# *In Vitro* Toxin Production by *Fusarium solani* f. sp. *piperis*

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## ABSTRACT

*Fusarium solani* f. sp. *piperis* (teleomorph: *Nectria haematococca* f. sp. *piperis*), causal agent of root rot and stem blight on black pepper (*Piper nigrum*), produces secondary metabolites with toxigenic properties, capable of inducing vein discoloration in detached leaves and wilting in transpiring microcuttings. Production of *F. solani* f. sp. *piperis* (Fsp) toxic metabolites reached a peak after 25 days of static incubation on potato sucrose broth at 25 °C under illumination. Changes in the pH of the culture filtrate did not alter the effect of toxic metabolites. However, when the pH was changed before the medium had been autoclaved, a more intense biological response was observed, with an optimum at pH 6.0. Isolates that produced red pigments in liquid cultures were more efficient in producing biologically active culture filtrates than those which produced pink coloured or clear filtrates suggesting that these pigments could be related to toxigenic activity. Detached leaves of seven black pepper cultivars and *Piper betle* showed symptoms of vein discoloration after immersion in autoclaved and non-autoclaved Fsp culture filtrates indicating the thermostable nature of these toxic metabolites.

**Additional keywords**: Secondary metabolites; resistance; black pepper; *Piper betle*.

## RESUMO

**Produção de toxinas *in vitro* por *Fusarium solani* f. sp. *piperis***

*Fusarium solani* f. sp. *piperis* (teleomórfico: *Nectria haematococca* f. sp. *piperis*) agente causal da podridão-das-raízes e do secamento-dos-ramos da pimenteira-do-reino (*Piper nigrum*) produz metabólitos secundários com propriedades tóxicas capazes de induzir descoloração das nervuras em folhas destacadas e murcha em micro-estacas. A produção de metabólitos tóxicos alcançou o pico após 25 dias de incubação, sob iluminação. Variações no pH do filtrado da cultura não aumentaram o efeito tóxico, entretanto, quando o pH foi ajustado antes da esterilização do meio de cultura, uma resposta biológica mais intensa foi observada, atingindo o máximo em pH 6,0. Isolados que produziram pigmentos vermelhos no meio de cultura foram mais eficientes em produzir filtrados biologicamente ativos do que aqueles que produziram filtrados de coloração rósea ou branca sugerindo que estes pigmentos podem estar relacionados com atividade toxigênica. Folhas destacadas de sete cultivares de pimenta-do-reino e *Piper betle* exibiram sintomas de descoloração das nervuras após imersão em filtrados autoclavado e não-autoclavado indicando a natureza termoestável destes metabólitos tóxicos.

## INTRODUCTION

During their development on host tissues or in culture, fungi can produce a wide range of toxic compounds with varied biochemical structures and modes of action. These include polypeptides, glycoproteins, aminoacid derivatives, polyketides, terpenoids, sterols and quinones (Kono *et al.*, 1981; Stoessl, 1981). Such compounds have been named pathotoxins or simply toxins.

The role of a toxin in a plant disease is not always easy to elucidate. A range of criteria is used to evaluate a substance before it is regarded as a factor in pathogenicity or virulence. It is important that a toxin should be detectable in infected plants, induce typical symptoms of the disease itself and that toxin production across a range of pathogen isolates correlate with their pathogenicity. These criteria have been satisfied for very few putative toxins (Yoder, 1980; Isaac, 1992). Nevertheless, there is considerable evidence that pathogenicity of certain fungi is closely related to the production of host selective toxins by the invading organisms (Arntzen *et al.*, 1973).

*Fusarium solani* (Mart.) Sacc. f. sp. *piperis* Albuq. and other species of *Fusarium* from the group *Martiella* Wollenweber, to which *F. solani* f. sp. *piperis* belongs, produce several phytotoxic red pigments with a common naphthazarin structure. Six compounds with different functional groups and biological activity have been isolated (Kern, 1972; Drysdale, 1984).

Since long time ago, it has been shown that black pepper (*Piper nigrum* L.) stems and stem cuttings, when infected by *F. solani* f. sp. *piperis* turn yellowish before becoming necrotic. It has also been noticed that fresh isolates

of *F. solani* f. sp. *piperis*, which are highly virulent to black pepper plants produce red pigments into culture medium. However, isolates maintained *in vitro* for two or more years, although pathogenic, can lose their ability to produce pigments, giving rise to pink or clear coloured filtrates. Black pepper stem cuttings inoculated with these three types of isolates (red, pink or clear) exhibit different degrees of symptoms, leading to the supposition that coloured toxic metabolites might be associated with the virulence of *Nectria haematococca* Berk. & Br. f. sp. *piperis* Albuq. isolates (Duarte & Albuquerque, 1979a; 1984). These observations hint that the pathogen may be toxigenic when growing in its natural substrate and provide a starting point for the present investigation.

