Toxin Production by an Isolate of Dothiorella phaseoli (Maubl.) Pet. et Syd., Syn. Macrophomina phaseoli (Maubl.) Ashby, from Crucifer host [1])

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Zusammenfassung

Dothiorella phaseoli (Maubl.) Pet. et *Syd.* bildet ein Toxin, das auf Blättern von Cruciferen in vivo und in vitro nekrotische Flecken hervorruft. Gleichzeitig steigt die Respirationsrate. Es handelt sich um ein aliphatisches Gemenge, das in Aether am besten löslich ist.

Introduction

A number of plant pathogens have been reported to produce toxic metabolites which are injurious to the host plants and accumulating information on this aspect supports the view that the toxic metabolites are somehow involved in the pathogenesis. Literature on this topic has been ably reviewed and discussed by various workers from time to time (Dimond and Waggoner, 1953; Brian, 1957; Sadasivan, 1958; Braun, 1959; Braun and Pringle, 1959; Ludwig, 1961; Wheeler and Luke, 1963; Beckmann, 1964; Pringle and Scheffer 1964; Brown, 1965; Allen, 1966; Kalayansundaram and Charudatan, 1966; Goodman et al. 1967; Wood, 1967).

The polyphagous parasite *Dothiorella phaseoli* has been found causing root-rot and leaf-spot diseases of various crucifers (Rai et al. 1969) and preliminary studies on the toxin production by an isolate of this pathogen indicated the production of a thermostable toxic metabolite that could cause the disease symptoms in the host plants.

Investigations on the toxin production by *Dothiorella phaseoli* have been carried out by a few workers and contradictory observations have been reported by them. West and Stuckey (1931) could not get any indication of the toxin production by this organism. Bremer (1954) observed that culture fluids of *D. phaseoli* proved injurious to beans but did not effect pepper, egg plant and potatoes.

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Ghosh and Mukerjee (1966—1967) were unable to detect the presence of any toxic metabolite. However, Mathur (1968) reported the production of toxin by isolates of sunflower and bean while Chan and Sackston (1967; 1969) indicated the production of metabolite from a cotton isolate.

In the light of these observations the problem appeared to be a fascinating one and a detailed investigation of in vitro and in vivo production of toxic metabolite(s) by the crucifer isolate of *D. phaseoli* causing leaf spot of cauliflower was undertaken. This particular isolate was taken up for the studies because of experimental convenience and relatively quick results.

Materials and Methods

The leaf-spot isolate of *D. phaseoli* was grown on Czapek's broth at 30 ± 1° C and after 10 days of inoculation the culture filtrate was Seitz filtered and collected. Unless otherwise stated, the culture filtrate was autoclaved at 15 lbs for 15 minutes and was analysed for the toxin production. The presence of toxin was assayed by putting drops of filtrate on the leaf surface or by dipping shoot-cuts in jars containing the original filtrates or its different dilutions made by using sterile distilled water.

The effect of different incubation periods on the toxin production was studied with filtrates obtained from 5, 10, 15, 20, 25 and 30 days old broth cultures. The toxin assay was done as mentioned above.

Effect of culture filtrate on the seed germination was studied by two methods, (i) by keeping the surface sterilized cauliflower seeds in petri-dishes containing filter paper and the culture filtrate, (ii) seeds were sown in petri-dishes containing acid washed sand and these petri-dishes were given post- and pre-emergence treatment with the culture filtrate.

Effect of soaking the seeds in the culture filtrate was studied by placing the seeds in filtrate for different periods, washing them with sterilized water and allowing them to germinate as mentioned above.

In the control sets, in all the experiments sterilized Czapek's broth and distilled water were used.

For isolation of the toxin, the culture filtrate of the fungus was treated with various organic solvents viz., chloroform, ether, benzene, petroleum ether etc. The solvent layer was taken and evaporated to dryness at room temperature. The residues were assayed for the presence of toxin. Similarly treated controls of uninoculated medium were also run.

The ether soluble portion showed maximum activity and for further purification the ether extracted residue was subjected to thin layer chromatography. Of the various methods tried for separation,

benzene and methanol as solvent and ceric sulphate in 2 n H_2SO_4 as developer gave encouraging results. This analysis resulted in separation of three fractions. The elutes from these fractions were tested for their toxic activity against the host.

