Eur. J. For. Path. 22 (1992) 432–440

ISSN 0300-1237

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In vitro host-pathogen interactions of Pinus elliottii calli and Fusarium moniliforme var. subglutinans

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Abstract

Establishment of dual cultures of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) with vegetative propagules of *Fusarium moniliforme* var. *subglutinans* (FMS) was only partially successful. In contrast, the response of slash pine callus to FMS culture filtrate was significant. The *in vitro* selection for FMS resistance using culture filtrate and slash pine callus or plantlets merits further investigation.

Key words: *Pinus elliottii* – *Fusarium moniliforme* var. *subglutinans* – Host-pathogen interaction.

1 Introduction

In vitro dual culture offers a controlled and simplified system for studying some host-pathogen interactions (Lepoivre et al. 1986; Meulemans et al. 1986). This technique may be an efficient tool for developing new crop varieties with enhanced disease resistance (Benke 1979). Interactions between organisms can be examined using selection agents such as pathogen propagules, purified or partially purified phytotoxins and, in some instances, culture filtrates of the pathogen (Tomas and Bockus 1987; Hartman et al. 1986; Stermer et al. 1984). The use of pathogen propagules as a selection agent, although feasible in some systems where resistance is expressed *in vitro*, is generally difficult because microbial agents colonize and utilize tissue culture media and overgrow plant cells (Jacobi et al. 1982).

Dual cultures have been established for several fungi and pine (*Pinus*) host tissues (Diner et al. 1984; Jacobi 1982a). The technique depends on the growth medium supporting growth of both host and pathogen without altering their genetic and/or physiological characteristics (Miller 1985; Hřib and Rypáček 1983). Environmental conditions and growth medium components, particularly growth regulators, can cause changes in cellular interactions and reactions to the disease (Michniewicz 1987). Growth medium effects on the host and the pathogen should, therefore, be determined prior to any pathogen interaction studies.

Slash pine (*P. elliottii* Engelm. var. *elliottii*) is an economically significant species which is highly susceptible to *Fusarium moniliforme* Sheld. var. *subglutinans* (FMS) Wollenw. and Reink. resulting in pitch canker (Kelley and Williams 1982; Kuhlman et al. 1978). Dual cultures of these organisms will permit evaluation of some mechanisms of disease resistance in a controlled environment and uniform host cell type (Stermer et al. 1984). Moreover, FMS resistant families of slash pine can be selected for genetic resistance in the field. Callus culture of slash pine has been achieved by Brown and Lawrence (1968). Similar to other *Fusarium* spp., FMS can be grown in axenic culture and reproduces by

conidia (Delucca et al. 1982; Cole et al. 1973). However, dual cultures of slash pine callus and FMS are not reported.

The role of toxins in disease induction and/or development is well documented in many host-pathogen relationships (Bains and Tewari 1987; Hartman et al. 1986; Lepoivre et al. 1986). *Fusarium* species produce toxic metabolites in culture (Solel and Bruck 1987; Cole et al. 1973). The mechanisms of disease resistance or susceptibility in the FMS-pine pathosystem is not fully elucidated. Delucca et al. (1982) tested the phytotoxicity of extracts from several *Fusarium* species, including FMS, grown on corn and rice substrates. These extracts, although initiating toxicity to chicken and mice embryos did not induce dieback or disease symptoms in pine seedlings. In contrast, Solel and Bruck (1987) reported that culture filtrates from four isolates of FMS induced epinasty, wilting and necrosis of loblolly pine (*Pinus taeda* L.) seedlings. The toxicity of FMS culture filtrates has not been demonstrated using tissue culture of pine. *In vitro* techniques for selection for disease resistance and subsequent plantlet regeneration have been demonstrated previously for potato (Meulemans et al. 1986), and may be applicable for FMS and slash pine.

The objectives of this study were to: 1. assess the influence of media formulation on single and dual cultures of *P. elliottii* calli with and without FMS, 2. characterize host-pathogen interactions *in vitro*, and 3. evaluate growth of calli on media containing FMS culture filtrate.