The objectives of this study were to determine the conditions required for toxin production and the host response to fungal culture filtrates.

## MATERIAL AND METHODS

### Plant material

Three species of the genus *Piper* were used in this study. black pepper cultivars 'Bragantina' (ecotype of 'Panniyur-1' hybrid), 'Cingapura' 'Guajarina' (ecotype of 'Arkulan Munda'), 'Iaçara-1', 'Karimunda', 'Kottanadan-1' and 'Kuthiravally'; *P. attenuatum* Buch.-Ham. ex Miq. and *P. betle* L.

### Fungal isolates

*Nectria haematococca* f. sp. *piperis* (Nhp) or *F. solani* f. sp. *piperis* (Fsp) were isolated from diseased tissues at the base of infected plants and from young lesions formed on stems, respectively. Fungal isolates were named according to their geographical origin or hosts as follows: Altamira -1, Altamira-2, Altamira – 3, Bonito, Castanhal-1, Castanhal-2, Igarapé-Açu, Jaburu, Ourem, Santarem Novo, Karimunda and Panniyur-1 from state of Para and São Mateus from the state of Espírito Santo, Brazil. Isolates from Jaburú, São Mateus, Altamira-3 and Castanhal-2 were used in the tests for producing strong pigmented cultures.

### Fungal culture filtrate

To obtain filtrates of the fungal cultures, flasks containing 250 ml potato-sucrose broth were inoculated with one 10-mm-diameter mycelial disk of red coloured culture of Fsp. Flasks were incubated statically up to 30 days at 25 °C under continuous illumination provided by two 40 watt fluorescent tubes at a distance of 40 cm or under darkness. Filtrates were harvested by filtering first through a nylon net cloth, twice through Whatman no. 1 paper circles before being passed three times through a re-sterilizable Nalgene® filtration unit containing, successively, cellulose ester membrane filters of 0.1 µm, 0.65 µm and 0.2 µm pore. Filtrates were then stored at 5 °C, in sterile bottles.

### Experiments

Culture filtrates of the isolate Altamira-3 were used for testing the effect of incubation time and concentration of Fsp culture filtrate on the induction of symptoms. Two flasks were harvested at ten, 15, 20, 25 and 30 days, repeatedly filtered and stored at 5 °C, until use. Culture filtrates were diluted with sterile distilled water to give concentration of 10%, 20% and 50%. Detached leaves of cultivar Kottanadan-1 were immersed in these solutions and infiltrated with crude culture filtrate to test the biological activity of these filtrates. Four replicates were used per treatment.

To test different defined culture media on the production of toxic metabolites, Fsp isolate 'São Mateus' was cultured in Czapek-Dox solution salts, Czapek-Dox half strength, Czapek-Dox quarter strength, Richard's solution salts, Richard's solution half strength, Richard's solution quarter strength, potato-sucrose broth and Sabouraud broth (Tuite, 1969). The pH of all culture media was adjusted to 5.8 by adding drops of 1M NaOH or 1M CH$_3$COOH solution. Stationary flasks were incubated at 25 °C under continuous illumination for 25 days, when liquid cultures were harvested, filtered and diluted to 1%, 10%, 20% and 50% concentration. After immersion of transpiring microcuttings of cultivar Kottanadan-1 in diluted culture filtrates, vials were placed inside gear boxes with moistened paper towel under continuous illumination at 25 °C.

The effect of pH on Fsp toxin production was studied after growing Jaburu isolate in PS culture medium with pH adjusted to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 prior to sterilisation. Culture filtrates were tested at concentrations of 10%, 20% and 50% in a detached leaf bioassay using cultivar Kottanadan-2 under the same incubation conditions. Distilled water (pH 4.7) was used as control. Toxicity was measured on a six-point graded scale and data transformed to give the average severity index (ASI)

The relative production of toxin by 11 isolates of Fsp was detected after growing those isolates in 250 ml PS medium under the same conditions as used in the previous experiments. Those filtrates were tested at different concentrations on a transpiring microcutting bioassay. Quadruplicate records were made for each treatment. Liquid cultures yielded clear and red filtrates, which were stored at 5 °C before use.

In order to determine the effect of autoclaving culture filtrate on symptom development on *Piper* spp., leaves of 'Bragantina', 'Cingapura', 'Guajarina', 'Iaçara-1', 'Karimunda', 'Kottanadan-1', 'Kuthiravally' and *P. betle* were harvested at an intermediate stage of development (third or forth leaf from apex of the main branch) and the cut petiole was immersed in diluted culture filtrate either autoclaved and non-autoclaved at concentrations of 10%, 20% and 50% and incubated at 25 °C. Each treatment was replicated four times. Symptoms were evaluated and recorded after 48 h of immersion, and again after 96 h when symptoms were well evident.

