Investigations on the Toxin Produced by Gloeosporium Fructigenum Sacc.

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GLOEOSPORIUM FRUCTIGENUM, SACC. attacks a variety of plants of economic importance. Of these the more important hosts are the apple, pears, quinces; spots occur on the leaves and bitter rot sets in the fruits. In the palms this fungus has been associated with premature nut falls.

The causal organism is *Gloeosporium, a* conidial stage reported as the imperfect form of a number of ascomyeetes, *Gnomoniopsis cingulata* Hon; *Glomerella rufomaculans* Spauld and Schrenk; *Venturia inaequalis* (Cooke) Wint. Mycelium is hyaline when young, producing dark, yellowish brown chlamydospores; conidia are subhyaline, golden, enmasse; oblong or cylindrical even slightly curved, 3.5-3 x 10-14 u. Dark appressoria are produced at the ends of the germ tubes.

The fungus has been grown on malt agar where the conidial production is maximum after a fortnight under normal room temperature. The present author has carried out toxicity tests on the fungal filtrates on test plants as tomatoes, cotton, bean and pelargonium, in the Federal Institute of Technology, Zurich.

A vast amount of literature has accumulated on the toxins produced by different parasjtes. The effect of the toxins is observed as wilting of the leaves and shoots of the host plant. (Gaumann 1949, 1951 a, 1951 b, 1952 b, 1952 c, 1953). These toxins are generally given the term wilt toxins.

Wilt toxins produce characteristic symptoms of brown, vascular discolorations on the host stems; in the advanced stages of the fungal attack the leaves of the host turn yellow, curl at the edges. In later stages the chlorosis spreads to the entire foliage which dries up. On the leaves in addition to the chlorosis are noted certain clear, water-soaked areas grouped towards the veins and the veinlets.

Conflicting opinions are expressed as to the nature of the wilt toxins. The American School headed by Walker, Waggoner and Scheffer (1946, 1953, 1954) hold the view that the wilt toxins are only enzymatic metabolites produced by the host or the parasite. The Swiss School is of the opinion that the Wilt toxins are responsible for the necrosis and browning.

Many scientists have recorded toxins produced by various pothogenic fungi-Gibberellin from *Gibberella fujikuvioi* (Saw) Wall (Tamura et al 1941, 1943 a, 1943 c), *Nectria Cinnabarina* (Koe-bel, 1951) Diaporthin (Bazzchiger 1953). It has been noted that one fungus is capable of producing more than one wilt toxin (1953, Gaumann).

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Materials and methods

Sterile 500 ml. Erlenmeyer flasks were filled with 100 ml. of the basal media and inoculated with 5 ml of spore suspensions in sterile, distilled water. Various media were tried for maximum efficacy.

1. Ri 5 with 5% glucose

Ammoniumnitrate	1%
Primary potassium sulphate	5%
Magnesium sulphate	25%
Ferric Chloride	0.002%
Water, Distilled	1000ml.

2. Czapek'smedium with the addition of 10 gm of cotton seed extract.

Sodium nitrate	0.3%
Primary potassium phosphate	0.1%
Magnesium sulphate	0.05%
Potassium chloride	0.05%
Ferric sulphate	0.001%
Cotton seed extract 10 gm.	

3. *Knop Yeast extract*

Calcium nitrate	0.1%
Primary potassium phosphate -	0.025%
Magnesium sulphate -	0.025%
Potassium chloride	0.025%
Ferric chloride	0.05%
Distilled water	1000 ml.
Yeast extract	0.5%

