

The Effect of Gums Induced by *Fusarium oxysporum* f. sp. *tulipae* in Tulip Bulbs on In Vitro Growth of the Pathogen and Its Production of Gum Degrading Enzymes

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Abstract

In tulip bulbs infected by *Fusarium oxysporum* f. sp. *tulipae*, and also in healthy bulbs stored together with bulbs with symptoms of fusariosis induction of gums take place. Ethylene is a common factor involved in the induction of gummosis. Physiological role of gums in plants is unknown but is believed that gums have a function in limiting the spread of pathogens by isolating the infected tissues. It was shown that addition of tulip gums to solid Czapek-Dox-Agar medium (CzDA) greatly stimulated mycelium growth and sporulation of *F. oxysporum* f. sp. *tulipae*. Supplementation of liquid Czapek-Dox-Broth medium (CzDB), both containing sucrose and the mineral medium (m-CzDB), increased secretion by the pathogen of some enzymes connected with degradation of tulip gum polysaccharides. It is possible that polysaccharides of tulip gum may act mainly as elicitor and/or partially as substrate in regulation of mycelium growth and sporulation of *Fusarium oxysporum* f. sp. *tulipae*.

INTRODUCTION

It is well known that tulip bulbs infected by *Fusarium oxysporum* f. sp. *tulipae* can produce considerable quantities of ethylene, enough to cause gummosis in diseased and healthy bulbs stored in the same conditions. Gummosis in tulip bulbs can be easily induced in healthy bulbs by exogenously applied ethylene or the ethylene-releasing compound ethephon (Kamerbeek and De Munk, 1976). The physiological role of gums in plants is unknown. It is believed that gums have a function in limiting the spread of fungal and bacterial pathogens by isolating the infected tissues (Boothby, 1983). Gums are a complex of different substances, but their most important constituent are polysaccharides. The composition of gum polysaccharides shows large variation, among various species (Boothby, 1983). Polysaccharides of tulip gums mainly consist of xylose, arabinose and uronic acid(s) (Saniewski et al., 2000).

The aim of the present work was to study the effect of tulip gums on the in vitro growth and development of *Fusarium oxysporum* f. sp. *tulipae* and on the secretion of some enzymes to the culture medium.

MATERIAL AND METHODS

The gums induced by *Fusarium oxysporum* f. sp. *tulipae* in tulip bulbs (Fig. 1), at final concentration 5 mg·cm⁻³ were dissolved in 5 cm³ distilled and sterilized water and added to Czapek-Dox-Agar (CzDA) before sterilization. Five mm diam. plugs taken from 7-day-old culture of *Fusarium oxysporum* f. sp. *tulipae*, were placed in the middle of 90 mm Petri dishes containing above-mentioned media supplemented with gums. Control plates constituted the culture growing on CzDA. The diameter of *Fusarium oxysporum* f. sp. *tulipae* colony was measured within a 7-day-incubation at 25°C in darkness.

After 6, 8 and 12 days of mycelium incubation on CzDA medium and CzDA supplemented with gums, colonies of *Fusarium oxysporum* f. sp. *tulipae* were used for estimation of the effect of gums on sporulation of the pathogen. From mycelium colony of the pathogen, 6 cm² of colony fragments were cut out and transferred to clean Petri

dishes containing 10 cm³ sterilized water. These fragments of colony were smoothed by glass bagette for liberation of spores; after 30 min. spores were separated from mycelium using filter paper. Density of spores in 1 cm³ of suspension was determined under microscope using Bürker's camera. From each of Petri dishes there were analyzed 2 fragments of mycelium.

Five dishes were used for each treatment and the experiment was repeated 3 times. The data were subjected to an analysis of variance and Duncan's multiple range test at 5% of significance was used for means separation.

For measurements of enzymes production *F. oxysporum* f. sp. *tulipae* was grown on liquid Czapek-Dox-Broth (CzDB) containing sucrose at concentration of 3% and on mineral CzDB without sucrose (m-CzDB), and supplemented with tulip gums at a concentration of 0.5% and 0.1%. After sterilization ninety milliliters of the liquid medium in 300 cm³ Erlenmayer flasks were inoculated with 10 cm³ of suspension with 22.5×10^6 spores of *Fusarium oxysporum* f. sp. *tulipae* and cultured for nine days at 25°C under constant agitation (140 r. min⁻¹).

In other experiments after 20, 44, 192 hrs of culturing density of spores in 1 cm³ of suspension of media was determined under microscope using Bürker's camera. The experiment was repeated 3 times. The data was subjected to an analysis of variance and Duncan's multiple range test at 5% of significance was used for means separation.

Measurements of enzyme activities were made 24, 48, 96, 144 and 192 hrs after *F. oxysporum* f. sp. *tulipae* incubation.

