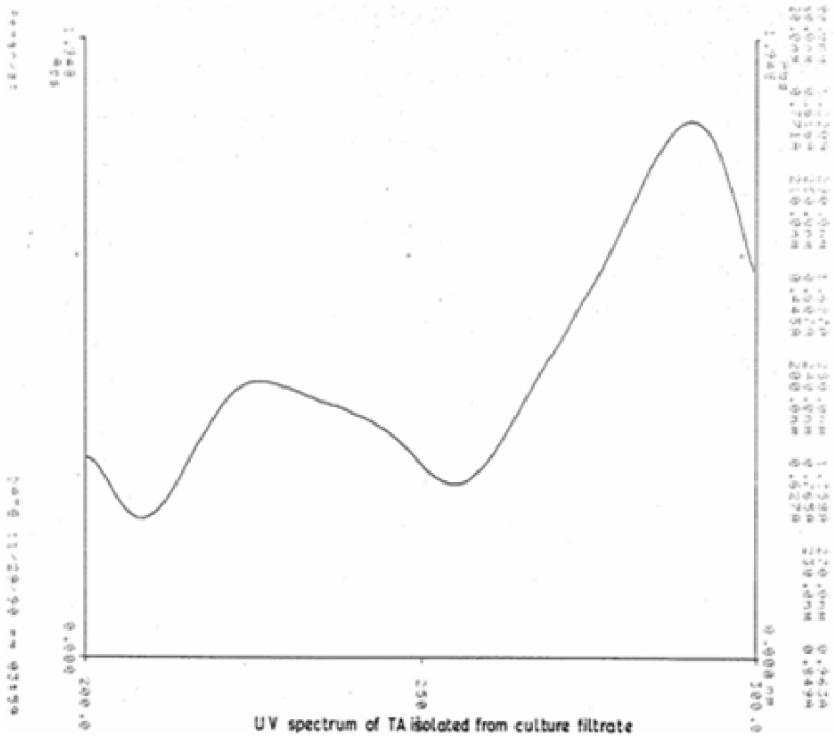

Figure 3c. UV absorption peaks of TA isolated from culture filtrate at 226 nm

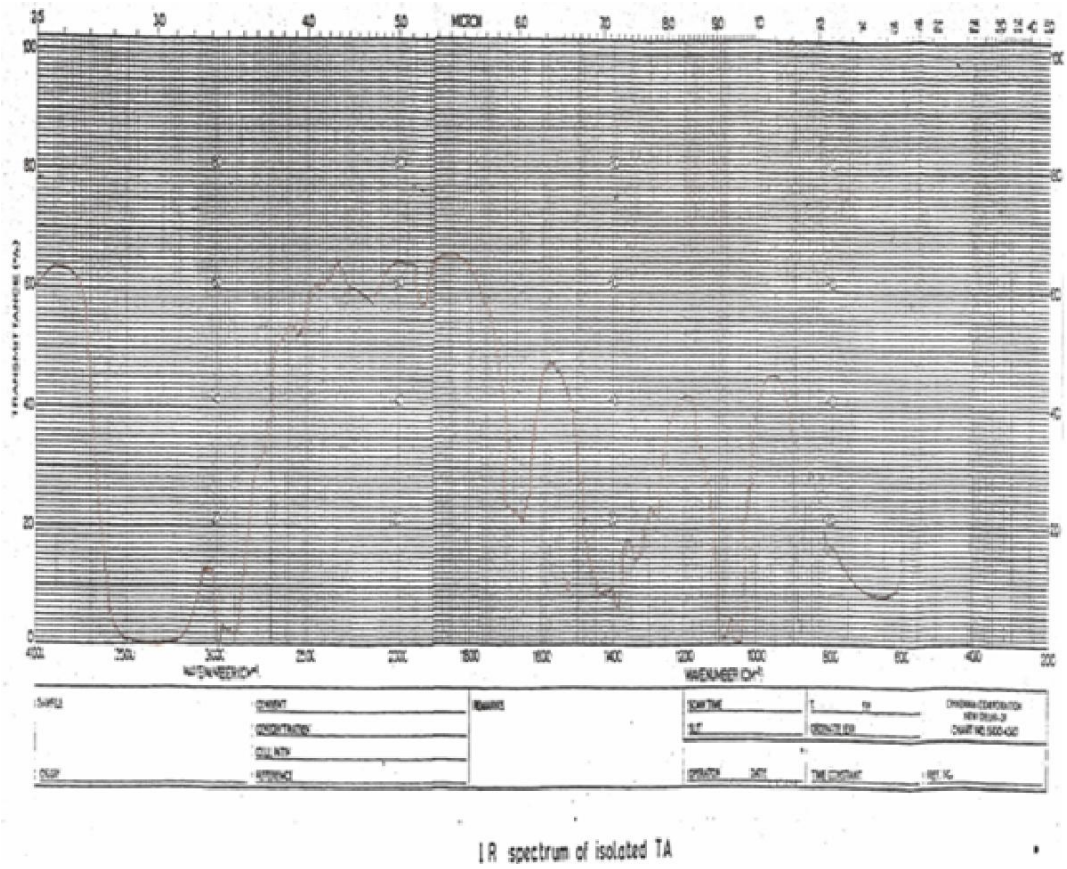

Figure 4a. IR Spectrum of Tenuazonic acid from isolated culture filtrate

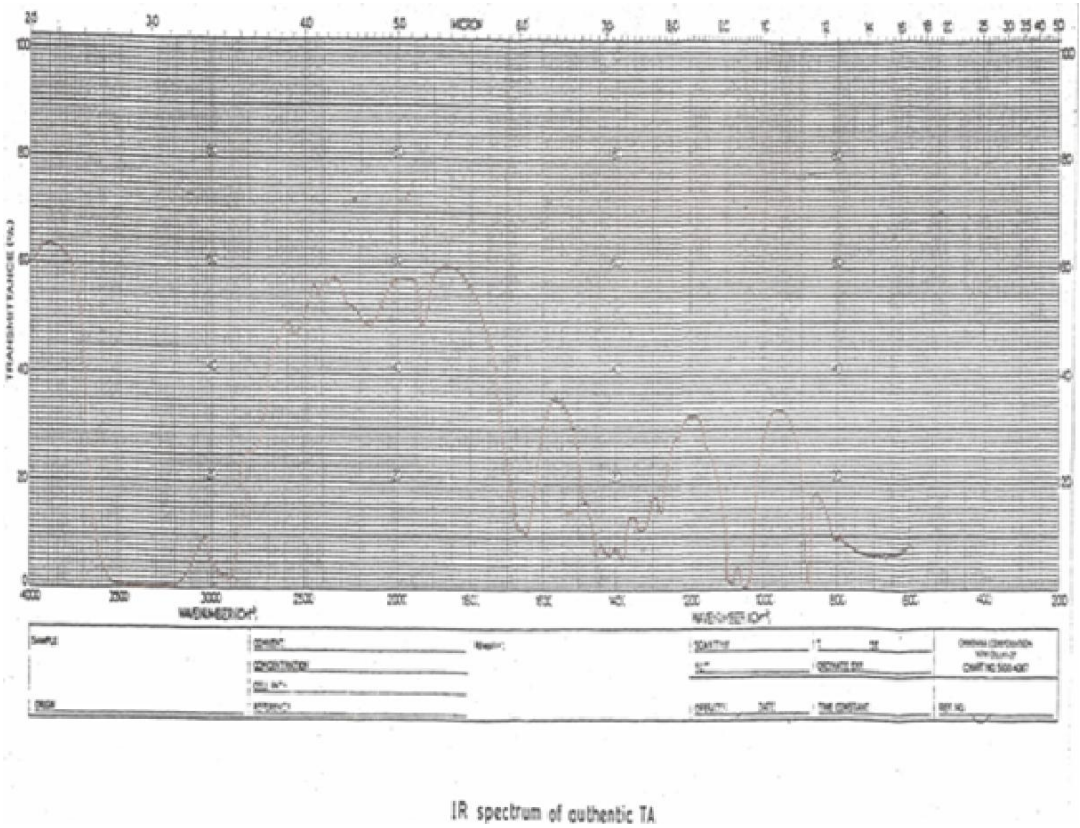

Figure 4b Figure 4a. IR Spectrum of authentic Tenuazonic acid

Alternaria alternata, *Alternaria.tenuissima* and *phoma sorghina* as a mycotoxin. Davis et al. (1976) and Kinoshita et al. (1972) screened 185 strains of *Alternaria* species and found the wide-spread occurrence of tenuazonic acid (TA). Thus, it was thought to be a characteristic metabolite of this genus and not a pathogen-specific toxin. Several other workers also observed the production of TA from *Alternaria* species isolated from different host plants (Chulze et al*.,* 1995; Hasan, 1996; Ozcelik and Ozcelik, 1997. Based on the present study finally we conclude that isolation of tenuazonic acid from infected, groundnut leaves indicate that the toxin was produced by the pathogen during pathogenesis. Thus tenuazonic acid can be considered as a vivotoxin in the case of Alternariosis of groundnut. The results also support the findings of Mikami et al. (1971) on leaf blight of *Datura innoxia*.

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