A fourth medium 'C' containing calcium citrate, potassium nitrate and calcium carbonate in addition was tried. Of the different media tried, maximum growth was recorded in the Czapek's medium so that in the later stages of the investigations only this medium was used. Standard cultures of the fungus were grown on agar slants, incubated at 17°C. Profuse, white, cottony, mycelium covered the medium after 4—5 days. Formation of conidial bodies was noted as black spots dotting the white mat, visible to the naked eye. The conidial production was at its peak, in the second week; 10 ml of sterile distilled water was introduced into the tube under sterile conditions. The tube was shaken thoroughly for half an hour so as so ensure the uniform distribution of the conidia in the water. With a 10 ml. pipette, quantities of this conidial suspension were introduced into the flasks containing the basal media. This work was done in the hand inoculation chamber. (This is a square glass chamber, previously sterilised with formaldehyde). Care had to be taken that no contamination occurs during all these operations as it was absolutely necessary that a pure culture grows in the basal media. The cultures were allowed to stand in an incubating room with a temperature of 21°C; half the number of Erlenmeyer flasks were kept in stands while the other half were fixed on shaking machine, operated by electric motors providing both horizontal and rotatory motions. The initial pH of the basal media in all the cultures was 4. The flasks were kept for twenty days in still as well as shaking cultures; at the end of this period the fluid surface was entirely covered by the fungal mat.

The mycelial mats are thick, dark brown to pale brown (depending on the basal medium) tough, slimy coverings; The basal medium was poured off; flasks were shaken thoroughly to disseminate the fungal metabolites. The solution was then filtered to make it cell-free by the filter paper method. Culture filtrate was dark brown in colour. Final pH readings were taken. The filtrate was diluted with sterile distilled water in proportions 1:2 and 1:5. Undiluted cultures were also used in further trials. Test plants used in these experiments were tomato, pelargonium, cotton and bean. These were grown in the glass houses; when the tomato seedlings were at the four-leaf stage, pelargonium, cotton and bean at the two—leaf

stage, they were used in the experiment, stems were cut flush with a blade from the root system. Wooden stands with fitted in aluminium holders for small 3 in. test tubes were kept in a sterile chamber; culture filtrate of 1: 2 dilution was pipetted into the tubes (1 ml of filtrate to 1 gm wt. of the plant) and the stems with 4 leaves in case of tomato, two in the rest of the test plants, cut flush from the root system were introduced into these solutions. Adequate controls were kept in ordinary tap water. The test plant were kept under constant fluorescent light. Readings were made at 72 hr. intervals. The following index was fixed on after a series of tests:—

0. . . .no symptom of wilt.

1. . . .Primary wilting of leaves.

2. . . .Complete wilting of petiole.

3. . . .Chlorosis and wilting.

4. . . .Chlorosis, wilting and necrotic spots.

In addition to the wilt assay, test organisms used were *Ustilago* spores, *Botrytis and Alternaria spores;* bacterial plates of *B. subtilis, B. Candida, and M. tuberculosus.*

In the ustilago method of testing, a spore suspension was prepared in distilled water, (10 ml. to a scoop of the ordinary laboratory scalpel) and shaken thoroughly. Examining a drop of this suspension, the density of the fluid was adjusted to 100 spores under the low power of the raicroseope. 1 cc of the suspension was added to diluted and graded solutions of the culture filtrate kept in small petri dishes. They were covered and incubated at (21 °C.)

The same procedure was adopted in the case of Botrytis and Alternaria spores. Best results were obtained in the case of the smut spore. The assay in this case was made by measuring the diameter of the fungal mats in the different dilutions and from a standard logarithemic graph count efficiency of the filtrate in spore inhibition calculated.

In the bacterial plates methods the following technique was adopted. Malt agar sterile plates were treated to a suspension of bacterial spores and incubated at 21°C, pieces of sterile filter paper (the paper was punched and the round pieces were used for the purpose of the experiment) were dipped in the culture filtrate and planted on the agar plates in the form of a ring for better arrangement and easier assaying. The plates were incubated at °C. The antibacterial action was measured by the clear halo around the filter paper (inhibition) discs.

Apart from these methods precipitation methods with ethyl alcohol (95%) methyl alcohol, and 1% trichloracetic acid; and ammonium sulphate, glycine and sodium tungstate were tried for the protein fraction in the filtrate.

10-12 days culture filtrate was shaken with equal amounts of the above alcohols, ammonium sulphate and trichloracetic acid. The precepitate was washed with sterile distilled water; the clear supernatant was centrifuged at 3000 r.p.m. and further precipitate along with the first lot were resuspended in sterile water. The supernatant was recovered by vacuum distillation of the alcohol.