2 Material and methods

2.1 Pathogen

Five FMS isolates were used in the experiments: L-01 and L-61 originally were isolated from loblolly pines in Craven Country, North Carolina, while the other isolates were originally collected from slash pine in North Florida, USA. All isolates were maintained on Potato Dextrose Agar (PDA) slants and were passed through the host immediately prior to use to document pathogenicity following the procedures of Kelley and Williams (1982). Fungi were recultured from single spores of each isolate. Pathogenicity of the isolates was tested on 7 to 12-day-old seedlings grown on modified Gresshoff and Doy (GD) medium following the procedures of Frampton (1984) and Gresshoff and Doy (1972). A spore suspension (50–100 spores) derived from PDA cultures of fungus was placed on wounded seedling hypocotyls which were incubated (16-hr photoperiod at $24 \pm 1\,^{\circ}C$). Cultures from PDA were used as the source of inoculum for subsequent experiments.

2.2 Host

Slash pine callus cultures were initiated, maintained and propagated using the techniques of Bronson and Dixon (1991) and Brown and Lawrence (1968). Callus cultures were derived from two half-sib slash pine families. Calli were initiated from seedling hypocotyl tissues collected from Wayne County, GA and Jackson County, MS, USA, respectively.

2.3 Media

Growth of slash pine callus and FMS was assessed on four media: modified Brown and Lawrence (BL), water agar (WA) (Jacobi 1982a), GD and PDA. The BL medium was supplemented with 0.23 μm 6-furfurylaminopurine (kinetin) and 4.5 μm 2,4-dichlorophenoxy acetic acid (2,4-D) (Brown and Lawrence 1968). Modified GD medium was supplemented with 0.01 mg L^{-1} α-napthalene acetic acid (NAA) and 10 mg L^{-1} 6-benzylaminopurine (BAP). No supplements were added to the PDA and nu-

trient deficient medium, WA. The BL and GD media were both prepared with and without 2% sucrose.

The BL medium containing 2% sucrose was used to assess the effect of sodium deoxyphenolic acid (sodium salt) on slash pine callus and FMS growth *in vitro*. Sodium salt was added to the BL medium at 0, 1, 10, 100, 200, 400 mg L^{-1}. All media contained 1% agar, adjusted to pH 5.6–5.7, then autoclaved (121 °C), and poured into plastic culture dishes (100 × 20 mm) in 25 ml aliquots. All treatments were replicated 10 times and all experiments were repeated at least once.

2.4 Dual cultures of FMS and slash pine callus

Radial growth of FMS in the presence of slash pine *calli* was evaluated using techniques modified from Hřib and Rypáček (1983) and Jacobi (1982b). Two callus lines (Georgia and Mississippi) were established *in vitro* on modified BL medium supplemented with kinetin and 2,4-D. After 21 days, fresh weights of callus averaged approximately 5 ± 0.5 mg. Propagules of FMS (isolate S-LG) on BL medium, equivalent in radial diameter, weight and age, were added to culture dishes containing the callus. The pine callus and FMS plug were placed 50 mm apart in the culture dishes. Radial growth of FMS was recorded after 2, 6, 10, and 14 days. All treatments were replicated 10 times and the experiment twice.

Slash pine *calli* were grown on BL medium supplemented with kinetin and 2,4-D for approximately 3 weeks as described. A spore suspension of FMS (isolate S-LG) was prepared from a 1-week-old culture on PDA medium and adjusted to approximately 1×10^5 microconidia per ml. One microliter (50–100 microconidia per loop) of the inoculum was placed on 50 ± 5 mg of the callus. Twenty replicates of the dual culture were established and fungus growth and colonization were observed for 7 days. Callus tissue was fixed in FAA, dehydrated in ethyl alcohol and tertiary-butyl alcohol, and embedded in paraffin for histological observations (Jensen 1962). Ten to 12 μm sections of the callus-FMS culture were sectioned and stained using the periodic acid-Schiff's Reaction (PAS) for starch and glycogen (Jensen 1962).

2.5 Culture filtrate production

S-LG and L-61 isolates of FMS were grown on asparagine medium (AM) (Solel and Bruck 1987) in 250-ml culture flasks without shaking. Cultures were established with a 1-ml spore suspension (1×10^5 spores ml^{-1}) of FMS. Sterile uninoculated AM was used as a control. The liquid cultures were maintained under an 8 hr photoperiod using cool white fluorescent lamps (300 $\mu Em^{-2}s^{-1}$) at 24 ± 1 °C. Filtrates of the FMS cultures were harvested 1, 3 and 5 weeks after inoculation by filtration through sterile cheesecloth and Nalgene filter units, 0.45 μm pore diameter.