### Assessment of experiments

All toxicity experiments involving Fsp culture filtrates were evaluated qualitatively by a graded scale. If transpiring microcuttings were used as the biological indicator, the effect of toxic metabolites was recorded on a 1 - 4 scale where: 1 =

healthy with green leaves and white adventitious roots; 2 = flaccid foliage, but still green or with black punctuation on stem epidermis immersed into filtrates; 3 = flaccid and yellowish leaves, epidermis immersed into solution darkened; 4 = shoot tip wilting or completely desiccated, discoloured adventitious roots.

When detached leaves were used in the bioassay the results were recorded using the following scale: 1 = no symptoms; 2 = yellowish or flaccid tissues; 3 = less than 15% vein discoloration; 4 = 16% to 25% vein discoloration; 5 - 26% a 50% vein discoloration; 6 = 51 a 75% vein discoloration. Scale notes recorded were transformed into an Average Severity Index (ASI) by the formula: ASI = ∑ (nki)/ N in which, n = number of microcuttings or detached leaves ascribed to each disease score N= total number of inoculated tissues, and i = symptom severity score (i = 1 to 4 or i = 1 to 6) (Hartman et al., 1984).

## Statistical analysis

The significant effect of each treatment was analysed in an extension of the Kruskal-Wallis two-way analysis of variance (Zar, 1999) and treatment means were compared using rank means, which parallel the Tukey test at $\alpha = 0.05$ (Miller, 1966). Since three factors were tested in the detached leaf bioassay, a three-factor ANOVA was performed using the ranks of the data instead of raw data to calculate the square sums and degrees of freedom (Sokal & Rohlf, 1981; Zar, 1999).

## RESULTS

### Effect of incubation time and concentration of Fsp culture filtrate on induction of symptoms

The first signs of uptake of toxic metabolites by black pepper tissues, characterised by discoloured secondary veins were observed 48 h after immersion in 50% and 20% filtrate solutions harvested at five-day intervals.

The most intense symptoms were associated with filtrates harvested at 20 days. Symptoms were more severe by 96 h and with concentrated filtrates (Table 1). Non-inoculated culture medium had no effect on black pepper tissue suggesting that the symptoms on detached leaves were caused by fungal metabolites.

Culture filtrates harvested at different ages also showed some differences, supported by analysis of variance when the means of ranks were compared (Table 1). Significant differences were observed among filtrates harvested at 15, 20 and 25 days when compared with ten and 30 days (p<0.01). The intensity of vein discoloration was highly dependent upon the concentration of culture filtrate in solution, but no relationship was observed between age at harvest and culture filtrate concentration.

Approximately 48 h after infiltration of crude culture filtrate, lesions were observed on black pepper detached leaves. From the central puncture, lesions spread throughout the central area of the lamina. These lesions were irregular, black

and soaked, surrounded by a yellow halo in which do not consider discoloured secondary and tertiary veins could be seen. Large lesions were formed after infiltration of culture filtrates harvested after 20, 25, and 30 days (Figure 1).

### Effect of different culture media on the production of Fsp toxic metabolites

Although the first symptoms appeared by 72 h, ratings were recorded five days after immersion of microcuttings (Table 2). Significant differences were observed between different culture media and concentrations of culture filtrates, with Sabouraud and potato-sucrose (undefined medium) being best. Analysis of variance showed that Sabouraud medium was more suitable for secondary metabolite production causing extensive necrosis even at 10%, followed by PS. The defined culture media did not differ significantly from each other except that Czapek-Dox at half and quarter strength was the least effective. Changes in initial pH from 4.6 (Czapek-Dox) and 4.1 (Richard's solution) to 5.8 may have prevented secondary metabolite production.

Non-inoculated and sequentially filtered culture media were tested at 10%, 50% and 100% (undiluted) in order to observe the possible interference of culture media constituents. No symptoms were observed in the bioassay suggesting that no constituents of the culture media tested caused symptoms on detached leaves.

### Effect of pH on Fsp toxin production

The first signs of damage were observed 24 h after immersion of leaves in a 20% filtrate solution, pH 6.0. After 96 h, symptoms were evident in most other treatments at 50% concentration (Table 3).