Glycosidase Determination

At different time intervals of culturing the samples were taken out, centrifuged and supernatants were used for enzyme activity assay. p-Nitrophenyl derivatives of sugars were used as substrates and the enzyme activity was assayed by measuring the amount of p-nitrophenyl liberated (Lehtinen, 1993). The activity of the following enzymes was assayed with the use of the following substrates:

β-1,4-glucosidase - p-nitrophenyl-β-D-glucopyranoside,
α-1,4-galactosidase - p-nitrophenyl-α-D-galactopyranoside,
β-xylosidase - p-nitrophenyl-β-D-xylopyranoside,
β-1,4-galactosidase - p-nitrophenyl-β-D-galactopyranoside,
β-1,4-glucuronidase - p-nitrophenyl-β-D-glucuronide,
α-1,4-xylosidase - p-nitrophenyl-α-D-xylopyranoside,
α-1,4-arabinosidase - p-nitrophenyl-α-D-arabinoside,
α-1,4-L-arabinosidase - p-nitrophenyl-α-L-arabinoside,
β-L-arabinosidase - p-nitrophenyl-β-L-arabinopyranoside,
α-1,4-mannosidase - p-nitrophenyl-α-D-mannopyranoside,
α-1,4-rhamnosidase - p-nitrophenyl-α-L-rhamnopyranoside, and
β-1,4-galacturonidase - p-nitrophenyl-β-D-galacturonide.
p-Nitrophenyl derivatives were from Sigma.

The reaction mixture consisted of 5 mM substrate in 0.2 ml of 0.04 M citrate buffer, pH 5.0 and 0.2 ml of culture supernatant. After 1 h incubation at 30°C the reaction was stopped by adding 1.6 ml of 0.5 M sodium carbonate, the absorbance was measured at 420 nm, and the p-nitrophenol released was calculated by reference to a calibration graph of the standard compound. One unit of glycosidase activity was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per 60 min.

Polygalacturonase Determination

At different time intervals of culturing the samples were taken, centrifuged and the supernatants were dialysed for 20 h at 4°C against 0.01 M acetate buffer, pH 5.0 and assayed for enzyme activity. The polygalacturonase activity was determined against sodium polypectate (Sigma) as substrate. Reaction mixture consisted of 0.5% substrate in 0.2 ml of 0.04 M acetate buffer, pH 5.0 and 0.2 ml culture filtrate. After 1 h incubation at 30°C releasing reducing groups were assayed by the colorimetric method of Nelson

(1944) using the reagent of Somogyi (1952). One unit of polygalacturonase activity was defined as the amount of enzyme liberating 1 μ mol of reducing groups per 1 h at 30°C.

RESULTS AND DISCUSSION

Tulip gums added to CzDA medium containing sucrose substantially stimulated mycelium growth of *Fusarium oxysporum* f. sp. *tulipae* (Fig. 2) and also stimulated sporulation of mycelium of the pathogen (Fig. 3). Thus, it is clear that tulip gums are not antifungal substances, but they have an evident stimulatory effect on the growth and development of *F. oxysporum* f. sp. *tulipae*. On the basis of these results it can be suggested that polysaccharide of tulip gums which is a glucuronoarabinoxylan may act mainly as elicitor which regulate some processes connected or responsible for mycelium growth and sporulation of *F. oxysporum* f. sp. *tulipae*. It is well known that different kind of oligosaccharides can function in plants as molecular signals (elicitors) that regulate growth, development and survival in the environment, through elicitation of various physiological and biochemical processes (Ebel and Mithöfer, 1998; Côte and Hahn, 1994; Aldington et al., 1991; Darvill et al., 1992).

The stimulatory role of polysaccharide of tulip gums as substrate for mycelium growth of the pathogen is also probable. Tulip gums contain also many other unidentified compounds which may have stimulatory effect on mycelium growth and sporulation of *F. oxysporum* f. sp. *tulipae*.

F. oxysporum f. sp. *tulipae* grown on liquid Czapek-Dox-Broth medium containing sucrose (CzDB) did not secrete to the medium all analyzed enzymes. However, on the CzDB medium supplemented with tulip gums *F. oxysporum* f. sp. *tulipae* clearly secreted β -1,4-glucosidase, α -1,4-galactosidase, β -xylosidase and polygalacturonase (Table 1).

Incubation of *F. oxysporum* f. sp. *tulipae* on mineral liquid Czapek-Dox-Broth medium (m-CzDB) caused secretion of β -1,4-glucosidase, α -1,4-galactosidase, and polygalacturonase to the medium and supplementation of the medium with tulip gums greatly increased secretion of those enzymes and caused secretion of β -xylosidase (Table 1).

