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Effect of environmental factors on toxin production of *Drechslera bicolor,* a causal agent of leaf blight in bell pepper

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***Drechslera bicolor* (Mitra.) Subram. and Jain, cause of leaf blight in bell pepper [*Capsicum annuum var. grossum* (L.) Sendt.], leads to necrotic lesions, early leaf senescence and yield losses. Detailed physiological analysis can contribute to an improved understanding of bell pepper disease interaction and cultivar improvement. *D. bicolor* produced maximum toxin in Richards' medium and toxin was found most active at 25±2°C, pH 6.0 and 100% relative humidity. Different hosts were screened for disease resistance and it was observed that the partial purified toxin showed some degree of host specificity. Fungal toxin was able to produce symptoms in all the tested hosts with the main infected host being bell pepper. Prominent symptoms of chlorosis and wilting were observed on chilli followed by tomato, brinjal and lady finger. The detached leaf dip method followed by carborundum abrasion method was found to be the best inoculation methods in the production of wilting and chlorosis in bell pepper. These results indicated that the maintenance of physiological function during leaf blight infection could result in improved bell pepper yields under diseased conditions.**

Key words: *Capsicum annuum var. grossum*, leaf blight, physiology, phytotoxin, bioassay.

INTRODUCTION

Sweet or bell pepper (*Capsicum annuum* var. *grossum*, L.), a member of the family Solanaceae is regarded as one of the most popular and nutritious vegetable crops. During cultivation, the crop is affected by a large number of diseases caused by fungi, bacteria, viruses and mycoplasmas, which reduce yield drastically. These pathogens also attack during transit and storage (Chadha, 2003). Phytotoxins are important metabolic products produced by fungi, which partially or fully express characteristic disease symptoms (Samaddar and Scheffer, 1971). The earliest effects of phytotoxins has been reported to cause lesions on cell membranes hampering the cell permeability thereby triggering increased leakage of electrolytes from the susceptible tissues in low concentration dosages (Thatcher, 1939). Partially purified toxins from *Verticillium dahliae,* a wilt

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pathogen, were used to detect the losses of cell permeability in the susceptible tissues of cotton hosts (Gour and Dube, 1985). Interestingly, Gour and Agarwal (1988) has successfully demonstrated that partially purified toxins from wilt pathogen of *Fusarium oxysporum* f. sp. *cumini* did not induce wilt symptoms in resistant varieties, whereas the susceptible cultivars showed the development of characteristic symptoms, establishing the fact that the toxins can be used in screening the varieties for disease resistance. Considering this basic phenomenon, fungal toxins have been widely exploited for disease screening. The present experiment was undertaken for isolation of toxins and their bioassay on seed and intact plants to demonstrate the toxic efficacies.

MATERIALS AND METHODS

Isolation, identification and pathogenicity

Isolation was done from diseased leaves collected from Hi-tech Horticultural Farm, MPUAT, Udaipur and monoconidial culture maintained on potato dextrose agar slants for further studies. Identification of the fungus was confirmed by ITCC, New Delhi (Identification No. 279/6513-07). Koch's postulates were proved on cv. Bombay red plants before the fungus was tested for its potential to secrete the toxic metabolite.

Fungus characterization and host range

The number of conidia in the resultant suspension was determined using a haemocytometer, and expressed as number of conidia mm^2 of medium 1-20 spores per microscopic field- poor (+), 21-40 spores- good (++) and 41 and above spores abundant (+++). Spore size (length and width) measurements were taken by measuring 50 spores of each isolate using stage and ocular micrometer and septa were also counted in 50 spores of each isolate. Conidial development and variation in conidial morphology of isolates were studied by "slide culture" method. The blight development on different host leaves and fruits were recorded 5 and 6 days post inoculations, respectively.