Measurements of pH after 25 days of incubation showed some changes from initial values. At pH 3.5 although no symptoms were induced on the lamina, petioles collapsed and were blighted probably as a direct result of the low pH alone. It is clear that PS culture medium at an initial pH 6.0 seems to be

**TABLE 1** - Effect of *Fusarium solani* f. sp. *piperis* culture filtrates of different ages and concentrations on detached leaves of black pepper (*Piper nigrum*), cultivar Kottanadan-1 after 96 h immersion (mean of four replicates)

| Source and age of Culture filtrate at harvest (days) | Filtrate concentration and scored symptoms | | | Contrast of Means for age at harvest |
|---|---|---|---|---|
| | **10%** | **20%** | **50%** | |
| 10 | 1.25 | 1.25 | 3.25 | 15.33  b |
| 15 | 3.00 | 3.25 | 4.50 | 36.79  a |
| 20 | 3.00 | 3.25 | 4.50 | 39.58  a |
| 25 | 2.00 | 4.50 | 4.50 | 32.36  a |
| 30 | 1.25 | 2.25 | 4.50 | 28.42  b |
| Control (PS) | 1.0 | 1.00 | 1.00 | - |

Figures are Average Severity Index derived from the following symptoms scale: 1 = no symptoms; 2 = yellowish or flaccid tissues; 3 = less than 15% vein discoloration; 4 = 25% vein discoloration; 5 = 50% vein discoloration and 6 = 75% vein discoloration. [1]Means followed by the same letter do not differ significantly (p<0.01).

most suitable for toxin production and biological activity, although when harvested at 25 days this has dropped to pH 4.6, the value at which the bioassay was performed. A second lesser optimum seems to occur at around pH 4.0 to 4.5. These views were confirmed by the analysis of variance, which indicated that significant differences did exist (Table 3).

Vein discoloration was more intense at a concentration of 50% (p<0.001) but there was no interaction between pH and concentration of culture filtrate (Table 3).

### Relative production of toxin by isolates of Fsp

Different isolates of the pathogen produced different quantities of toxic metabolites as indicated, indirectly, by the intensity of symptoms induced in transpiring microcuttings of cultivars 'Guajarina' and 'Kottanadan-1'. Symptoms were observed five days after immersion of microcuttings, but in this experiment obvious symptoms were observed (score 4) in 50% of the culture filtrates tested at 50% of the initial
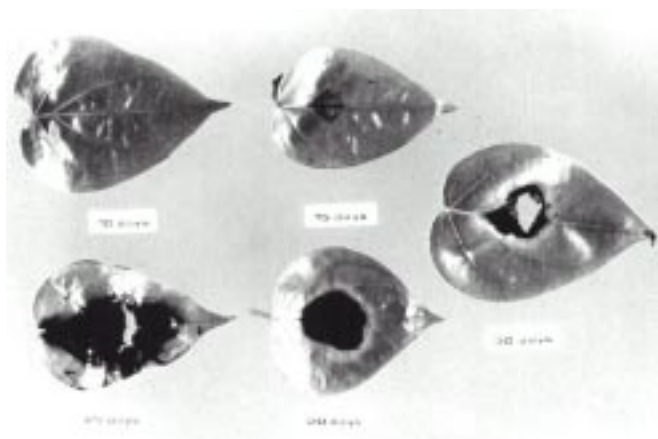


**FIG. 1** - Lesions formed on punctured leaves of black pepper (*Piper nigrum*) after infiltration of crude culture filtrate produced by Altamira-3 isolate, of various ages at harvest. Black pepper leaves, cultivar Kottanadan-1, photographed five days after infiltration.

**TABLE 2** - Response of black pepper (*Piper nigrum*) 'Kottanadan-1' to diluted filtrates from six defined culture media in comparison with potato-sucrose (undefined medium), 96 h after microcutting inoculation (mean of five replicates)

| Culture medium | Diluted culture filtrate | | | | | Contrast of means for |
|---|---|---|---|---|---|---|
| | Cont. | 1% | 10% | 20% | 50% | culture medium |
| Sabouraud | 1.0 | 1.2 | 4.0 | 4.0 | 4.0 | 121.55 a |
| Potato -sucrose | 1.0 | 1.4 | 2.2 | 3.2 | 4.0 | 103.90 b |
| Czapek -Dox | 1.0 | 1.4 | 1.8 | 2.2 | 2.4 | 79.40 c |
| Richard's solution/2 | 1.0 | 1.2 | 1.4 | 2.2 | 3.0 | 76.95 c |
| Richard's solution | 1.0 | 1.0 | 1.4 | 2.2 | 3.0 | 74.13 c |
| Richard's solution/4 | 1.0 | 1.2 | 1.6 | 2.0 | 2.6 | 70.35 c |
| Czapek -Dox/2 | 1.0 | 1.3 | 1.0 | 1.6 | 2.2 | 5 8.68 d |
| Czapek -Dox/4 | 1.0 | 1.0 | 1.0 | 1.4 | 2.4 | 53.05 d |

Figures are Average Severity Index (ASI) scored as follow: 1 = green leaves and white adventitious roots; 2 = flaccid but green foliage black punctuation on epidermis immersed into filtrate; 3 = flaccid and yellow leaves, epidermis immersed darkened; 4 = shoot wilting, discoloured adventitious roots. [1]Means followed by the same letter do not differ significantly (p<0.001). Cont. = Control.