2.6 Callus production and testing

The test media contained culture filtrates of FMS mixed with modified BL medium in a 50:50 ratio. The medium was prepared using previously described procedures (Section 2.3). Calli (approximately 25 ± 5 mg) of both families were placed on the test medium (BL medium with S-LG culture filtrate aged 1, 3 and 5 weeks). Both autoclaved and non-autoclaved culture filtrates were tested. The cultures were incubated under controlled environmental conditions described in Section 2.5 for 4 weeks. Fresh weights of the colonies were used to compare callus from the medium with and without culture filtrates of the two isolates. Browning and necrosis of calli were assessed. Treatments were replicated 15 times.

2.7 Statistical analysis

Data were subjected to analysis of variance. Treatment means were evaluated using Duncan's multiple range test where applicable (STEEL and TORRIE 1980).

3 Results

3.1 Callus growth

In vitro cultures of slash pine callus were established on BL medium supplemented with 2,4-D, kinetin and sucrose (Table 1). The GD, WA and PDA media did not support the growth of slash pine callus. In the absence of sucrose as a carbon source, no media supported continued callus growth. Browning or necrosis of the calli became apparent after about a week. By the third and fourth week all calli on sucrose-free media were dead.

Table 1. **In vitro growth of two half-sib families of P. elliottii callus after 30 days, and FMS colony diameter and growth index after 7 days on six culture media**

Media	*P. elliottii* family[1]		FMS colony diameter (mm)					FMS mycelia growth index
	GA	MS	L-01	L-61	S-LG	S-W	S-M	
BL + sucrose	+[2]	+	34b[4]	36b	40b	37b	37b	3[3]
BL − sucrose	−	−	69a	72a	75a	75a	70a	2
GD + sucrose	−	−	55a	56ab	61a	66a	62a	3
GD − sucrose	−	−	51ab	60a	62a	65a	64a	2
WA − sucrose	−	−	53a	61a	64a	64a	60a	2
PDA − sucrose	−	−	67a	74a	74a	75a	72a	4

[1] GA = Georgia family, MS = Mississippi family.
[2] Growth of callus was indicated as + (callus remained green and growth not inhibited) or − (browning, necrosis and inhibition of growth).
[3] FMS mycelial growth index was rated as follows: 1 = poor, complete inhibition of radial growth, 2 = fair, mycelial growth thin and sporulation sparse, 3 = moderate to good, thick mycelial growth with slight inhibition of radial growth, and sporulation moderately abundant, and 4 = abundant, mycelial growth thick and no inhibition of radial growth, sporulation abundant.
[4] Means followed by a common letter are not significantly different by Duncan's multiple range test (P = 0.05).

3.2 Fungus growth

All media, with or without a carbon source, supported the growth of FMS (Table 1), However, FMS radial growth and mycelial density (as observed with 20 to 40X magnification) differed with media. Mycelial diameter growth was greatest on PDA and poorest on the BL medium. The addition of sucrose reduced the growth of FMS on BL media. Sporulation of FMS occurred on all media.

3.3 Dual cultures of callus and pathogen

Advanced establishment of slash pine callus on BL medium reduced the radial growth of FMS (Table 2). Callus cultures of the Georgia family limited FMS growth after 14 days. FMS growth was not signficantly reduced by the callus from the Mississippi family. Decreased FMS mycelial density occurred near the callus and mycelial growth was relatively dense in cultures containing the Georgia callus line (data not shown).

When established simultaneously, FMS colonized slash pine calli 3–5 days following

Table 2. **In vitro colony diameter growth (mm) of FMS (isolate S-LG) in the presence of P. elliottii. Callus lines were established for 21 days on BL medium before inoculation with FMS**

P. elliottii family	Colony Diameter (days)			
	2	6	10	14
Georgia	16a	32a	44a	56b
Mississippi	16a	31a	39a	65ab
control (no callus)	16a	35a	48a	76a

Means within a column sharing a common letter are not significantly different by Duncan's multiple range test (P = 0.05).

inoculation (data not shown). Calli from the Georgia and Mississippi families did not differ in the amount of fungus colonization. Histological examination revealed extensive mycelial growth external to the pine cells, but no evidence of intracellular penetration by fungal hyphae was observe. No cell necrosis was observed in dual culture of the host and pathogen.