In all analyzed variants of used media after incubation of *F. oxysporum* f. sp. *tulipae* there were not detected activity of the following enzymes: β -1,4-galactosidase, β -1,4-glucuronidase, α -1,4-xylosidase, α -1,4-arabinosidase, α -1,4-L-arabinosidase, β -L-arabinosidase, α -1,4-mannosidase, α -1,4-rhamnosidase and β -1,4-galacturonidase.

Tulip gums added to liquid Czapek-Dox-Broth medium both containing sucrose or only mineral compounds substantially stimulated sporulation of *F. oxysporum* f. sp. *tulipae* (Table 2).

Fusarium oxysporum is very capable as an autotroph, requiring only carbon source, for structure and energy, and inorganic compounds. Sources of carbon useful to *F. oxysporum* include most sugars, alcohols, pectins, cellulose, and amino acids, and a wide variety of additional carbon compounds (Woltz and Jones, 1981). This organism processes a very highly developed versatility in utilization of compounds permitting growth and survival under many chemical-physical environments. Enzymatic adaptation is readily accomplished by *F. oxysporum* and facilitates growth and pathogenicity. Enzyme production is conservative, generally being formed as required; if not needed, such as when alternates are available, enzyme synthesis may be at very low level (Woltz and Jones, 1981). Exposure to tulip gums as carbon source activates enzymes or enzyme synthesizing mechanisms in *F. oxysporum* f. sp. *tulipae*.

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Tables

Table 1. Activity of some enzymes secreted by *Fusarium oxysporum* f. sp. *tulipae* grown on Czapek-Dox-Broth (CzDB) medium containing sucrose and without sucrose (mineral compounds only, m-CzDB), and supplemented with tulip gums, after different time of incubation.

Enzyme	Time of incubation (hrs)	CzDB + suc.	CzDB + suc. + gums	m-CzDB	m-CzDB + gums
β-1,4-glucosidase	24	0	0	0.405	0.139
	48	0	0.262	3.314	2.433
	96	0	0.379	4.288	8.582
	144	0	0.258	4.603	9.045
	192	0	0.220	4.547	7.701
α-1,4-galactosidase	24	0	0	0	3.534
	48	0	0.414	0.207	12.606
	96	0	0.748	0.276	19.004
	144	0	1.008	0.310	19.890
	192	0	1.926	0.310	11.172
β-xylosidase	24	0	0	0	0.129
	48	0	0.319	0	0.496
	96	0	0.552	0	1.608
	144	0	0.733	0	1.926
	192	0	0.845	0	1.327
polygalacturonase	24	-	-	-	-
	48	0	0.322	0.142	6.375
	96	0	4.460	0.393	11.153
	144	0	4.631	0.204	4.493
	192	0	4.299	0.052	0.384

Table 2. The effect of gums added to liquid Czapek-Dox-Broth medium containing sucrose (CzDB) and without sucrose (mineral compounds only, m-CzDB) on sporulation of *Fusarium oxysporum* f. sp. *tulipae*. Initial level of spores in cm³ - 1.5 x 10⁶.

Kind of medium	Number of spores in cm ³ of suspension after different time of incubation (hrs)		
	20	44	192
CzDB + sucrose	1.8 x 10 ⁶ bc	1.4 x 10 ⁶ ab	11.4 x 10 ⁶ c
CzDB + sucrose + gums 0.5%	2.7 x 10 ⁶ d	3.5 x 10 ⁶ c	17.7 x 10 ⁶ d
m-CzDB	1.6 x 10 ⁶ b	1.8 x 10 ⁶ b	3.7 x 10 ⁶ b
m-CzDB + gums 0.5%	1.7 x 10 ⁶ bc	11.8 x 10 ⁶ d	16.4 x 10 ⁶ d
m-CzDB + gums 1.0%	1.9 x 10 ⁶ c	12.5 x 10 ⁶ e	22.7 x 10 ⁶ e
Water	1.4 x 10 ⁶ a	0.9 x 10 ⁶ a	0.9 x 10 ⁶ a
Water + gums 0.5%	1.5 x 10 ⁶ a	1.5 x 10 ⁶ ab	0.6 x 10 ⁶ a
Water + gums 1.0%	1.5 x 10 ⁶ a	1.5 x 10 ⁶ ab	0.7 x 10 ⁶ a

Means in rows followed by the same letters are not significantly different at P=0.05 according to Duncan's test.