**TABLE 3** - Response of detached leaves of black pepper (*Piper nigrum*) 'Kottanadan-1' to *Fusarium solani* f. sp. *piperis* culture filtrate at different pH 96 h after inoculation (mean of five replicates)

| pH of culture media | pH of culture filtrate | Vein discoloration [1] | | | Contrast of means for |
|---|---|---|---|---|---|
| | | 10% | 20% | 50% | pH of culture media |
| 4.0 | 4.5 | 3.5 | 3.5 | 5.8 | 61.08 b |
| 4.5 | 4.6 | 4.0 | 4.3 | 5.8 | 60.93 b |
| 5.0 | 4.4 | 1.3 | 2.3 | 5.5 | 43.88 c |
| 5.5 | 4.5 | 1.0 | 1.3 | 3.8 | 25.42 c |
| 6.0 | 4.6 | 4.5 | 5.3 | 5.8 | 77.58 a |
| 6.5 | 5.0 | 1.0 | 3.8 | 5.3 | 46.6 7 c |
| 7.0 | 4.5 | 2.0 | 2.3 | 4.3 | 39.63 c |
| 7.5 | 5.1 | 1.5 | 2.3 | 3.8 | 32.83 c |
| 4.7 (Water) | - | 1.0 | 1.0 | 1.0 | - |
| Contrasts of means for concentration | | 28.59 c | 43.03 b | 73.99 a | |

[1]Data represent Averaged Severity Index (ASI) derived from the following symptoms scale: 1 = no symptoms; 2 = yellowish or flaccid tissues; 3 = less than 15% vein discoloration; 4 = 25% vein discoloration; 5 = 50% vein discoloration and 6 = 75% vein discoloration. Means followed by the same letter do not differ significantly (p<0.001).

concentration by day four. After ten days all microcuttings immersed in culture filtrates diluted to concentrations of 10%, 20% and 50% had dried completely. In general, isolates whose culture filtrates were deep red coloured seemed to produce the largest quantities of toxic metabolites with the exception of 'Karimunda' which produced clear culture filtrate but incited symptoms comparable to 'Altamira-1', 'Panniyur-1' and 'São Mateus`. The 'Karimunda' culture filtrate incited symptoms even at 1% concentration. Isolates producing pink or clear filtrates were in general less toxic (Table 4).

Statistical analysis of the data revealed significant differences among isolates in terms of production of toxic metabolites (p<0.005). The isolates which produced deep red coloured culture filtrates like 'São Mateus', 'Ourém', 'Altamira-1' and 'Panniyur-1' were confirmed as belonging to a single group. Isolates that produced pink coloured culture filtrates formed another group with the exception of 'Bonito', which was different from other pink-isolates. Karimunda, a clear-filtrate-producing isolate produced quantities of toxic metabolites comparable to those of red-isolates (Table 4). The quickest and most intense symptoms were induced by solutions diluted to 50%. No differences were observed between 20% and 10% concentrations but these dilutions were different from the 1% filtrate solution (p<0.001).

**The effect of autoclaving culture filtrate on development of symptoms in *Piper* spp.**

Discoloration of secondary and tertiary veins was first observed in the cultivar Kottanadan-1 at 50% concentration, 48 h after immersion of detached leaves. Discoloration began in the primary veins and spread from base to apex, and only then to secondary and tertiary veins. The culture filtrate solution in most cultivars became viscous. Symptoms of vein

discoloration were recorded at 96 h. Although *P. betle* is regarded as non-host of the pathogen, it showed vein discoloration and responded in the assay in a fashion similar to the cultivated host species *P. nigrum*.

Analysis of data revealed no significant differences between autoclaved and non-autoclaved culture filtrates. All cultivars of black pepper and *P. betle* responded similarly to both types of culture filtrate. Although no significant effect has been detected, culture filtrates seem to have their effect slightly weakened by autoclaving when data on intensity of symptoms are compared (Table 5). Significant differences were only observed between dilutions, in which 50% and 20% differed from the 10% dilution (p<0.01).

Although there were exceptions, the main effect of autoclaving the filtrates was to reduce slightly the intensity of the observed symptoms. The reduction was, however, small and it is clear that most of the toxic metabolites of Fsp is thermostable.

**DISCUSSION**

During its phase of rapid growth in liquid culture Fsp produces secondary metabolites with toxigenic properties, capable of inducing symptoms such as vein discoloration on detached leaves, wilting in transpiring microcuttings, and necrosis in tissues of black pepper plants. Suitable conditions for the production of Fsp toxic metabolites were established together with optimisation of the bioassay procedures.

Production of Fsp toxic metabolites on PS-broth reached a peak after 25 days of static incubation at 25 °C under illumination. The effect of the inoculum concentration on the time needed for maximum toxin production has previously been described for the enniatins (Rudolph, 1976).