Suitable medium, temperature, pH and relative humidity for growth, sporulation and toxin production

Twelve different natural, synthetic and semi-synthetic sterilized liquid media were examined under similar conditions of pH, temperature and incubation period, to find out the most suitable medium for all further studies. The amount of sporulation was determined on the basis of number of spores per microscopic field at 100x magnification. The effect of six different temperatures viz., 15, 20, 25, 30, 35 and 40 ± 2°C on the growth, sporulation and production of toxin was examined. Observations of sporulation and weight of mycelial growth were also recorded. Evaluation of suitable pH for maximum growth and toxin production was done using flasks containing 25 ml Richard's medium adjusted to six different pH levels. The growth of fungus at different pH was measured by determining mycelial dry weight and sporulation was measured under low microscopic field. The different humidity levels were first prepared by adding equal volume of concentrated H_2SO_4 and distilled water and further dilutions were made as par Buxton and Mellanby (1934). Observations were recorded for mycelial weight and sporulation.

Toxin study

Extraction of toxins

250 ml Erlenmeyer flasks each containing 30 ml of Czapek-Dox medium were inoculated with 4 mm diameter fungal plugs of 7-day old cultures of *D. bicolor.* After 15 days of growth under stationary conditions at room temperature (25±2°C), a cell free clear culture filtrate was obtained by filtration of fungal growth through Whatman filter paper no 42 (Gour and Agrawal, 1988).

Purification of toxin(s)

The culture filtrate was centrifuged at 3000 rpm for 20 min. The clear supernatant solution was collected in a clean sterilized conical flask and pellet sedimented at the bottom of the centrifuge tube was discarded. The clear supernatant solution served as a sample of crude toxin preparation produced by *D. bicolor in vitro*. The crude toxins were partially purified for its active factors using ethyl alcohol (1:2), ammonium sulphate (1:2) and acetone (1:2) fractionation (Gour et al., 1992). Chilled solvents were added to each of the 50 ml of culture filtrates and kept at 4°C. The precipitate collected was dissolved in double-distilled water. Since the solution obtained from ammonium sulphate solvent was comparatively found more toxic, all further steps of purification of toxins were carried out with only ammonium sulphate fractionated solutions following the method described by Kumar et al. (2013).

Bioassay tests

Different methods viz., detached twig dip, pin prick, carborundum abrasion, toxin(s) spray and injection were used for bioassay of toxin. All the methods were run in four replications under similar conditions. The leaves were observed for symptom development after 24 h of treatment. A control of sterile distilled water was kept for each treatment (Kumar et al., 2013). The disease symptoms were rated on a disease rating scale: For chlorosis (0-5): 0 = no chlorosis; 1 = slight chlorosis occurs and covers >/= 1% leaf area; 2 = slight chlorosis with slight vein clearing; 3 = chlorosis increase and primary lesions formed; 4 = light brown patches formed; 5 = chlorosis increase and covers > 50% leaf area. For wilting (0-5): 0 = no wilting; 1 = slight wilting occurs; 2 = wilting increased but leaves contained some water; 3 = leaves curled by wilting; 4 = leaves dried and completely curled; 5 = leaves completely dried and dead.

RESULTS

Initial symptoms appeared as yellowing of leaves near the tip of young leaves. This was followed by a rapid increase in leaf diseased area. Large straw or brown blight patches were formed which covered the whole leaf area leading to leaf coalescence and drop. The apical portion of sweet pepper fruit was found rotten with rapid discolouration and ultimate progression of internal decay. Later on, the rotted fruits became completely deformed.

Fungus characterization

The fungal colony was bottle green to whitish grey black in colour, hyphae with olivaceous dark green septate (width 10.73 µm); conidiophores were single or in groups,

Table 1. Host range studies of *D. bicolor*.