Where glycine was used, filtrate was treated with 5 gm of glycine to 100 ml of culture filtrate. The flasks were in rooms maintaining a constant temperature of i5 ° for 7 hrs. The filtrate was again precipitated with 0,3-0.6% of ammonium sulphate solution in distilled water.

RESULTS.

Ustilago Spore Tests

Calcium citrate medium.

			Ustilago count		*pH*	
	5 days	Richard's medium	7.5	E	6.9	
		Czapek's medium	6.5	R	5.8	14 E–5.1 pH
		HGF medium	5.5	E	7.3	
	7 days	R 1	7	E	5.4	
		CW 4	27	E	6.0	15 E–7.0 pH
1		HGF	0	E	6.8	
	9 days	R 1	10	E	5.4	
		CW 4	27	E	8.0	6 E-7.2 pH
		HGF	15	E	7.3	
	9 days	R 1	7.1	E	6.5	
		CW 4	17	E	6.0	
		HGF	7.5	E	8.0	
	12 days	R 1	0	E	8.0	
		CW 4	0	E	8 1	
		HGF	0	E	8.0	

Bacterial Plate Tests

Results were not satisfactory in all the plates.

Qualitative Tests

Incubation period	R1 Medium				Medium CW4				KHG			
	I	8	Be	Pe gon								
5 days	0/0	1/0	1/o	O/O	l/o	2/o	2/o	l/o	l/o	2/o	l/o	l/o
7 days	2/o	2/o	2/o	2/o	3/o	2/o	3/o	l/o	l/o	2/o	3/o	l/o
9 days	2/o	2/o	2/o	o/o	4/o	3/o	4/o	l/o	3/o	3/o	3/o	l/o
9 days	3/o	4/0	4/o	l/o	3/o	3/o	3/o	l/o	2/0	2/o	3/o	l/o
12 days	4/o	l/o	3/o	l/o	3/o	4/o	2/o	4/o	2/o	3/o	l/o	l/o
16 days	2/o	2/o	2/o	2/o	4/o	4/o	4/o	4/o	l/o	3/o	2/o	l/o
20 days	2/o	3/0	2/o	o/o	2/o	3/o	3/o	3/o	O/O	1/o	O/O	1/0

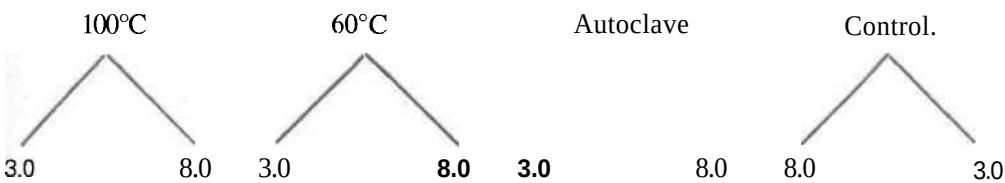
Chemical and Physical Tests

Experiment I.

After 9 days shaking culture filtrate at a pH of 8 was divided into 3 equal parts ; these aliquots were heated at 60°C, and 100°C, and autoclaved for 8 hrs. The solutions were then tested for activity by the various methods described above.

Autoclaved aliquot	Ustilago
100°C - heated	5 E
60°C – heated	5 E
Control	27 E

The pH in the final stages after heating the culture filtrate was found to be 3 ; this was brought again to 8.



After heat treatment pH of the medium did not affect the assays. In qualitative tests activity was not noted in cotton and tomato but a 2/0 was noted on the beans.

Experiment II.

The culture filtrate after centrifuging was kept in cellophane bags under water and dialysed at 2°C for 12 hours against ordinary tap water; both the solution and the residue were tested for activity.

Ustilago Test

Solution O E
Residue 5 E ?
Filtrate 4 E

This result was rather confusing; whether the decreased activity of the residue was due to the effect of cold and darkness or whether the toxic subsiances are decomposed during pialysis is not very clear.