**TABLE 4** - Response of black pepper (*Piper nigrum*) cultivar 'Kottanadan-1' to diluted culture filtrates from different fungal isolates 96 h after microcutting inoculation (mean of four replicates)

| Pathogen isolate | Filtrate colour | Culture filtrate concentration | | | | Contrast of means for isolate |
| --- | --- | --- | --- | --- | --- | --- |
| | | 1% | 10% | 20% | 50% | |
| São Mateus | Red | 1.25 | 2.50 | 3.25 | 4.00 | 112.31 a |
| Karimunda | Clear | 1.50 | 2.25 | 3.00 | 4.00 | 111.69 a |
| Ourém | Red | 1.50 | 2.25 | 2.75 | 4.00 | 109.25 a |
| Altamira -1 | Red | 1.50 | 2.50 | 2.75 | 4.00 | 108.00 a |
| Panniyur -1 | Red | 1.00 | 2.25 | 3.00 | 4.00 | 104.31 a |
| Bonito | Pink | 1.00 | 2.25 | 11.25 | 3.00 | 87.86 b |
| Santarém Novo | Pink | 1.75 | 1.75 | 2.00 | 2.00 | 77.50 c |
| Castanhal -1 | Pink | 1.00 | 1.50 | 2.00 | 2.75 | 71.94 c |
| Jaburú | Pink | 1.00 | 1.50 | 2.00 | 2.25 | 66.44 c |
| Igarapé Açu | Pink | 1.00 | 1.25 | 1.50 | 3.25 | 66.36 c |
| Altamira -2 (Nhp) | Cream | 1.25 | 1.25 | 1.75 | 2.00 | 56.0 c |
| Control (water) | - | 1.00 | 1.00 | 1.00 | 1.00 | - |
| Contrasts of means for concentration | | 41.02 c | 76.05 b | 100.82 b | 133.18 a | |

Data are expressed as Average Severity Index (ASI) scored at: 1 = green leaves and white adventitious roots; 2 = flaccid but green foliage, black punctuation on epidermis immersed into filtrate; 3 = flaccid and yellow leaves, epidermis immersed into filtrate darkened; 4 = shoot wilting, discoloured adventitious roots. Means followed by the same letter do not differ significantly at 1% level (p<0.001).

Defined culture media such as Richard's solution or Czapek-Dox did not induce greater production of toxic metabolites when compared with PS. In spite of the presence of nitrogen in both forms ($NO_3^-$ and $NH^+$) and salts containing zinc ions, which are regarded as essential for production of toxins (naphthazarins and fusaric acid) in *Fusarium* spp. (Kern *et al.*, 1972), no beneficial response was noticed. In fact, toxin production was minimal in these defined media (Table 3). A complete inhibition of pigment formation was observed when cultures of *F. solani* were grown on Czapek-Dox (Claydon *et al.*, 1977).

Sabouraud's medium was also unsatisfactory, because the symptoms produced by culture filtrates based on this medium were atypical; and of the substrate tested Sabouraud's was the only non-inoculated medium to cause symptoms on black pepper. Taken together, these results suggest interference with the bioassay by Sabouraud's medium components. Culture media containing metal ions or complex organic substances can disguise the phytotoxicity of fungal products in filtrates (Blain *et al.,* 1991). Blain et al. (1991) noticed that eightfold dilutions of *Fusarium roseum* (Link) Snyder & Hans culture filtrate and non-inoculated filtrate inhibited red clover (*Phleum pratense* L.) seedling germination and stem elongation by 78% and 50% respectively, crediting this results to interference by culture medium constituents.

Changes in the pH of the culture filtrate did not increase the effect of toxic metabolites during assays. However, when the pH was changed before the medium was autoclaved, a more intense biological response was observed, peaking at pH 6.0, suggesting that a higher initial pH of the culture medium may stimulate the production of toxic metabolites. Culture media adjusted to pH 6.7 and even 7.0 are used to promote *Verticillium dahliae* and *Curvularia lunata* toxins respectively (Nachmias *et al.,* 1987; Patel *et al.*, 1987). The optimum pH for *Corynespora cassiicola* toxin production is pH 6 - 7, and toxin production was markedly reduced at pH 5.5 or 8.0 (Onesirosan *et al.*, 1975).

Orenstein *et al*. (1989) observed maximal toxin-induced inhibition of root elongation on tomato seedlings when culture filtrates were adjusted to pH 6.0.

