TOXICITY OF CRUDE AND PURIFIED MYCOTOXINS FROM ALTERNARIA SP - C0067/A0087

S. Panigrahi, Livestock Section, Natural Resources Institute

SUMMARY

The chick embryo, brine shrimp and moong seed germination bioassays were used to screen numerous solvent extracts of rice substrate-based Alternaria sp culture, with a view to locating the toxic fractions, determine LC50 or LD50 values, and determining any interactions between the active extracts. A feeding trial was also carried out with broiler chicks to determine the effects of graded levels of Tenuazonic Acid (TA) on growth and health parameters.

TA was found to be toxic in all tests, LC50 being around 75 µg/ml in the brine shrimp bioassay, 1.4 mg/ml in moong seed germination bioassay and about 150 mg per egg in the chick embryo bioassay. The LC50 of Alternariol, Altertoxin I and Altenuene for brine shrimp larvae were around 100, 200 and 375 µg/mg respectively. TA produced severe haemorrhages in chicks and although growth rates in TA containing diet groups were not significantly lower than control, they were numerically lower; it is likely that if a longer feeding period had been allowed growth rates would have been reduced significantly.

Three other non-TA containing extracts were particularly toxic to brine shrimp larvae and inhibited moong seed germination with two of these extracts also being toxic to chick embryos. Results of TLC revealed that the compounds responsible for these toxicities could have been Alternariol and Altenuene.

Although Alternariol monomethyl ether was found not to be toxic in the brine shrimp and chick embryo bioassays, these tests were unsatisfactory due to the poor solubility of the compound in ethanol, chloroform and water.

INTRODUCTION

Since Alternaria species produce a number of metabolites which may be toxic to one or several livestock, several bioassays involving different species are required to screen extracts from a culture of Alternaria to determine toxicity. The range of bioassays recommended for use in a routine screening procedure (Panigrahi, S. 1993) include: (a) the brine shrimp bioassay, (b) a bacterial or yeast bioassay, (c) chick embryo bioassay, (d) a plant bioassay, and (e) an insect bioassay. The purpose of this study was to use a combination of brine shrimp, chick embryo and moong seed germination bioassays to locate toxic compounds in a range of TLC fractions provided by the Food Safety Section. In addition, a three week feeding trial was designed to study the effect of dietary Tenuazonic Acid (TA) on chicks.

MATERIALS AND METHODS

Test extracts and compounds: The following samples were provided (see Figures 1 and 2). All extracts, except where concentrations are given, were dissolved in 5 mls of the solvent appropriate for bioassays:

Code	Description of Test Material
F	chloroform
A	ethanol (absolute alcohol)
XC	4.26 mg Alternariol (AOH) in chloroform
YC	5.026 mg Altertoxin I (ATX) in chloroform
ZC	4.05 mg Altenuene (ALT) in chloroform
CC	- impure TA in chloroform
CA	- impure TA in ethanol
DC	- 56.4 mg TA/ml in chloroform
DA	- 56.4 mg TA/ml in ethanol
EC	20 mg AME/ml in chloroform
EA	20 mg AME/ml in ethanol
AA	4.45 mg AME/ml in acetone
A3	impure AME (large amount) in chloroform (soot type particles present)
A4	impure AME (large amount) in ethanol (soot type particles present)
A5	impure AME (small amount) in chloroform (soot type particles present)
A6	impure AME (small amount) in ethanol (soot type particles present)
A7	impure AME (small amount) + Altenuene in chloroform (soot type particles present)
A8	impure AME (small amount) + Altenuene in ethanol (soot type particles present)
A9	impure AME (small amount) + Alternariol in chloroform (soot type particles present)
	impure AME (small amount) + AOH in ethanol (soot type particles present)
	impure AME/AOH + pigment in chloroform (clear solution)
A12	impure AME/AOH + pigment in ethanol (clear solution)
A13	- Altenuene in chloroform (pink solution)
A14	- Altenuene in ethanol (pink solution)
A15	- impure AME/AOH in chloroform (golden solution)
A16	- impure AME/AOH in ethanol (golden solution)

Chloroform solvent was considered the appropriate solvent to use for the brine shrimp and moong seed germination bioassays, whilst ethanol was selected for the chick embryo bioassay.

Moong seed germination.
Quantities ranging between 100 and 500 µl of test compounds in chloroform were placed in 100 ml glass beakers. After 2 hours at room temperature, all the chloroform had

evaporated. 2 mls of distilled water were then added to each beaker and left standing for 15 minutes with periodic mild agitation. Dried moong seeds (*Phaseolus radiatus*) were selected for similarity of size and colour, rejecting abnormal seeds. 20 seeds were placed in each beaker (each treatment in duplicate) and agitated by shaking for a 10 seconds. The seeds were placed in the dark, in a Gallenkamp incubator at 30°C.

After 20 hours seeds were placed on petri dishes and germination noted. The beakers were then filled two-thirds with Homebase Multipurpose compost after the latter had been sterilised by placing in an oven at 105°C for 2 hours. The germinated seeds were planted on the compost surface and covered with 1 cm of compost. The beakers were kept near a window (natural day length) at room temperature until both leaves had opened in 80% of cases. (This took between 6 or 7 days). During this time the compost was kept just moist. The two-leaf plants were then examined for radicle length (primary root), hypocotyl length, pedicle length (defined as length between cotyledon and leaf node), and leaf length. The proportion of stunted plants and plants with numerous primary roots were also noted.