3.4 Response of callus and FMS to sodium salt

The addition of sodium deoxyphenolic acid to BL media reduced or limited the growth of slash pine calli (Table 3). The Mississippi callus line was sensitive to sodium salt concentration as low as 1 mg L^{-1}, whereas the Georgia family tolerated sodium salt at 10 mg L^{-1}. Radial diameter growth of 2 FMS isolates (L-01 and S-M) was not significantly reduced. Isolate S-LG tolerated 200 mg L^{-1} salt whereas L-61 and S-W tolerated 100 mg L^{-1} salt without significant reductions in colony growth.

Table 3. **In vitro growth of P. elliottii callus from two families after 30 days, and colony diameter of 5 FMS isolates after 7 days on Brown and Lawrence (BL) medium containing 2% sucrose and amended with sodium deoxyphenolic acid**

Sodium deoxyphenolic acid concentration (mg/L)	*P. elliottii* family[1]		FMS colony diameter (mm)				
	GA	MS	L-01	L-61	S-LG	S-W	S-M
0	+[2]	+	38a	41a	43a	42a	41a
1	+	−	34a	38ab	41ab	36ab	36a
10	+	−	30a	35ab	40ab	36ab	36a
100	−	−	30a	33ab	37ab	32ab	34a
200	−	−	30a	30b	35ab	30b	34a
400	−	−	30a	31b	33b	31b	33a

[1] GA = Georgia family. MS = Mississippi family.
[2] Callus tissue green with active cell division (+) or brown with no growth (−).
[3] Means within a column sharing a common letter are not significantly different by Duncan's multiple range test (P = 0.05).

3.5 Callus response to FMS culture filtrate

The callus cultures from the Georgia and Mississippi provenances responded differently to the culture filtrates of the two FMS isolates (Table 4). After 4 weeks, the L-61 FMS isolate reduced callus growth of the Georgia family relative to the control (AM medium without FMS), and the S-LG isolate significantly reduced callus growth of the Mississippi family. Callus response to autoclaved and non-autoclaved culture filtrate was the same. Browning and necrosis of callus ranged from 50–85% and 75–95%, for the Georgia and Mississippi families, respectively (data not shown).

Table 4. **Fresh weight (mg) of P. elliottii callus from two families after 4 weeks on Brown and Lawrence (BL) medium amended with FMS filtrates from two differing fungal isolates. Age of filtrate was 3 weeks. The asparagene medium (AM) was used for the control treatment**

P. elliottii family	FMS isolates		
	S-LG	L-61	Control
Georgia	97.2ab[1]	69.3b	124.5a
Mississippi	18.7b	28.1ab	36.2a

[1] Means within a row (family) sharing a common letter are not significantly different by Duncan's multiple range test (P = 0.05).

Table 5. **Fresh weight (mg) of P. elliottii callus from two families after 4 weeks on Brown and Lawrence (BL) medium amended with FMS (isolate S-LG) culture filtrates ages 1, 3, and 5 weeks**

P. elliottii family	Culture age (weeks)		
	1	3	5
Georgia	87.3a[1]	21.8b	25.8b
Mississippi	26.9a	21.6ab	18.7b

[1] Means within a row (family) sharing a common letter are not significantly different by Duncan's multiple range test (P = 0.05).

The age of FMS culture filtrate significantly influenced the fresh weight of slash pine callus culture (Table 5). Filtrates of FMS isolate S-LG, 5 weeks of age, reduced callus weights in both pine families. The callus growth reduction was most pronounced in the Georgia family, where growth was reduced with both 3 and 5-week-old FMS filtrates.

4 Discussion

The nutritional requirements of slash pine callus and FMS on culture media differed significantly. *In vitro* cultures of slash pine calli require inorganic salts, specific ammonia:nitrate ratio, vitamins and a carbon source (Brown and Lawrence 1968). In contrast, FMS grew well and reproduced on most of the basal media evaluated. The approximate 50% inhibition of FMS growth by 2% sucrose in the BL medium suggests the carbon:nitrogen ratio was inadequate. Jacobi et al. (1982a) suggested a higher concentration of nitrogen was inhibitory to colonization of *Cronartium fusiforme* Hedg. and Hunt ex Cumm. of loblolly pine callus. It is not known if the nitrogen content or the growth factor levels were inhibitory to FMS growth. No reduction of FMS growth occurred on the GDI medium containing sucrose. The relationship of sucrose to other constituents in the BL medium and inhibitory effect on FMS growth remain to be elucidated.