The extent of toxin production in culture depends upon the pathogen isolate. Isolates which produced red pigments in liquid cultures were more efficient in producing biologically active culture filtrates than those that produced pink coloured or clear culture filtrates, suggesting that these pigments may have toxigenic activity. The only exception was the isolate from Karimunda, which lost its ability to produce pigments *in vitro* after successive transfers (Knoche & Duvik, 1987) but was capable of producing an active culture filtrate. Although it has been classified as *F. solani* and is pathogenic to black pepper, it is less virulent and shows some morphological degeneration when compared with others isolates, as previously observed by D. Brayford (personal communication). A lack of correlation between the ability of two isolates of *Botryosphaeria obtusa* (Schwein) Shoemaker to produce toxins and their pathogenicity on apple (*Malus domestica* Borkh.) foliage has previously been recorded (Venkatasubbaiah *et al*., 1991). It has also been shown that the production *in vitro* of fusaric acid by *Fusarium oxysporum* Schlecht.: Fr. f. sp. *lycopersici* (Sacc.) Snyder & Hans varies considerably with the composition of the culture medium and virulent and avirulent strains behave differently (Rudolph, 1976).

Detached leaves of seven black pepper cultivars and *P. betle* showed symptoms of vein discoloration after immersion in both autoclaved and non-autoclaved Fsp culture filtrate, indicating the thermostable nature of these metabolites. Some slight reduction of biological activity was observed suggesting that if some substances were inactivated by heating, most of the toxic components remained active. All germplasm tested showed sensitivity to both culture filtrates, even *P. betle* which is resistant to pathogen colonisation. Metha & Brogin (2000) also observed that culture filtrate produced by *Stemphylium solani* Weber was shown to be stable during autoclaving

**TABLE 5** - Effect of heat on culture filtrate produced by *Fusarium solani* f. sp. *piperis*, isolate Altamira-3, on vein discoloration of detached leaves of seven black pepper (*Piper nigrum*) cultivars and one wild species (mean of four replicates)

| Host cultivar   or species | Autoclaved culture filtrate | | | Non -autoclaved culture filtrate | | |
|---|---|---|---|---|---|---|
| | 10% | 20% | 50% | 10 % | 20% | 50% |
| *Piper nigrum* | | | | | | |
| Bragantina | 2.3 | 3.5 | 4.5 | 2.8 | 4.0 | 5.7 |
| Cingapura | 2.5 | 3.8 | 3.3 | 2.3 | 4.3 | 5.8 |
| Guajarina | 2.8 | 3.0 | 4.5 | 1.5 | 3.3 | 3.3 |
| Iaçara  -1 | 3.0 | 3.5 | 5.0 | 3.3 | 3.3 | 4.8 |
| Karimunda | 2.5 | 2.8 | 3.5 | 2.8 | 3.0 | 4.0 |
| Kottanadan   -1 | 2.5 | 3.3 | 3.3 | 2.5 | 3.0 | 5.0 |
| Kuthiravally | 3.0 | 4.0 | 5.8 | 3.5 | 3.0 | 4.8 |
| *Piper betle* | 2.0 | 3.8 | 3.0 | 3.3 | 3.0 | 4.8 |
| Contr ast of means for concentration | 105,75 c | 191,92 b | 275,31 a | | | |

Figures are  Average Severity Index, recorded at 96 hours immersion and scored as follow: 1 = no symptoms; 2 = yellowish or flaccid tissues; 3 = less than 15% vein discoloration; 4 = 16% to 25% vein discoloration; 5 = 26% to 50% vein discoloration; 6 = 51% to 75% vein discoloration. Means followed by the same latter do not differ significantly at 1% level ($p<0.01$).

however, the 10-fold concentration of toxin under partial vacuum at 45 °C did not show ten-fold increase in activity, compared with the original toxin-containing culture filtrate. This suggests that the concentration process under partial vacuum may have inactivated some of the toxins. Thermostability has been observed for many host-specific and non-host specific toxins (Hartman *et al.*, 1984; Patel *et al.*, 1987).

Although the toxic metabolites produced by Fsp in culture have not been identified chemically, the biological and physiological aspects studied throughout the research lead us to suppose that they are similar to the naphthazarin pigments (Kern, 1972), since all species of *Fusarium* belonging to the section *Martiella* produce these toxins. Further research is needed to isolate and identify structurally the toxic metabolites produced by *F solani* f. sp. *piperis in vitro*, since the chemical characterisation can contribute to an understanding of the physiological role of a toxin (Rudolph, 1976).

## LITERATURE CITED

ARNTZEN, C.J., KOEPPE, D.E., MILLER, R.J. & PEVERLY, J.H. The effect of pathotoxin from *Helminthosporium maydis* (race T) on energy-linked process of corn seedlings. Physiological Plant Pathology 3:79-89. 1973.

BLAIN, F., BERNSTEIN, M., KHANIZADEH, S. & SPARACE, S. A. Phytotoxicity and pathogenicity of *Fusarium roseum* to red clover. Phytopathology 81:105-108. 1991.