Brine shrimp bioassay (Artemia salina)
Brine shrimp medium (BSM) was prepared using the following per litre of distilled water: sodium chloride 30 g; calcium chloride dihydrate 0.3 g; magnesium chloride hexahydrate 1.5 g; magnesium sulphate hexahydrate 0.5 g; potassium chloride 0.8 g; magnesium bromide hexahydrate 0.1 g; glycine 6 g. The BSM was autoclaved at 121°C for 15 minutes.

A conical flask with BSM was placed in a water bath, set at 30°C. Brine shrimp ova were sprinkled into the flask, which was shaken automatically. After 15 hours, hatched larvae were poured in the outer compartment of a brine shrimp hatcher. The lid was replaced and hatcher illuminated with a 8 watt fluorescent lamp. After 4 hours clean larvae were collected in a beaker. This was diluted with BSM to achieve a concentration of 45-80 larvae per 375 µl.

For each test material, antibiotic assay discs were mounted on dissection pins supported in a plasticine base. The discs were inoculated with up to 100 µl of test compounds dissolved in chloroform. After the solvent had evaporated (facilitated by placing pins in an oven at 40°C for 10 minutes) the discs were placed in the wells of a Falcon 3047 multiwell, low-evaporation lid, tissue culture plates. 375 µl of BSM containing the larvae were placed in each well. Each test was replicated 4 times, and solvent controls were included.

Chick embryo development
The method involves administration of extracts of commodities, dissolved in ethanol via a hole in the shell into the air sac. After incubation for 22 days death and abnormalities in chicks were noted.

Chick feeding trial with TA
The effects of feeding broiler chicks on a diet contaminated with (mg/kg) 0, 3.75, 7.5 and 15.0 Tenuazonic acid (TA), isolated from cultured Alternaria sp was investigated.

A commercial 225 g crude protein/kg chick starter feed (H.E. Chicken Starter Crumbs) containing a coccidiostat (Robenidine) was obtained from BOCM-Pauls Ltd. The feed was analysed for the presence of the following mycotoxins: aflatoxins, fumonisin, deoxynivalinel, etc (details to be filled in by Food Safety Section). Batches of feed found to contain any of these mycotoxins were rejected until a clean batch found. The toxin, which was stored as a copper salt was dissolved in chloroform and then mixed with the feed on a weekly basis, to reduce the risk of isomeric transformation or degradation. Thus, the toxic feed were no more than 7 days old before they were consumed by chicks. In each case, the quantity of chloroform used was in the ratio of 250 mls/23 kg of feed. The control diet was treated with chloroform only. Mixing was done over 10 minutes, the chloroform was allowed to evaporate before feeding to chicks.

Each of the experimental diets was fed to 32, 1-day-old, Ross 1-broiler cockerels for 19 days. The birds were housed as groups of 8 in wire cages., i.e., 4 replicates per treatment. The temperature of the environment-controlled room was set at 33°C initially but reduced by 2°C every 6 days; relative humidity was maintained at 50%. The food intake and weight gain of chicks were recorded. Water intake was recorded during week 2, and the behaviour of the chicks was monitored daily. After 19 days the birds were starved for 20 hours (water provided) before being sacrificed. Three of the four cages of chicks per treatment were selected at random and a post-mortem examination carried out in which particular attention was given to occurrence of the following symptoms: (a) appearance of haemorrhages under the skin and musculature (breast and thigh) - scored on a 0-4 order of severity; (b) incidence and type of haemorrhages on liver; pale coloured liver (icteric). The weights of liver, pancreas and spleen were also recorded. The gizzard was opened from the proventriculus end and internal appearance noted. Gizzard erosion was scored on a 0-4 scale of severity. Growth performance data were treated to analysis of variance using the ANOVA programme of SPSS (1988), whilst organ weights were examined by analysis of covariance.

RESULTS AND DISCUSSION

Brine shrimp bioassay
The results are summarised in Tables 1-6. Tests with brine shrimp bioassay using the disc inoculation method showed Tenuazonic Acid-containing fractions to be highly toxic to brine shrimps, the LC50 concentration being between 50 and 100 µg/ml. Three other non-TA fractions proved toxic to brine shrimps: A7, A13 and A15, although the precise toxicity in terms to LC50 values could not be ascertained because the purity of the compounds were not determined;

dose-response curves were however obtained for these fractions.

In decreasing order of the toxicity, pure Alternariol, Altertoxin I and Altenuene killed brine shrimp larvae, the LC50 being approximately 100, 200 and 375 µg/ml respectively. Thus, the toxicity of Alternariol appears only slightly lower than that of TA to the larvae.

Moong seed germination bioassay
The results are summarised in Tables 7-11. The results show Tenuazonic Acid containing fractions to be toxic, the minimum effective dose for inhibition of germination being 141 mg/ml, with an ID50 (inhibition dose 50) of around 1.4 mg/ml. Radicle and hypocotyl development were also affected, although expressing the toxicity of compounds reliably on the basis of these parameters is not possible. In decreasing order of toxicity, fractions A15, A7 and A13 also reduced seed germination.