For demonstrating the presence of the toxin in the host, freshly infected leaves were harvested, thoroughly washed, homogenised in distilled water (1 : 5 W/V) and then filtered. The filtrate obtained was centrifuged for 30 minutes at 3200 r.p.m. and supernatent was boiled. This final extract was assayed for toxin activity against the host in similar manner as described earlier. For control, a similar treatment with the extract obtained from healthy leaves was also run.

For studying respiration, manometric techniques of Umbreit et al. (1957) were used. Approximately equal amount of toxin (isolated from chromatography) was applied in the form of droplets on the leaves at different intervals. The spot areas produced due to the activity of toxin were cut out after 24, 48 and 72 hours and were utilized for the study. The experiments were carried out in triplicates at 37° C in Warburg's apparatus.

Results

The culture filtrate on the host leaves produced lesions which clearly indicated the presence of a thermostable toxic substance in it. The spots developed within 48—60 hours. The spots in the beginning showed yellowing followed by browning of the middle region, later they showed necrosis finally becoming dry and papery. Internally, as seen in the sections, the mesophyll and palisade tissue first showed browning and later collapsed. The metabolite also caused lesions on various other cruciferous and noncruciferous hosts, viz., *Brassica campestris var. sarson, B. campestris var. toria, B. juncea, B. oleracea var. capitata, B. oleracea var. caulorana, B. rapa, Eruca sativa, Raphanus sativus, Lycopersicon esculentum* and *Solanum melongena.*

The metabolite present in the filtrate also caused wilting (fig. 1) of cauliflower shoot-cuts which in early stages showed sagging, followed by partial drooping and drying of the leaf margins (fig. 2). Some necrotic spots also developed scattered over the leaves. The leaves finally droop, show chlorosis and become dry in wilted twigs. The twigs wilted in 24—28 hours after dipping in pure culture filtrate. The dilutions (1 : 5, 1 : 10, 1 : 20) of the culture filtrate also caused wilting of shoot-cuts although the time taken for wilting increased with increase in dilution. Wilting of shoot-cuts of *B. campestris var. sarson, B. campestris var. toria, B. juncea, Eruca sativa, Lycopersicon esculentum* and *Solanum melongina* also occurred when cut twigs were dipped in culture filtrate. The sequence of the symptom appearance was almost similar in all the plants.

Sections cut at different stages of experiment showed that the browning of vascular region occurs even up to leaf veins. In wilted plants, the tissue surrounding the vascular region also showed browning. In the controls such browning was not observed.

The observations on the effect of incubation period on toxin production showed that the pathogen starts producing toxin from an early stage and 5-day old culture filtrate was active in producing lesions on the leaves and also checked the germination of seeds of cauliflower. Optimum toxin production took place in 10 day old cultures after which there was no major difference in the toxic activity of the filtrates obtained from the cultures incubated upto 30 days.

The metabolite also affected the germination of seeds (Table I). The pure culture filtrate showed about 80—85 percent inhibition in germination of seeds. The dilutions of the culture filtrate also affected seed germination and it has been observed that with the increase in dilution of the metabolite in the filtrate the percent germination and the growth of the seedlings also increased. In pure filtrate only small protuberences of radicle came out. There was a relative decrease in length of radicle or root with increase in concentration of the filtrate. Similar results were obtained when seeds of some oil yielding crucifers were taken instead of cauliflower.

On sand also, the metabolite affected seed germination. The pre-emergence treatment resulted either in the failure of the seeds to germinate or only emergence of radicles (Fig. 3). The post emergence treatment resulted in wilting of the seedlings within 24—30 hours. The cotyledons in the early stages show marginal drying and curling which gradually spreads over the whole surface. The development of root-system was poor in the treated seedlings in comparison to the control ones.

The same type of results could be obtained when soil was taken instead of washed sand.

The results of the effect of soaking of seeds in culture filtrate on seed germination indicated (Table II) that the increase in the time of soaking of seeds in culture filtrate decreased the percentage germination, length of radicle or root and development of root hairs. The development of radicle and root hairs have been found to be inversely proportionate to the time of soaking.