CLAYDON, N., GROVE, J.F. & POPLE, M. Fusaric acid from *Fusarium solani*. Phytochemistry 16:603. 1977.

DRYSDALE, R.B. The production and significance in phytopathology of toxins produced by species of *Fusarium*. In: Moss, M.O. & Smith, J.E. (Eds.) *The Applied Mycology of Fusarium*. B.M.S. Symposium Nº 7. Cambridge University Press. 1984. pp.95-105.

DUARTE, M.L.R. & ALBUQUERQUE, F.C. Estudo da variabilidade de isolamentos de *Nectria haematococca* (*Fusarium solani* f. sp. *piperis*) de diferentes regiões do Estado do Pará. Fitopatologia Brasileira 4:103-104. 1979.

DUARTE, M.L.R. & ALBUQUERQUE, F.C. Secamento dos ramos da pimenta-do-reino. Simpósio do Trópico Úmido, 1., 1984. Belém, PA. **Anais**. Brasília: Embrapa-DID, 1986. v.4. pp.339-342.

HARTMAN, C.L., MCCOY, T.J. & KNOUS, T.R. Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxin(s) produced by *Fusarium oxysporum* f. sp. *medicaginis*. Plant Science Letters 34:183-194. 1984.

ISAAC, S. *Fungal-Plant Interactions*. Chapman & Hall, London. 1992.

KERN, H. Phytotoxins produced by Fusaria. In: Wood, R.K.S., Balili, A. & Graniti, A. (Eds.) *Phytotoxins in Plant Disease* Academic Press, New York. 1972. pp.35-48.

KEEN, N.T., LONG, M. & ERWIN, D.C. Possible involvement of a pathogen-produced protein-lipopolysaccharide complex in *Verticillium* wilt of cotton. Physiological Plant Pathology 2:317-331. 1972.

KNOCHE, H.H. & DUVICK, J.P. The role of fungal toxins in plant disease. In: Pegg, G.F. & Ayres, P.G. (Eds.) *Fungal Infection of Plants*. Cambridge University Press. 1987. pp.158-192.

KONO, Y., KNOCHE, H.W. & DALY, J.M. Structure: Fungal host-specific. In: Durbin, R.D. (Ed.) *Toxin in Plant Disease*. Academic Press. 1981. pp.221-257.

METHA, Y.R. & BROGIN, R.L. Phytotoxity of a culture filtrate produced by *Stemphylium solani* of cotton. Plant Disease 84:838-842. 2000.

MILLER, R. G. Jr. *Simultaneous Statistical Interference*. McGraw-Hill, New York. 1966.

NACHMIAS, S., BUCHNER, V., TSROR, L. BURSTEIN, Y. & KEEN, N. Differential phytotoxicity of peptides from culture fluids of *Verticillium dahliae* races 1 and 2 and their relationship to pathogenicity of the fungi on tomato. Phytopathology 77:506-510. 1987.

ONESIROSAN, P., MABUNI, C. T., DURBIN, R.D., MORIN, R.B., RICH, D.H. & ARNY, D.C. Toxin production by *Corynespora cassiicola*. Physiological Plant Pathology 5:298-295. 1975.

ORENSTEIN, J., NACHMIAS, A. & LIPS, H. Inhibition of tomato root growth in culture by a *Verticillium dahliae* phytotoxin. Phytoparasitica 17:269-278. 1989.

PATEL, J.D., PATIL, M.R. & SAPKAL, P.N. Toxic effect of culture filtrate of *Curvularia lunata* (Wakker) Boedijin and *Alternaria tenuis* Auct. PKV Research Journal 11:189. 1987.

RUDOLPH, K. Non-specific toxins. In: Heitefuss, R. & Williams, P.H. (Eds.) *Encyclopedia of Plant Physiology, New Series*. vol. IV: *Physiological Plant Pathology*. Springer-Verlag. Berlin. 1976. pp.279-315.

SOKAL, R.P. & ROHLF, F.J. *Biometry*. W. H. Freeman & Co., New York. 2nd Edition. 1981.

STOESSL, A. Structure and biogenetic relations: Fungal nonhost-specific. In: Durbin, R.D. (Ed.) *Toxin in Plant Disease*. Academic Press. London. 1981. pp.110-197.

TUITE, J. *Plant Pathological Methods. Fungi and Bacteria*. Burgess Publishing Co. Minnesotta. 1969.

VENKATASUBBAIAH, P., SUTTON, T.B. & CHILTON, W.S. Effect of phytotoxins produced by *Botryosphaeria obtusa*, the cause of black rot of apple and frogeye leaf spot. Phytopathology 81:243-247. 1991.

YODER, O.C. Toxins in pathogenesis. Annual Review of Phytopathology 18:103-129. 1980.

ZAR, J. *Biostatistical analysis*. 4th Edition. Prentice Hall. New Jersey. 1999.

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