Low level activity was detected with extract AA (AME) in Experiment 4 (Table 10) and Experiment 5 (Table 11) although this was not observed in Experiment 3 (Table 9). Low level inhibition of germination was also detected with extracts A5, A9 and A11 (Table 11), although dose-response relationships were not always apparent. This leaves open the possibility that in these latter cases, activity could at least partly be attributable to the crude nature of the extracts rather than purely to the effects of the toxin. Further studies are required with pure extracts to ascertain the nature and severity of the biological activity of the compounds from Alternaria sp.

In the cases of A15 (AOH) and DC (TA) there was a qualitative difference between the effects of the compounds in that A15 produced an abnormally high proportion of seeds that did not absorb any water (marked VHU in the Tables 10 and 11), whereas with DC all seeds absorbed some water and therefore inhibition of germination was caused by a different mechanism.

A general point concerning the moong seed bioassay is that development to the two leaf stage was variable, producing a high proportion of stunted plants and plants with numerous root branches under the conditions used in this study. The data obtained was therefore not consistent with the results of germination test after overnight soaking. It is therefore concluded, that Phase 2 (plant development) is unsuitable for detecting low level toxicity and emphasis should be placed on greater replication of Phase 1 treatments. For highly active compounds, such as TA, Phase 2 proved to be highly suitable bioassay (Table 7).

Chick embryo bioassay
The results are summarised in Tables 12-14. Results of

chick embryo bioassay also showed Tenuazonic Acid to be toxic, with LD50 appearing to be around 150 mg per egg. The only other fraction found to be toxic was A16. Overall, chick embryo bioassay did not prove a successful method because of its high cost in terms of time and finance. Only 3 experiments were possible with a 90 egg incubator over a 4-month period: this proved inadequate for detecting toxic fractions and then determining dose-response relationships.

Although AME proved non-toxic in brine shrimp and chick embryo bioassays, this might have been due to AME's very low solubility in chloroform, ethanol and acetone and water, the media used in the bioassays. It is therefore possible that an inaccurate picture of its toxicity was obtained - the tests may be considered unsatisfactory.

Results of the thin layer chromatography indicated A7 to contain AME and Altenuene, A13 to contain large amount of Altenuene, and A15 to contain AME and a large amount of Alternariol. Since pure AME was found to be non-toxic to shrimps, the results indicate that Altenuene and Alternariol could have been the compounds responsible for toxicity.

Chick feeding trial.
The growth parameters and postmortem observations are summarised in Tables 15 and 16 respectively. Whilst weight gains of chicks fed TA after 19 days were not significantly different from controls, (P=0.21), the former chicks were in general lighter. Interestingly, the variation in weight gain as indicated by the standard deviations increased with the quantity of toxin in the diet. Such an effect is not uncommon where a low level of toxicity is observed in feeding trials with day old chicks.

On necropsy, carcases from the TA groups showed haemorrhagic disease on liver characterised by petechia, ecchymoses and subcapsular haematoma. Increased petechial haemorrhages were also apparent on breast and thigh musculature. Spleen weights and appearance were unaffected, but liver weights increased and with some becoming icteric. There was also an increased incidence of gizzard erosion. These results are in accordance with previous reports of the toxicity of TA to broiler chicks (Forgacs et al., 1962), (Forgacs and Carll, 1955) and (Giambrone et al 1978). However, the latter workers showed that Tenuazonic acid depressed weight gain significantly in three week old broiler chicks at 10 mg/kg of feed.

The results of these bioassays and chick feeding trial indicate that at least 4 of Alternaria sp metabolites are toxic. Further studies are required to determine the combined effects of Alternariol, Altenuene, Tenuazonic Acid, Altertoxin I, and possibly other Alternaria toxins, to fully understand the hazards of these fungal toxins to non-ruminant livestock.

REFERENCES

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Figure 1. Tenuazonic Acid (TA) extraction summary