Plant	Scientific name	Plant part	Reaction (blight/wilt)	Incubation period (Days)
Bell pepper	*Capsicum annum var.grossum* L.	Fruit	+	6
Chilli (Morphological types)				
(a) Thin	*Capsicum annum var. frutescens* L.	Fruit	+	6
(b) Thick	*Capsicum annum var. chinense* L.	Fruit	+	6
(e) Yellow thick	*Capsicum annum var. annum* L.	Fruit	+	6
Lady finger	*Abelmoschus esculentus* (L.) Moch.	Fruit	+	8
Brinjal	*Solanum melongena* L.	Fruit	+	8
Tomato	*Solanum lycopersicum* (L.) Mill.	Fruit	+	8
Datura	*Datura stramonium*	Fruit	+	9
Chilli	*Capsicum annum* L.	Leaf	+	5 – 7
Bell pepper	*Capsium annum var.grossum* L.	Leaf	+	5 – 7
Brinjal	*Solanum melongena* L.	Leaf	+	5 – 7
Tomato	*Solanum lycopersicum* (L.) Mill.	Leaf	+	5 – 7
Lady finger	*Abelmoschus esculentus* (L.) Moch.	Leaf	+	8-10
Groundnut	*Arachis hypogaca* L.	Leaf	+	8-10
Cluster bean	*Cyamopsis tetragonoloba* L.	Leaf	+	8-10
Sorghum	*Sorghum bicolor* (L). Monech	Leaf	+	10-12
Maize	*Zea mays* L.	Leaf	+	10-12

+ = Visible symptoms.

sometimes swollen at the base up to (400 x 5-10 µm) and conidia were light brown to olive brown, straight, cylindrical, rounded with 5-12 septa, (104.03 X 13.43 µm); central cells of mature conidia were dark brown or smoky brown. On 15 different plant species, host range was studied and the fungus produced visible symptoms on all hosts within 5-12 days post inoculation (Table 1).

Media, temperature, pH and relative humidity for growth, sporulation and toxin production

Maximum toxic metabolites were present in the cultural filtrate obtained from inoculated Richard's liquid medium, where the susceptible twig not only expressed maximum chlorosis (3.1) but also maximum wilting (5.0). Similarly, malt extract liquid medium was also found better for the production of the maximum chlorosis and wilting. However, it is evident from this study that the toxic metabolites were present in all the culture filtrates tested from different synthetic media (Table 2). Maximum fungal growth, chlorosis, wilting and toxin production were found at 25±2°C followed by 30±2°C. Nevertheless, the quantity of toxin production correlated to the growth of the pathogen, that is, least chlorosis and wilting was obtained at 40±2°C (Table 3). The production of toxin was also influenced by different pH levels. Maximum toxic metabolites were produced at pH 6.0 as indicated by twigs dip in different treatment solutions after 72 h of toxin treatment and least activities of toxin was noticed at pH 4.0 and 9.0 (Table 4). Optimum relative humidity for the growth and sporulation of *D. bicolor* was between 90 and 100%. Maximum growth of the fungus was found at 100% RH as well as maximum chlorosis and wilting at this level, indicating maximum production of toxin which was followed by 90% RH (Table 5).

Toxin study

Fungal pathogens are known to synthesize various kinds of secondary metabolites, which play a key role during pathogenesis. Toxins are important metabolites to express symptoms of diseases after the post infection stages.

Toxin bioassay

D. bicolor produced metabolites on Czapek-Dox medium which by partial purification with ammonium sulphate, induced characteristic disease symptoms. Toxin-treated plant cuttings indicated yellowing of older leaves within 12 h, there was general yellowing after 25 h and the leaves dried completely after 48 h of treatment. Since the ammonium sulphate extracted toxin solutions caused severe chlorosis and wilt in bell pepper plant cuttings, ammonium sulphate was the most suitable solvent for isolation of toxins from culture filtrates. However, fractionation with acetone and ethyl alcohol was partly successful (Table 6).