Effect of ether extract: As already mentioned earlier in materials and methods, of the various organic solvents tried, chloroform and ether gave positive results of which ether proved to be a better solvent. The chloroform and ether extracted residues were redissolved in distilled water and when applied on the leaves gave prominent lesions within 24 to 48 hours. The spots first showed slight etiolation followed by necrosis which results in the formation of prominent

Table I

Effect of culture filtrate on the seed germination of *Brassica oleracea var. botrytes*

Dilutions of the Culture filtrate	No. of seeds taken	No. of seeds germinated	No. of seedlings with cotyledons	No. of seedlings with radicle only	Length of radicle (in cms)	Brown discolouration of radicle or root
Undiluted	100	22	3	19	0,2—0,8	—
1:5	100	49	21	28	0,2—1,4	—
1:10	100	59	40	19	0,2—1,7	—
1:20	100	70	59	11	0,2—2,6	—
Control	100	94	94	—	0,8—3,4	—

Table II

Showing effect of soaking of seeds of cauliflower in culture filtrates (for different hours) on their germination

Time taken for soaking (in hours)		No. of seeds placed for germination	No. of seeds germinated	No. of seedlings with cotyledons	No. of seedlings with radicle only	Length in cms. of radicle or root	Brown Discolouration in radicle or root
6	Test	100	81	70	11	0,2—3,2	—
	Control	100	92	92	0	0,5—3,4	—
12	Test	100	59	40	19	0,2—2,6	—
	Control	100	89	89	9	0,5—3,1	—
18	Test	100	43	30	13	0,2—1,7	—
	Control	100	91	91	0	0,5—3,6	—
24	Test	100	30	12	18	0,2—0,9	—
	Control	100	90	90	0	0,5—3,4	—