Qualitative Tests

Residue was diluted to 1:5 in distilled water (1 ml. to each gm. wt). Assays were made in beans and tomatoes.

Beans	Tomato
3/o	2/0

Experiment III.

The culture filtrate (12 days shaking) was shaken up with 0·3%, 0.6% and 1.0% of ammonium sulphate solution respectively in equal parts, very thoroughly; the precipitate was dissolved in water and this solution was tested for activity.

Ustilago Tests

1% soln.	0.6% soln.	0.3%soln.
12 E	18 E	36 E

Qualitative Tests.

Solution	Tomato	Beans
0.3%	2/o	2/o
0.6%	2/o	4/o
1.0%	1 *In*	3/o

Experiment IV.

The culture filtrate was precipitated with Barium hydroxide. Tests were made as before. No. activity was noted in any instance.

Experiment V.

A trial was made with different quantities of the Gloeosporium spore suspensions in the basal medium. Cultures were withdrawn at random intervals and pH of the basal medium adjusted in all cases to 6.1. Solutions were diluted with tap water 1 : 2 and tested for activity.

No great effect in the activity was noted by increasing the spore suspension.

Suspension	Ustilago	Tomato	Beans	Cotton	Pelargonium
2 ml.	14 E				
4 ml.	10½ E				
6 ml.	14 E				
8 ml.	0 E?				
10 ml.	22 E	2/o	2/o	1/0	1/o

Experiment VI.

The 10 days culture filtrate was adjusted to pH 6.1, treated witn 95% ethyl alcohol three times the filtrate volume; the mixture was shaken very thoroughly; the alcohol was removed after centrifuging at 3000 r.p.m. by vacum distillation. The remaining liquid was tested for activity.

Ustilago Test

OE

Qualitative Tests

Tomato	Bean	Cotton
2/9	1/0	0/0

Experiment VII.

The culture filtrate was shaken with methyl alcohol and the whole process repeated as in the previous experiment.

Ustilago Test

OE

Qualitative Tests

Tomato	Bean	Cotton
O/o	1/O	O/o

Experiment VIII.

The experiment was repeated with 1% trichloracetic acid.

Ustilago Test

OE

Qualitative Test

Tomato	Bean	Cotton
O/o	1/O	O/o

Experiment IX.

Culture filtrate with the basal medium containing calcium nitrate and sodium citrate, was at initial pH 4.9. Inoculum used in this experiment was of the concentration 2 ml. After 20 days the culture solution was deep, reddish brown and the mycelial mat very slimy and tough. Filtration by the ordinary method failed absolutely. Seitz's filter also gave negative results; so resort had to be taken to nylon cloth and the filtrate further cleared by centrifuging at 2000 r. p. m. The mycelial portion was dried with acetone and powdered with refined sand, using a pestle and mortar. This was treated with 10% glycine for 24 hours at —2°C. The clear solution was decanted off and this fluid was precipitated with 6% ammonium sulphate. A reddish white deposit was separated at the base of the flask- This deposit was dissolved in water and tested for activity (½ ml. to wt.)

Ustilago Test

41 E

Qualitative Tests

Tomato	Bean	Cotton
	2/0	3/o

Experiment XL

One month old fungus culture was grown innutrient solution 'C' as before; 300 ml. of this filtrate was taken picked at random from the Erlemeyer flasks; the pH was maintained at 6.5. The filtrate was now mixed with a solution (10% strength) of sodium tungstate in the ratio 1 : 9 and whole mixture shaken very thoroughly. The precipitate was washed in distilled water and tested as before. In the test seedlings there was very little of rolling up of the leaf edges; practically no necrotic spots were noticed.

Experiment XII.

The culture filtrate was treated with 95% alcohol; the mixture was shaken thoroughly; after centrifuging the precipitate was dried at 70°C in the thermostat; this was dissolved in distilled water and tested for activity.

Ustilago Tests

No activity.