Inhibition of FMS mycelial growth in dual culture with *P. elliottii* callus indicated substance(s) inhibitory to the fungus is extruded into the medium. In dual cultures of *C. fusiforme* and loblolly pine callus on GD media, a bioassay test of callus washes revealed the presence of an inhibitory factor that restricted radial growth of colonies of *C. fusiforme* (Jacobi 1982a). Hřib and Rypáček (1983) observed a reduction in the colony growth of the wood-destroying fungus, *Phaeolus schweinitzii* (Fr.) Pat. when dual cultures with several pine species were cultured on modified Murashigi and Skoog medium and modified White's vitamins. They suggested that the defense reaction of callus cultures to *P. schweinitzii* could indicate the degree of the resistance to the wood-destroying fungus.

The GD, WA and PDA media did not support dual cultures of slash pine calli and FMS. GD medium with kinetin and 2,4-D significantly reduced the growth of loblolly

pine (*P. taeda* L.) and *C. fusiforme* (Jacobi 1982a). Michniewicz (1987) showed varying effects of indole-3-acetic acid, gibberellic acid, kinetin, abscisic acid, and Ethrel on mycelial growth, sporulation and germination of *Fusarium culmorum* (W. G. Sm.). He concluded that there was no correlation between the pathogenicity of *F. culmorum* isolates to wheat (*Triticum*) seedlings and exogenous growth regulators.

Histological examination revealed FMS did not colonize slash pine. In contrast, FMS colonized and penetrated tissue and cells of inoculated seedlings. The differential response of calli and organized tissue to FMS suggests a biochemical rather than morphologicecal defense mechanism. Similarly, Jacobi (1982b) found that loblolly pine callus was resistant both to cell penetration and intercellular growth by *C. fusiforme*. Diner et al. (1984) reported western white pine (*P. monticola* Dougl.) cell cultures developed a hypersensitive response to *C. ribicola in vitro*.

One of the difficulties of *in vitro* dual culture is the rapid overgrowth of plant cells by the fungus (Jacobi et al. 1982a). Evaluation of slash pine callus reaction to the pathogen was difficult in this study because of the rapid growth of FMS. In an attempt to slow the colonization of FMS of slash pine callus, sodium deoxyphenolic acid, an anionic biological detergent was added to BL medium. Depending upon the fungal isolate, this agent was slightly inhibitory to fungal growth at approximately 200 to 400 mg L^{-1}. However, those levels were detrimental to slash pine callus. Thus, the potential benefit of using this substance for screening *in vitro* cultures of FMS and slash pine is limited. Preliminary assessments of the effectiveness of other fungistatic agents, such as Benlate and mycostatin, proved to be equally ineffective in dual cultures of FMS and slash pine (data not shown).

Autoclaved and non-autoclaved culture filtrates of FMS inhibited slash pine callus growth *in vitro*. The response of calli from two slash pine families to the toxic medium was markedly different. Callus growth of the Georgia family was robust after four weeks in the medium containing culture filtrate. Alternatively, the Mississippi family consistently showed decline in cell growth, and toxic effects of the filtrate were manifested by the appearance of necrotic colonies. Solel and Bruck (1987) suggested that mycotoxins may induce dieback symptoms in loblolly pine. Our results support this hypothesis, but the identity of the toxic metabolite(s) produced by FMS have not been determined. The suspected FMS toxic agent(s) are heat tolerant (121 °C) and could pass through a 0.45 μm filter. Stermer et al. (1984) demonstrated that toxins produced by *Hypoxylon mammatum* (Wahl.) Miller are significant determinants in canker disease development of *Populus tremuloides*.