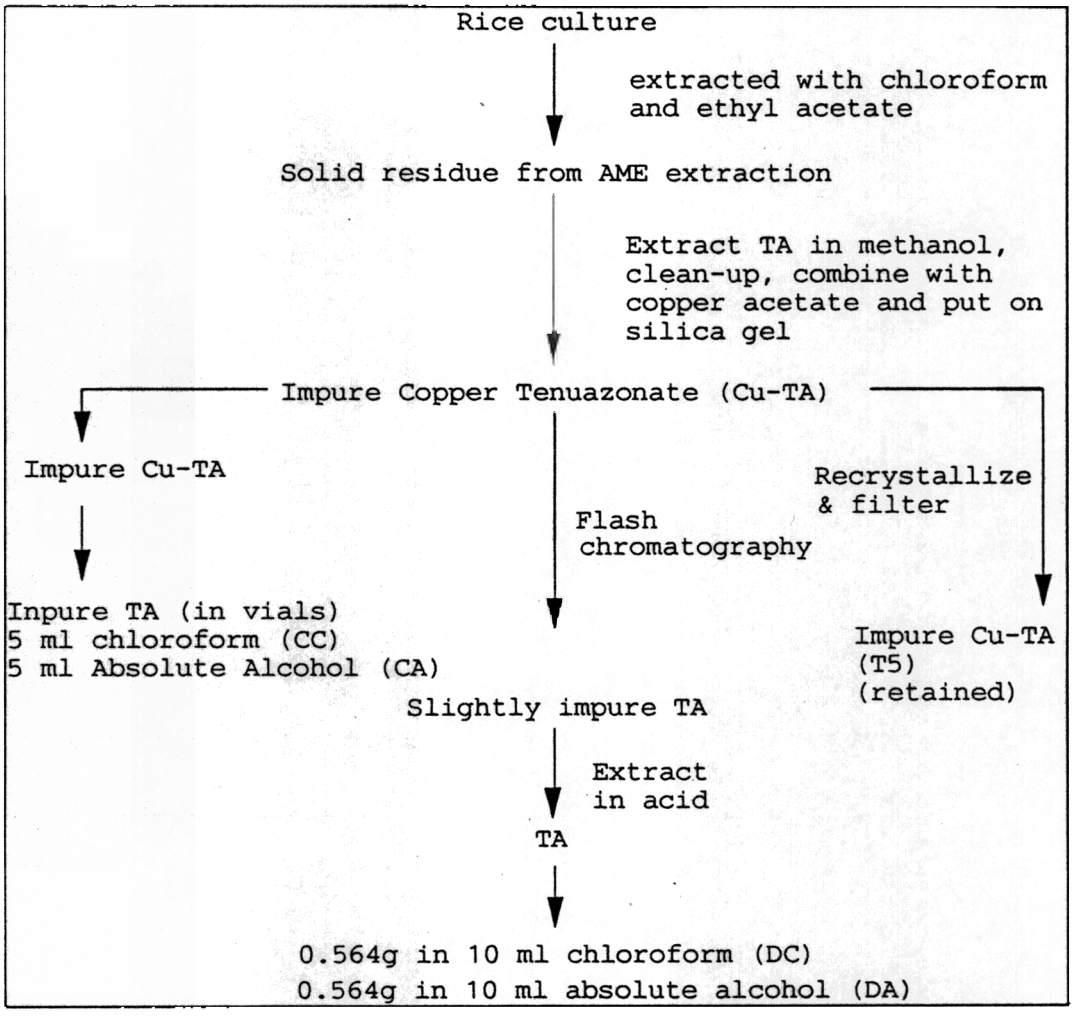

Figure 2. Alternariol mono methyl ether extraction summary

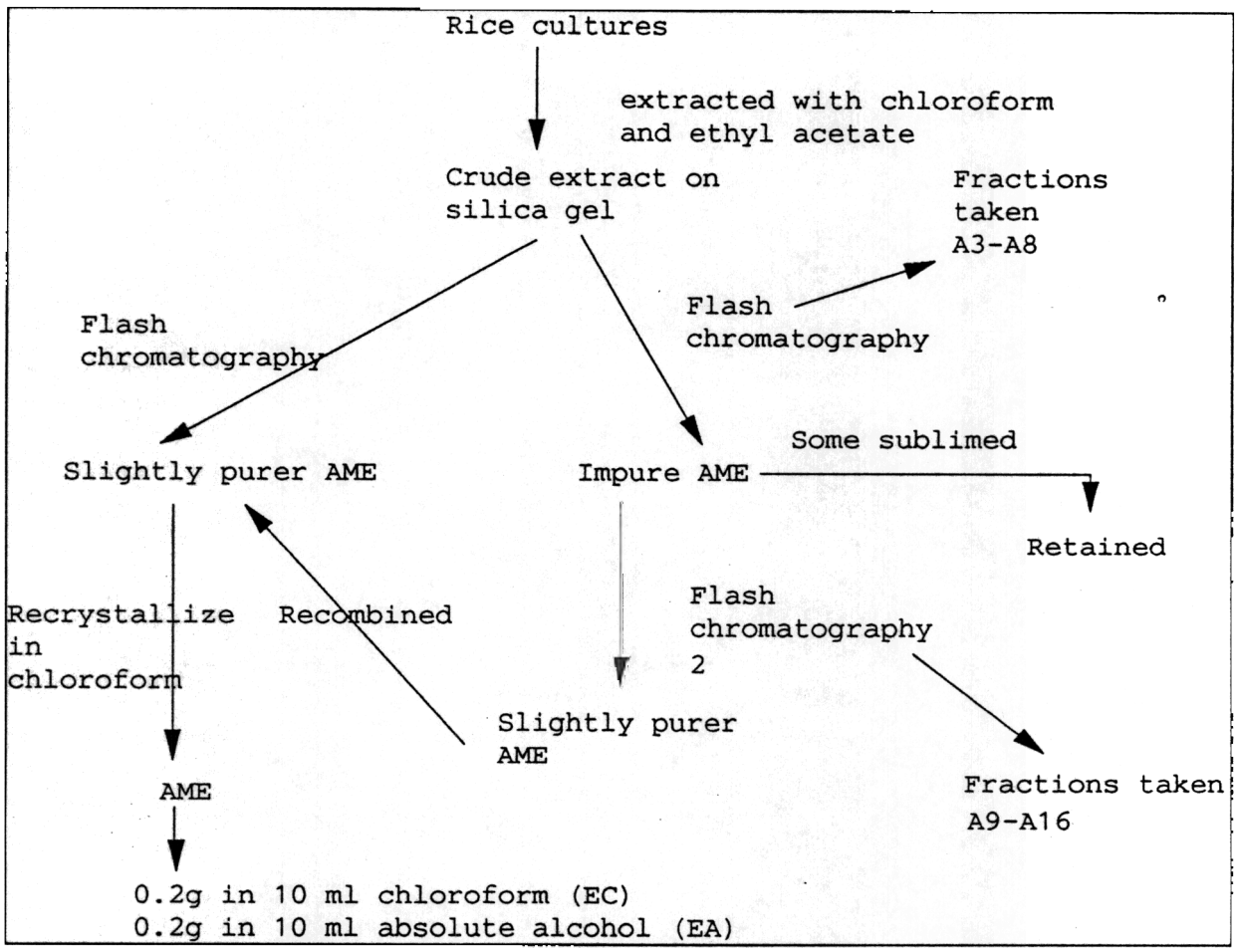

Table 1. Effect of pure Tenuazonic Acid (DC) on brine shrimp larvae.

Various dilutions of DC	Well conc. of DC (µg/ml)	% Mortality
7µl DC8	526.4	99.6
3.5 µl DC8	263.2	93.5
25 µl DC9	188.0	88.5
15 µl DC9	112.8	96.7
7 µl DC1	105.28	64.6
3.5 µl DC1	52.64	13.7
25 µl DC2	37.6	14.7
3.5 µl DC9	26.32	6.8
15 µl DC2	22.56	9.3
25 µl DC3	3.76	7.7
15 µl DC3	2.256	2.0
3.5 µl DC3	0.5264	4.9
3.5 µl F ($CHCl_3$)	0	4.1
7 µl F ($CHCl_3$)	0	5.0
15 µl F ($CHCl_3$)	0	7.0
25 µl F ($CHCl_3$)	0	2.1

Table 2. Effect of an impure fraction of Tenuazonic Acid (CC) on brine shrimp larvae.

Various dilutions of CC	Conc. of equal volumes of DC (µg/ml)	% Mortality
25 µl CC8	1880	100
15 µl CC8	1128	100
7 µl CC8	526.4	99.2
3.5 µl CC8	263.2	97.1
25 µl CC1	376	100
15 µl CC1	225.6	96.4
7 µl CC1	105.28	62.5
3.5 µl CC1	52.64	26.1
25 µl CC9	188	97.5
15 µl CC9	112.8	85.6
7 µl CC9	52.64	20.7
3.5 µl CC9	26.32	19.2
3.5 µl F ($CHCl_3$)	0	7.6