Table 2. Effect of different media on growth, sporulation and toxin production of *D. bicolor.*

Media	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
Sach's	84.75	+	0.00	0.50	0.75	0.00	0.25	0.50
Asthana & Hawkar's	107.50	+	0.25	0.50	0.75	0.25	0.50	1.00
Sabourd's	95.25	++	0.50	0.50	0.75	0.25	0.50	1.00
Czpek Dox	134.25	++	0.50	0.75	1.00	0.50	1.00	2.00
Richard's	361.25	++	1.00	2.00	3.10	1.75	3.00	5.00
Malt extract	288.00	+++	1.25	2.05	3.00	2.00	3.05	5.00
Yeast extract	144.00	++	0.50	0.50	0.75	0.50	1.00	2.00
Potato dextrose	207.50	+++	0.75	0.75	1.25	0.50	1.25	2.25
Bell pepper leaf	253.25	++	0.50	1.00	1.50	0.75	1.50	2.50
Basal medium (modified)	193.75	++	0.50	0.75	1.25	0.50	1.25	2.25
Elliot's' medium	144.50	+	0.50	0.50	1.00	0.50	1.00	2.00
Oat meal	146.00	++	0.50	0.75	1.00	0.50	1.00	2.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.51							
CD 0.05	7.19							

*Average symptom rating of five replications (0-5 rating scale); - = Nil, + = poor, ++ = good and +++ = abundant.

Table 3. Effect of different temperatures on growth, sporulation and toxin production of *D. bicolor.*

Temperature (± 2°C)	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
15	131.50	++	0.75	1.00	1.25	1.00	1.50	2.75
20	164.75	++	1.00	1.25	1.50	1.00	2.00	3.00
25	208.75	+++	1.00	1.50	2.00	2.00	3.75	5.00
30	187.50	+++	1.00	1.25	1.75	2.00	3.25	3.75
35	107.50	+	0.75	1.00	1.00	0.50	1.00	1.25
40	82.75	-	0.00	0.75	1.00	0.00	0.75	1.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.78							
CD 0.05	8.25							

*Average symptoms rating of four replications (0-5 rating scale); - = Nil, + = poor, ++ = good and +++ = abundant.

Table 4. Effect of different pH on growth, sporulation and toxin production of *D. bicolor.*

pH	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
4	85.75	++	0.50	1.25	1.50	1.00	1.25	1.75
5	169.75	++	0.75	1.50	1.75	1.50	3.00	3.25
6	183.75	+++	1.00	2.00	3.00	2.00	3.75	5.00
7	176.25	+++	1.00	1.75	2.00	1.75	3.50	4.50
8	99.50	++	0.50	1.00	1.25	1.25	1.75	2.00
9	80.25	+	0.00	0.00	0.50	0.75	1.00	1.50
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	1.821							
CD	5.410							

*Average symptoms rating of four replications (0-5 rating scale); - = nil, + = poor, ++ = good and +++ = abundant.

Table 5. Effect of different relative humidity on growth, sporulation and toxin production of *D. bicolor.*

Humidity (%)	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
40	144.75	-	0.25	0.50	1.00	0.75	1.00	1.50
50	165.50	+	0.75	1.00	1.50	1.00	1.25	1.75
60	174.25	++	1.00	1.50	1.75	1.25	1.50	2.00
70	182.75	++	1.00	1.75	2.00	1.50	1.75	2.50
80	199.50	++	1.25	2.00	2.50	1.75	2.00	2.75
90	285.75	+++	1.50	2.25	3.00	2.00	2.25	3.00
100	317.50	+++	1.75	2.50	3.75	2.25	3.50	5.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.127							
CD	6.255							

*Average symptoms rating of four replications (0-5 rating scale); - = nil, + = poor, ++ = good and +++ = abundant.

Table 6. Partially purification of toxin (s) from culture filtrates of *D. bicolor* with different solvents.

Solvents	Plant cuttings*	
	Wilting	Chlorosis
Ammonium sulphate	5.0	5.0
Ethyl alcohol	2.0	3.5
Acetone	2.2	3.0
Sterilized uninoculated Czapek-Dox medium	0.0	0.0
Distilled water	0.0	0.0

*Average symptom rating of five replications (0-5 rating scale).