Qualitative Tests

Tomato	Bean	Cotton
O/o	O/o	O/o

This result leads one to the view that there may be a polysaccharide fraction also in the culture filtrate. This fraction may have no effect on Ustilago spores and does not produce any visible symptom on bean and cotton. But on tomatoes a 1 : 3 diluted solution causes symptoms confined only to the leaflets; there is severe wilting on the tips and edges of the leaflets in certain cases. This is not confined however to all the test plants. Irregular shiny areas appeared on the upper surface of the leaves. Yellowing, flaccidity and discoloration of the stem and desiccation are particularly absent in this fraction tested.

The above results indicate that Gloeosporium fructigenum shows certain toxic characters in its fungal extracts; but there are a number of difficulties met with during the course of the investigations. Due to the slimy sheath around the fungus the culture filtrate contains a large amount of slime and it has not been possible to remove this slimy deposit from the filtrate. Precipitation methods showed that a toxic fraction is removed and this causes flaccidity, chlorosis and desiccation in the test seedlings. But the symptoms produced were not so acute as in the case of the untreated filtrate. This phenomenon may also be due to the small portions of the culture filtrates used.

When ammonium sulphate and glycol were use in the culture filtrate, the test plants wilted, dried up and exhibited chlorosis, Ustilago spores also showed germination inhibition. The supernatant liquuid had very little of the effects noted with the residue. Test seedlings showed a browning of the lower end of the stems, in the case of tomatoes. It is therefore possible that the culture filtrate consists of more than one toxic fraction; one of them causes wilting and the other the discolouration in the lower portions of the stems. Hence trials were made to precipitate out the protein fractions with the aid of sodium tungstate solution.

The potency of the toxins in the culture filtrate increases with the age of the culture. The initial pH of the basal medium is 4.8—5.1 and this is maintained constant for about 3 days. After this there is a gradual rise followed by the wilting effects noted in the test plants. After 10-11 days there is decrease in the toxic action of the filtrate. This is indeed to be expected due to the degeneration of the enzymes and interaction with the other products of metabolism excreted by the by parasite. The activity is practically nil after 18 - 20 days. This phenomenon goes a long way to prove that the toxin is not produced by the autoly sis of the fungal metabolites.

Extraction methods employed to separate the toxic fraction from the Culture Filtrate

The culture filtrate was filtered through a nylon net. The filtration process is a very tedious one due to the slimy consistency of the solution choking the pores. Filtration was therefore carried under vacuum. The filtrate was mixed with twice the volume of 0.4 to 0.5% Nornit - superactive Coal and the whole filtrate was mixed thoroughly with an electric blender. The filtration was now continued under water-vaccum. The process was repeated with fresh quantities of charcoal. The coal which has absorbed the toxic matter was eluted with 2.5 litres of methanol and the mixture kept standing at 4°C for 24-48 hours; the alcohol was evaporated to dryness by fractional distillation; other organic solvents tried were acetone, chloroform, ethanol etc. In all cases the above process was repeated - the solution after being thoroughly shaken evaporated to dryness.

The residue left behind was pale yellow; this was diluted with distilled water and the solution tested for toxicity. No conclusive results were obtained.

The extraction methods did not prove as successful as the precipitation methods.

From the investigations it is suggested that *Gloeosporiumfructigenum* when grown in liquid cultures, gave toxic substances which diffused into the basal culture medium. This culture filtrate produced positive tests of necrosis on tomato and bean plants. The toxins were precipitated to a greater extent by chemical re-agents than by extraction with organic solvents.

Purification of the toxic substances was not attempted. This work however gives evidence of the presence of a toxin leading to disease symptoms.

SUMMARY

1. *Gleosporium fructigenum Sac* was grown in Richards, Czapek's and KFG mediums. The best results were obtained in Czapek's medium.

2. The toxic metabolites were produced at the peak in 10-12 days cultures, kept in shaking cultures.

3. Test organisms used were snut spores and seedlings of tomato, bean, cotton and pelargonium. Positive results were obtained.

4. Precipation methods of extracting the toxic principles showed the possibility of more than one toxin.

5. Extraction by organic solvents did not give conclusive results.

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