7 µl F ($CHCl_3$)	0	9.7
15 µl F ($CHCl_3$)	0	5.0
25 µl F ($CHCl_3$)	0	7.2

Table 3. Toxicity of various extracts of Alternaria sp to brine shrimp larvae.

Test Compound and Volume	% Mortality after 18 hrs
Experiment 1	
F - 50 µl	4.8
AA - 50 µl	2.6
A3 - 50 µl	0
A5 - 50 µl	6.9
A7 - 50 µl	59.8
A9 - 50 µl	1.8
A11 - 50 µl	5.3
A13 - 50 µl	57.0
Experiment 2	
A7 - 25µl	56.0
A7 - 50µl	84.8
A7 - 100µl	99.1
A13 - 25µl	14.2
A13 - 50µl	59.6
A13 - 100µl	91.4
A15 - 25µl	30.2
A15 - 50µl	56.5
A15 - 100µl	88.5
F ($CHCl_3$) - 25µl	10.4
F ($CHCl_3$) - 50µl	15.3
F ($CHCl_3$) - 100µl	9.3

Table 4. Toxicity of Alternariol (AOH) to brine shrimp larvae.

Treatment	Well conc. of XC (AOH) (µg/ml)	% Mortality after 18 hrs
3.5µl XC (AOH)	39.76	10.7
7 µl XC (AOH)	79.52	36.5
15 µl XC (AOH)	170.4	91.4
25 µl XC (AOH)	284	97.0
40 µl XC (AOH)	454.4	98.8
3.5 µl of F ($CHCl_3$)	0	2.3
7 µl of F ($CHCl_3$)	0	3.6
15µ of F ($CHCl_3$)	0	4.3
25µl of F ($CHCl_3$)	0	5.8
40µl of F ($CHCl_3$)	0	0.6
Blank disc	0	6.8
No disc	0	0.9

Table 5. Toxicity of Altertoxin I (ATX) to brine shrimp larvae.