Table 7. Effect of different bioassay methods on production of symptoms by partially purified toxin (s) of *D. bicolor.*

Methods of toxin treatments	Detached		Intact	
	Wilting	Chlorosis	Wilting	Chlorosis
Detached leaf dip method **	5.00	4.00	-	-
Pin prick method	2.50	3.80	2.50	3.00
Carborundum abrasion method	2.75	3.90	3.00	4.00
Injection method	2.00	3.80	2.75	3.50
Spray method	1.00	1.00	2.00	2.00

* Average symptom rating of five replications (0-5 rating scale); ** only *in vitro* treatment.

Bioassay tests

Detached leaf dip method and carborundum abrasion method were the best inoculation method in the production of typical disease symptoms (Table 7). In the detached leaf twig method leaf chlorosis was followed by wilting and drooping.

DISCUSSION

Fungus characterization

Similar descriptions was reported by Pandey and Shukla (1978) who studied the host range of *Helminthosporium* spp. causing leaf spot diseases of sorghum.

Media, temperatures, pH and relative humidity for growth, sporulation and toxin production

Various workers studied different media, temperatures, pH and relative humidity levels against different plant pathogenic fungi in the laboratory and came with interesting results. Shukla and Husain (1987) reported Fries' medium, pH 5.0 and 21 days of incubation for production of toxic metabolites of *Drechslera maydis* causing severe leaf blight of *Costus speciosus*. The toxin was stable in acidic conditions (pH 3.5-7.0) but unstable under alkaline conditions. Raut and Wangikar (1974) reported Czpek's medium, temperature 25°C, 90-100% RH and pH 6 as best for *Drechslera tetramera,* leaf spot of barley. Kumar and Mishra (1993) found PDA with pH 6.8- 7.0 as best for *Drechslera oryzae* and Nagaraja et al. (1992) reported Richard's medium, 25°C temperature, pH 5.8 as best for *Drechslera sorokiniana* leaf spot of *Dioscorea* sp.

Toxin bioassay

Janardhanan et al. (1981) isolated toxin from the culture filtrate of *D. maydis* and induced necrosis and chlorosis on *Costus speciosus* leaves followed by severe yellowing and defoliation. It also produced symptoms on mono and dicot plants. It showed strong growth inhibiting activity, causing total inhibition of root elongation in germinating wheat seeds. Gour and Dube (1985) isolated partially purified toxins from *Verticillium dahliae,* a wilt pathogen, and detected the loss of cell permeability in the susceptible tissue of cotton hosts. Similarly, Gour and Agarwal (1988) isolated partially purified toxins from *Fusarium oxysporum* f. sp. *cumini* and demonstrated use of toxins in screening the varieties for diseases resistance. Kramer et al. (1989) in their study found that partially purified toxin preparation from culture filtrate of the pathogen *D. teres* caused concentrated specific necrotic lesions on leaf tips and margins of susceptible genotypes of barley.

Bioassay tests

Kumar et al. (2013) found that the plants which were spray inoculated with different dilutions of *A. alternata* partially purified toxin developed chlorosis initially followed by scattered black dotted spots on leaves sprayed with solution of 2:8 dilutions under pot conditions. Fungal pathogens are known to synthesize various kinds of secondary metabolites which play a key role in patho-genesis. Several workers have documented the properties and specificity of fungal toxins in host plants. Gilchrist and Grogean (1976) and Kramer et al. (1989) observed similar results with the toxins produced by *A. alternata* and *Drechslera teres*, respectively. Effects of phytotoxic metabolites of *A. solani* resulted in marginal and inter veinal leaf necrosis and subsequent wilting of tomato seedlings (Maiero et al., 1991).

Conflict of interests

The authors did not declare any conflict of interest.

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