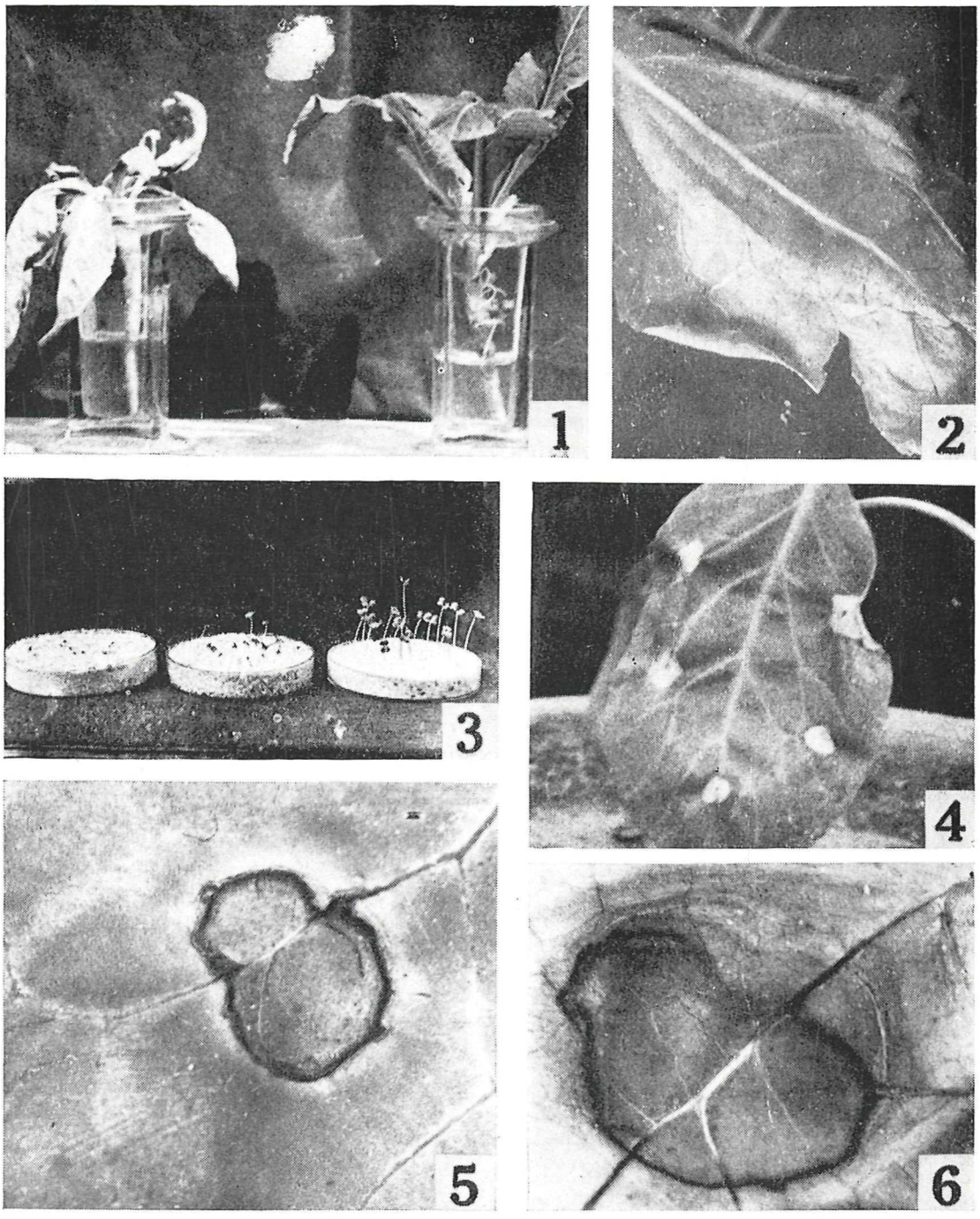

Fig. 1. Showing wilting of shoot-cuts of *Brassica oleracea var. botrytis* when kept in culture filtrate. Right: Control, Left: Treatment

Fig. 2. A close up of leaf of cauliflower showing marginal drying and curling.

Fig. 3. Showing wilting of seedlings of cauliflower in sand having been given pre- and post-emergence treatment with culture filtrate; from left to right: left one showing pre-emergence treatment, middle one: showing post-emergence treatment, extreme right: control.

Fig. 4. Showing formation of spots only by ether extracted fraction on the leaves of cauliflower.

Fig. 5—6. Spot formed by the isolated toxin (obtained after thin layer chromatography). 5. Spot in early stage. 6. a mature spot.

brownish papery spot (fig. 4) with irregular margins. Effect of further isolated fraction: One of the three fractions obtained by chromatography produced typical lesions on the leaves (Fig. 5, 6), thus indicating the presence of an active principle in it. The early symptoms of the spots where the fraction was applied was slight yellowing of the affected portion mixed with light brown discolouration nearly after 12 hours of inoculation. The etiolation slowly increased in the middle region showing gradual necrosis (Fig. 5). The whole tissue which was covered by the inoculated drop became necrotic with well defined spot. The surrounding tissue of the spot also showed etiolation and loss of turgidity, which ultimately dried up and finally became papery in nature. It also showed wrinkles which appeared in the form of faint concentric zonations. It was also observed that when the toxin was applied on the main vascular vein of the leaf, the whole leaf collapsed and symptoms like chlorosis and drooping were very well observed.

The cut twigs showed wilting and typical symptoms when dipped in the ether soluble fraction of the culture filtrate. The wilting occurred in about 20—22 hours in comparison to crude culture filtrate in which the twigs took about 28 hours to wilt. It was interesting to note that in this treatment small discoloured areas, somewhat similar to those produced by the toxin (ether fraction) developed on the leaves.

Histopathological changes caused by isolated toxin: Sections through the toxin treated tissue in the earlier stages showed light brown discolouration. Later, the discolouration became quite dense, the cells became irregular in shape and the tissue is seen nearly in collapsed condition. It was also observed that cell walls do not remain of same thickness, they become slightly thinner at places and it is at these points of weakness that the walls show bending in different directions resulting in irregular outline of the cells as well as the compression of the tissue. In early stages application of the toxin on the leaves showed shrinkage and plasmolysis of the cytoplasmic contents.