Treatment	Well conc. of YC (ATX) (µg/ml)	% Mortality after 18 hrs
3.5µl YC (ATX)	46.1	6.0
7 µl YC (ATX)	93.8	17.1
15 µl YC (ATX)	201.0	59.0
25 µl YC (ATX)	335 1	85.1
40 µl YC (ATX)	536.1	100
3.5 µl of F ($CHCl_3$)	0	9.5
7 µl of F ($CHCl_3$)	0	9.0
15µ of F ($CHCl_3$)	0	7.8
25µl of F ($CHCl_3$)	0	5.3
40µl of F ($CHCl_3$)	0	5.7
Blank disc	0	0
No disc	0	3.3

Table 6. Toxicity of Altenuene to brine shrimp larvae.

Treatment	Well conc. of ZC (ALT) (µg/ml)	% Mortality after 18 hrs
3.5µl ZC (ALT)	37.8	12.8
7 µl ZC (ALT)	75.6	13.9
15 µl ZC (ALT)	162.0	13.1
25 µl ZC (ALT)	270.0	46.4
40 µl ZC (ALT)	432.0	59.4
3.5 µl of F ($CHCl_3$)	0	10.3
7 µl of F ($CHCl_3$)	0	7.1
15µ of F ($CHCl_3$)	0	
25µl of F ($CHCl_3$)	0	7.7
40µl of F ($CHCl_3$)	0	8.3
Blank disc	0	9.0
No disc	0	2.6

Table 7. Moong Seed Germination Bioassay of Mycotoxins from Alternaria sp. (Experiment 1)

Germination - 24 hrs/30°C					Plant development - 6 days										
Treatments (µg/ml soaking solution)	% seeds root length >6mm	% seeds root length >0-6mm	% germintn inhibitn	Other comments	Radicle (mm) (A)	Hypocotyl (mm) (B)	(A+B)	Pedicle (mm) (C)	(B+C)	(A+B+C) (mm)	Leaf length (mm)	No of opened leaves	% of stunted plants	% of plants with many root branches	% plants fully opened leaves
(2820) DC	0	25.0	75.0	NAWA	14.4	10.5	24.9	0.60	11.1	25.6	4.1	0	2.6	0	0
(1410) DC-8	5.0	47.5	47.5	NAWA	71.6	34.0	105.6	6.25	40.2	111.9	18.0	0.22	0	8.3	12.5
(282) DC-1	40.0	50.0	10.0		85.7	43.5	129.2	11.3	54.8	140.5	23.1	1.38	22.5	8.3	67.5
(141) DC-9	55.0	37.5	7.5		93.2	47.2	140.2	11.6	58.9	152.3	214.9	1.55	7.7	5.1	76.3
(28.2) DC-2	87.5	12.5	0		68.8	45.4	114.3	12.8	58.2	127.1	24.5	1.65	10.0	12.5	82.5
(2.82) DC-3	90.0	10.0	0		83.1	56.9	140.0	19.0	75.9	159.0	29.1	1.85	24.0	7.3	92.7
(0.28) DC-4	90.0	10.0	0		84.5	47.4	132.0	13.2	60.6	145.1	26.0	1.66	12.2	2.4	80.5
(0.028) DC-5	82.5	17.5	0		79.1	47.5	126.6	14.7	62.2	141.3	25.7	1.59	7.7	23.1	79.5
(0.0028) DC-6	90.0	10.0	0		86.8	48.7	135.6	12.9	61.6	148.5	25.3	1.77	7.5	5.0	87.5
(0.0003) DC-7	87.2	12.8	0	1 VHU	69.0	47.1	116.2	10.7	57.8	126.9	25.9	1.59	20.5	20.5	79.5
(0) F-Control ($CHCl_3$)	77.5	22.5	0		71.3	40.7	112.0	12.3	52.9	124.2	22.7	1.49	23.1	17.9	74.3
CC	0	18.0	55.0	NAWA	27.7	13.8	41.5	1.62	15.1	42.5	7.1	0.06	2.6	0	2.7

NOTE: NAWA = not all water absorbed VHU = very hard, unviable. All figures are the mean from 40 seeds from 2 replicates.

Table 8. Moong Seed Germination Bioassay of Mycotoxins from Alternaria sp. (Experiment 2).

Germination - 24 hours			
Treatments (100µl)	% seeds with root length >6 mm	% seeds with root length >0-6 mm	% Germination inhibition
F	59.0	41.0	0
DC-7	47.5	50.0	2.5
DC-5	50.0	47.5	2.5
DC-3	60.0	40.0	0
DC-1	10.0	82.5	7.5

For concentrations, see Table 7. All figures are the mean of readings from 40 seeds from 2 replicates.

Table 9. Moong Seed Germination Bioassay of Mycotoxins from Alternaria sp. (Experiment 3).