Summary

In vitro growth of *Fusarium moniliforme* var. *subglutinans* (FMS) and slash pine (*Pinus elliottii* Engelm. var. *elliottii*) calli were evaluated on four media: modified Brown and Lawrence (BL), Gresshoff and Doy (GD), potato dextrose agar (PDA), and water agar (WA). Callus biomass on BL and GD media was significantly greater compared to PDA or WA after 30 days. Radial growth and sporulation of FMS occurred on all media tested after 21 days. Slash pine calli and FMS were established in a dual culture on BL medium. Callus of slash pine inhibited radial growth of FMS in dual culture. Propagules of FMS rapidly colonized callus tissues but no intracellular penetration of calli by fungal hyphae was observed. Sodium deoxyphenolic acid, added to dual culture media, slowed FMS colonization of calli. The sodium salt (200 mg L^{-1} significantly reduced mycelial growth of FMS and slash pine callus in dual culture on BL media. Calli derived from two slash pine families were subjected to FMS culture filtrate. Significant filtrate toxicity to callus growth was observed for both families. Moreover, significant differences between families to toxicity of different FMS isolates were observed. Callus symptoms of FMS filtrate toxicity were cell browning and necrosis. The suspected FMS toxin appears to be a heat tolerant molecule smaller than 0.45 μm.

Résumé

Interaction in vitro entre Fusarium moniliforme var. subglutinans et des cals de Pinus elliotii

La croissance *in vitro* de *F. moniliforme* var. *subglutinans* (FMS) et celle de cals de *P. elliottii* ont été évaluées sur quatre milieux: Brown et Lawrence modifié (BL), Gresshoff et Doy (GD), PDA et eau gélosée (WA). La biomasse des cals sur BL et GD était significativement plus grande après 30 jours. La croissance radiale et la sporulation de FMS avaient lieu sur tous les milieux en 21 jours. Les cals et FMS ont été implantés en culture double sur BL. Les cals inhibaient la croissance radiale de FMS. Les propagules des FMS colonisaient rapidement les cals mais aucune pénétration intracellulaire par les hyphes n'a été observée. L'acide désoxyphénolic de sodium, ajouté au milieu, relentissait la colonisation des cals. Ce sel (200 mgl^{-1}) réduisait significativement la croissance mycélienne de FMS et celle des cals en cultures doubles sur BL. Les cals issus de deux familles de pins ont été soumis à des filtrats de culture de FMS. Une toxicité significative vis-à-vis de la croissance des cals a été observée dans les deux cas. De plus, des différences significatives entre les deux familles pour la toxicité des filtrats de différents isolats de FMS, ont été observées. Les symptômes étaient le brunissement des cellules et la nécrose. La toxine supposée de FMS apparaît être une molécule thermotolérant inférieure à 0,45 μm.

Zusammenfassung

In vitro Wirt-Pathogen Wechselwirkungen von Pinus elliottii und Fusarium moniliforme var. subglutinans

Das Wachstum von *Fusarium moniliforme* var. *subglutinans* (FMS) und Kallus von *Pinus elliottii* Engelm. var. *elliottii* wurde auf vier verschiedenen Medien getestet: modifiziertes Medium nach Brown und Lawrence (BL), Medium von Gresshoff und Doy (GD), Kartoffeldextroseagar (PDA) und Wasseragar (WA). Die Biomasse von Kallus auf BL und GD war nach 30 Tagen signifikant größer als auf PDA oder WA. FMS-Kulturen, die nach 21 Tagen begutachtet wurden, zeigten für alle vier Medien radiales Wachstum und Sporulation. Kallus und FMS wurden in Dualkultur auf BL Medium angezogen. Der Kallus hemmte das radiale Wachstum von FMS. Konidien von FMS besiedelten den Kallus sehr rasch, es wurden jedoch keine intrazellulären Hyphen beobachtet. Zugabe von Natriumdesoxyphenolsäure zum Medium verlangsamte die Besiedlung von Kallus durch FMS. Das Natriumsalz (200 mgl^{-1}) reduzierte das Wachstum des Pilzes und des Kallus in Dualkulturen auf BL signifikant. Kallus von zwei verschiedenen Klonen von *Pinus elliottii* var. *elliottii* wurden Kulturfiltraten von FMS ausgesetzt. Dieses wirkte toxisch auf die Kalli beider Klone. Die toxische Wirkung der Kulturfiltrate auf den Kallus zeigte sich in Form von Verbräunungen und Nekrosen. Zwischen den beiden Klonen wurden zudem signifikante Unterschiede hinsichtlich ihrer Reaktion auf Filtrate von verschiedenen FMS Isolaten festgestellt. Das FMS Toxin scheint hitzebeständig zu sein und weist einen Moleküldurchmesser von weniger als 0,45 μm auf.

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Receipt of ms.: 18. 5. 1992