Effect of toxin on the respiration of the cauliflower leaves: As evident from the Table III the oxygen uptake in the initial stages of 24 hours increased to about 3.5 times that of the normal leaves. The rate of O_2-uptake decreased with increase in the time of treatment. The oxygen uptake came down to 1.6 and 1.28 times in 48 and 72 hours respectively. The decrease in the O_2-uptake indicated that action of toxin killed the tissue with increase in the age of treatment, and this might be one of the main factors in bringing down the rate of oxygen-uptake apart from other important metabolic changes.

Nature of toxin: Though it has not been possible so far to confirm the true nature of the toxin, the IR spectrum analysis of the fraction which caused lesions on the leaves showed absence of aromatic peak

indicating the aliphatic nature of the toxin. The toxic metabolite seems to be a mixture of substances as revealed by the spectrum. Further efforts to isolate the toxin in greater quantity and its characterisation are being made.

Table III

Effect of toxin of *Dothiorella phaseoli* on respiration of cauliflower leaves.

Time of harvesting	µl of O_2 taken up in 60 minutes *	
	Healthy	Toxin treated
After 24 hours	8,87	28,55
After 48 hours	9,17	15,245
After 72 hours	8,98	11,565

* µl of oxygen taken up per 100 mg fresh-weight of tissue.

Discussion

The results obtained during the present study clearly showed that an isolate of *D. phaseoli* which caused leaf-spot disease in cauliflower *(Brassica oleracea var. botrytis)* produced a thermostable toxic metabolite in vitro and in vivo. This toxic metabolite proved injurious to the host plant as it produced necrotic lesions on the leaves and also caused wilting of the shoot-cuts. The toxic substance was found effective even when diluted to various concentrations. These observations are in agreement with those of Mathur (1968) and Chan and Sackston (1967 and 1969) who reported the toxin production by the isolates of *D. phaseoli* pathogenic on sunflower and bean, and that of cotton respectively.

Among the various organic solvents tried for the isolation of toxic principle, ether was found to be the best. Differential isolation from the ether extract by thin layer chromatography helped in the separation of the active toxic principle, which quickly produced necrotic spots and lesions on the leaves of the host plant.

Ten days old culture filtrate showed maximum toxic activity after which there was no major difference in the activity upto 30 days old culture filtrates.

The culture filtrate containing toxic metabolite caused lesions on the leaves and also prooved injurious to the shoot-cuts of various cruciferous and non-cruciferous hosts used in the study and the appearance of the symptoms on the treated cut shoots was almost similar in all the cases. This observation clearly indicates that the toxic metabolite

is of non-specific nature. These observations are at variance with those of Bremer (1954) who reported the toxin produced by *D. phaseoli* to be host specific.

The culture filtrate also inhibited the germination of seeds of the host plant as well as of some other crucifers. It also affected the development of radicle and cotyledons. With increased dilutions of the culture filtrate the percentage of seed germination also increased. There was also a relative decrease in the length of the radicle or root and in the frequency of the root hairs with increase in the concentration of the culture filtrate. Inhibition of seed germination due to fungal toxins in various degrees has been reported by Mahadevan and Caroselli, 1965; Pringle and Scheffer, 1967; Lakshman and Vanterpool, 1967.

The toxic metabolite has also been observed in vivo and on isolation it produced necrotic spots and lesions on the host leaves that were similar to those of natural infection. Thus the presence of the toxic substance both in vitro and in vivo suggests that it is somehow involved in the pathogenesis. Moreover, the toxic metabolite appeared to be a product of actively growing hyphae as the perceptible toxic activity in the culture filtrate was observed even on 5th day after inoculations.

Treatment of the host leaves with the purified toxic substance besides causing necrotic spots also caused increase in rate of respiration of the affected tissue. The increase in respiration due to fungal toxins has been reported by several workers (Allen, 1953; Romanko, 1959; Grimm and Wheeler, 1963; Scheffer and Pringle, 1963; Wheeler and Luke, 1963; Dimond, 1970). Dimond (1970) has attributed the increase in respiration due to the toxins in plant diseases as an essential symptom of the disease physiology.

The preliminary observations made so far on the nature of the toxin have shown it to be of aliphatic nature.

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