Germination - 24 hrs					Plant development - 7 days										
Treatmnts	% seeds root length >6mm	% seeds root length >0-6mm	% germint inhibitn	Other comments	Radicle (mm) (A)	Hypocotyl (mm) (B)	(A+B)	Pedicle (c) (mm)	(B+C)	(A+B+C) (mm)	Leaf length (mm)	No of opened leaves	% of stunted plants	% of plants with many root branches	Other comments (% plants fully opened leaves)
F-250	70.0	30.0	0		88.0	44.2	132.3	11.4	55.6	143.7	25.8	1.42	20.0	32.5	62.5
F-500µl	70.0	30.0	0		103.4	45.6	149.0	11.8	56.2	160.9	24.6	1.52	20.0	40.0	63.1
DC3-250µl	72.5	27.5	0		116.5	48.9	165.4	13.8	62.7	179.2	26.9	1.79	17.5	15.0	75.0
DC3-500µl	80.0	20.0	0		107.8	48.5	156.3	13.9	62.4	170.2	27.4	1.48	25.0	30.0	72.5
EC-220µl	80.0	20.0	0		96.4	40.7	137.1	14.5	55.1	151.5	24.9	1.38	15.0	25.0	60.0
AA-250µl	67.5	30.0	2.5	1 VHU	96.7	51.6	148.4	13.5	65.2	161.9	26.6	1.71	10.0	20.0	82.5
AA-500µl	87.5	12.5	0		101.3	42.1	143.4	11.0	53.1	154.1	24.3	1.35	15.0	15.0	67.5
A3-250µl	67.5	30.0	2.5		85.2	39.1	124.3	9.65	48.7	133.9	23.6	1.54	22.5	12.5	60.0
A3-500µl	75.0	25.0	0		103.3	45.9	149.2	12.8	58.7	162.0	23.6	1.30	20.0	15.0	64.1
A7-250µl	47.5	50.0	2.5		108.5	44.7	153.1	11.2	55.8	164.3	25.0	1.53	9.8	9.7	70.7
A7-500µl	45.0	55.0	0		91.1	39.1	130.2	9.07	48.2	139.3	22.2	1.27	22.5	12.5	55.0
A13-250µl	39.0	60.1	0		95.3	41.3	136.6	10.5	51.8	147.1	22.5	1.30	19.5	21.9	58.5
A13-500µl	35.0	65.0	0		104.8	47.7	152.4	10.8	58.4	163.2	22.5	1.37	10.0	12.5	50.0
A15-250µl	40.0	22.5	37.5	NAWA 13 VHU	75.9	36.2	112.1	9.6	45.8	121.7	19.0	1.14	17.5	2.5	52.5
A15-500µl	56.0	29.3	14.6	NAWA 3 VHU	82.2	40.9	123.1	10.6	51.4	133.6	22.3	1.30	4.9	19.5	51.2

NOTES: NAWA = not all water absorbed. VHU = very hard, unviable. All figures are the mean of readings from 40 seeds from 2 replicates.

Table 10. Moong Seed Germination Bioassay of Mycotoxins from Alternaria sp. (Experiment 4).

Germination - 24 hours				
Treatments (100µl)	% seeds with root length >6 mm	% seeds with root length >0-6 mm	% Germination inhibition	% Plants with fully open leaves
F	77.5	22.5	0	75.0
	67.5	32.5	0	77.5
A5	80.0	20.0	0	76.9
A7	55.0	42.5	2.5	87.1
A9	72.5	27.5	0	72.2
	72.5	27.5	0	83.8
A13	70.0	30.0	0	82.5
A15	62.5	37.5	0	84.2
EC	57.5	42.5	0	70.0
AA	60.0	40.0	0	82.1

All figures are the mean of readings from 40 seeds from 2 replicates

Table 1. Moong Seed Germination Bioassay of Mycotoxins from Alternaria sp. (Experiment 5).

	Germination - 24 hrs				Plant development - 7 days										
Treatmnts	% seeds root length >6mm	% seeds root length >0-6mm	% germint inhibitn	Other comments	Radicle (mm) (A)	Hypocotyl (mm) (B)	(A+B)	Pedicle (c) (mm)	(B+C)	(A+B+C) (mm)	Leaf length (mm)	No of opened leaves	% of stunted plants	% of plants with many root branches	Other comments (% plants fully opened leaves)
Control	55.0	45.0	0		96.6	44.4	141.0	7.7	52.2	148.8	23.5	0.69	27.5	12.5	
F-250 µl	52.5	47.5	0		95.7	42.2	137.9	7.1	49.2	144.9	23.6	0.94	22.5	30.0	
F-500µl	48.7	48.7	2.6		102.2	46.7	148.9	7.9	52.6	156.8	24.7	1.01	20.5	12.8	
DC3-250µl	47.5	52.5	0		81.3	37.2	118.4	6.4	43.5	124.8	22.1	0.69	37.5	17.5	
DC3-500µl	50.0	50.0	0		105.8	46.0	151.8	9.8	55.8	161.6	25.3	1.11	25.0	20.0	
AA-250µl	27.5	72.5	0	1VHU	99.5	41.5	141.1	7.4	48.9	148.5	23.0	0.62	12.5	25.0	
AA-500µl	32.5	65.0	2.5	1VHU	85.2	42.4	127.7	8.0	50.4	135.7	23.4	0.88	7.5	32.5	
A5-250µl	20.0	80.0	0		87.5	41.0	128.4	7.3	48.3	135.7	22.0	0.72	25.0	10.0	
A5-500µl	25.0	75.0	0		90.4	43.8	134.2	6.3	50.1	140.6	21.7	0.68	15.0	20.0	
A9-250µl	35.0	62.5	2.5	1 VHU	92.7	41.1	133.8	6.9	48.1	140.8	23.0	0.76	25.0	25.0	
A9-500µl	35.0	62.5	2.5		91.4	41.4	132.8	6.6	48.0	139.5	24.4	0.91	20.0	25.0	
A11-250µl	40.0	60.0	0		93.7	41.5	135.2	7.6	49.1	142.8	23.6	0.93	22.5	20.0	
A11-500µl	40.0	57.5	2.5		86.3	39.0	125.3	8.1	47.1	133.5	22.6	0.76	20.0	27.5	
A15-250µl	25.0	60.0	15.0	NAWA 3 VHU	100.2	41.8	142.0	7.1	48.9	149.1	22.7	1.16	5.0	10.0	
A15-440µl	40.0	40.0	20.0	NAWA 4 VHU	105.3	41.6	146.9	9.0	50.6	155.9	22.8	1.19	15.0	15.0	17.5

NOTES: Control = no solvent, VHU = very hard, unviable. NAWA = not all water absorbed. All figures are the mean readings from 40 seeds from 2 replicates, except A15 which are the mean of 20 seeds.

Table 12. Effect of Tenuazonic Acid and Alternariol monomethyl ether on chick embryo development (brown eggs 59-68 gm range).

Test compounds (25µl)	Mortality
Fertility Control	1/10
Ethanol Solvent Control	1/10
EA1 250µg/egg	2/10
EA 500µg/egg	2/10
DA6 - 0.0014µg	2/8
DA5 - 0.0141µg	0/9
DA4 - 0.141µg	5/9
DA3 - 1.41µg	4/9
DA2 - 14.1µg	2/7
DA1 - 141µg	6/7

NOTE: 10 Eggs were used per treatment and results are expressed as a proportion of fertile eggs.

Table 13. Effects of various extracts from Alternaria sp culture on chick embryo development in Experiment 2 (white eggs, 59-68 gm range).

Test compound (40µl	Mortality
Ethanol solvent control	2/6
A4	1/9
A6	1/6
A8	2/7
A10	1/8
	2/8
	2/6
	5/6
DA2 (22.5 µg/egg)	2/10

NOTE: 10 eggs were used per treatment and results expressed as a proportion of fertile eggs.

Table 14. Effects of various combinations of extracts from Alternaria sp culture on chick embryo development in Experiment 3 (brown eggs, 59-66 gm range).

Treatment Test Compounds	Mortality 15 days	22 days
Ethanol solvent control (50 µl)	1/9	2/9
DA9 (50 µl) - 141 µg/egg	3/10	4/10
DA9 (25 µl) - 70.5 µg/egg	2/5	3/5
DA9 (25 µl) + A8 (25 µl)	2/10	4/10
DA9 (25 µl) + A14 (25 µl)	2/9	5/9
DA9 (25 µl) + A16 (25 µl)	3/8	3/8
A16 (25 µl) + A14 (25 µl)	3/10	4/10
A8 (25 µl) + A14 (25 µl)	2/9	3/9
A16 (25 µl) + A8 (25 µl)	2/10	4/10

NOTE: 11 eggs were randomly used for treatment and results are expressed as a proportion of fertile eggs.

Table 15. Performance of chicks 0-19 days.

Treatments (mg TA/kg)	0 (Control	3.5	7.0	15.0	SEM[1]	Significance (P=)
Initial body weight (g)	41	42	42	42	0.4	0.6637
Weight gain (g)[2]	685±9.2	648±11.0	676±16.2	664±14.7	13.1	0.2722
Coefficient of variation (%)	2.70	3.38	4.78			
Food intake (g)	922[ac]	882[a]	936[c]	906[ac]	14.8	0.1182
Efficiency of food utilisation[3]	0.74	0.73	0.72	0.73	0.007	0.2530
Water:food intake (ml/g)[4]	2.1	2.1	2.0	2.0	0.077	0.7723

NOTE: 1 standard error of means. 2.±standard error; 3 - weight gain:food intake; 4 - for week 2. Values in each horizontal line with different superscripts are significantly different (P<0.05)

Table 16. Symptoms observed in autopsied chicks

Treatments (mg TA/kg)	0	3.5	7.0	15.0	RMSE[1]	Main effects	Covariate diet
TA intake per bird (mg)	0	3.07	6.66	13.74			
Organ weights:							
Body weight (g)	671	633	672	657			
Liver weight	19.7	18.6	22.1	21.3	2.29	0.0001	0.0001
% of birds with enlarged liver[2]	42	37	79	79			
Pancreas weight	1.55	1.48	1.55	1.57	0.184	0.0001	0.7173
Spleen weight	0.46	0.46	0.48	0.45	0.114	0.001	0.8835
Haemorrhages:							
Under skin	0.3	0.7	0.2	0.4	0.65	-	0.0393
On musculature[3]	1.3	1.1.	1.3	1.9	0.81	-	0.0106
On liver[4]	8.3	12.5	16.7	37.5			
Other symptoms:							
Icteric liver[4]	4.2	8.3	4.2	25			
Gizzard erosion	1.4	1.2	2.0	2.0	1.01		0.0163

NOTE 1. Root mean square error; residual degrees of freedom 92. 2. defined as birds with a relative liver weight higher than the average of controls. 3.- on thigh & breast. 4. percentage of carcasses affected.