Figures

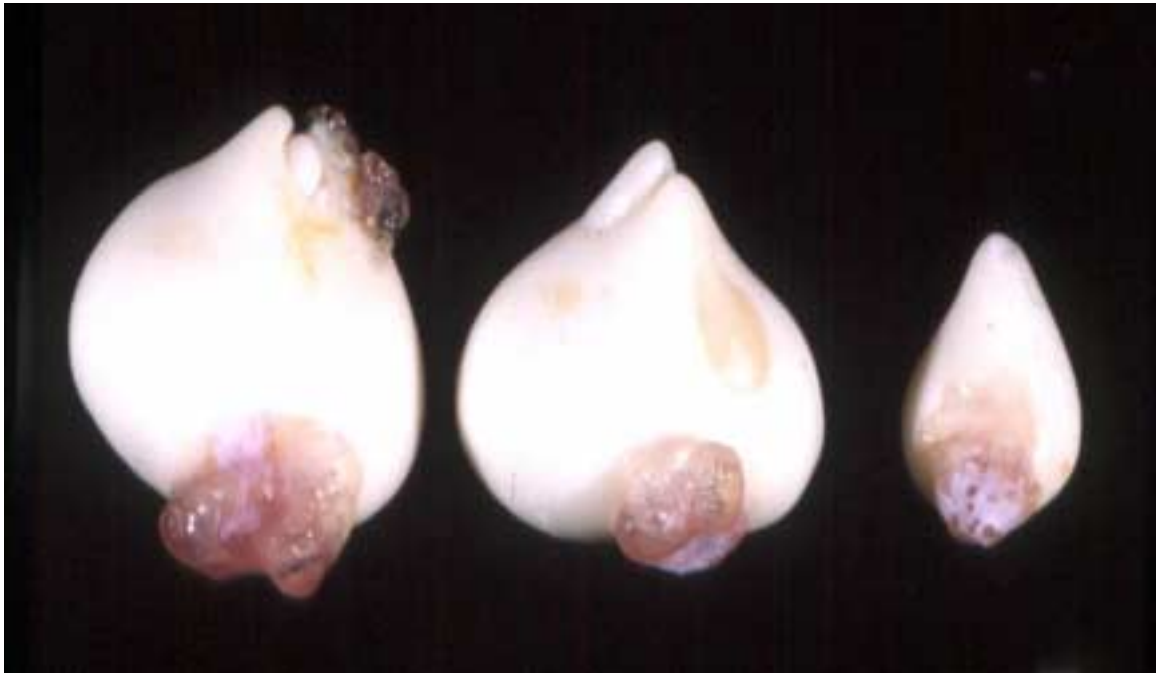


Fig. 1. Gums induced in tulip bulbs by *Fusarium oxysporum* f. sp. *tulipae*

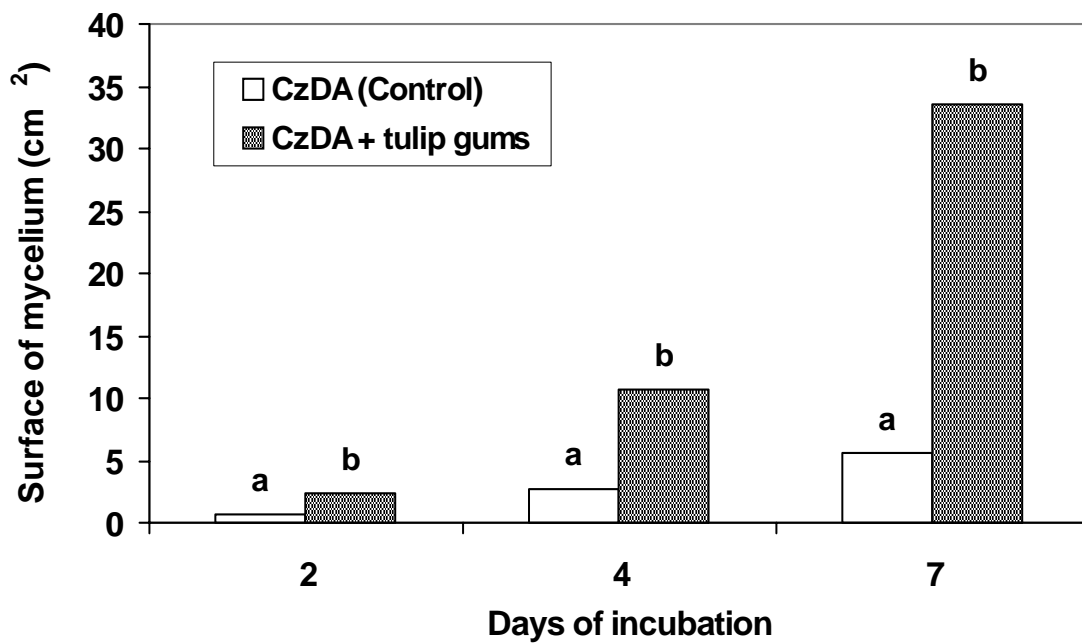


Fig. 2. Influence of tulip gums (5 mg.cm^{-3}) on the in vitro growth of *Fusarium oxysporum* f. sp. *tulipae* cultured on Czapek-Dox-Agar (CzDA). In days of incubation, means followed by the same letter are not significantly different at $P=0.05$ according to Duncan's test; values are calculated separately for each used media.

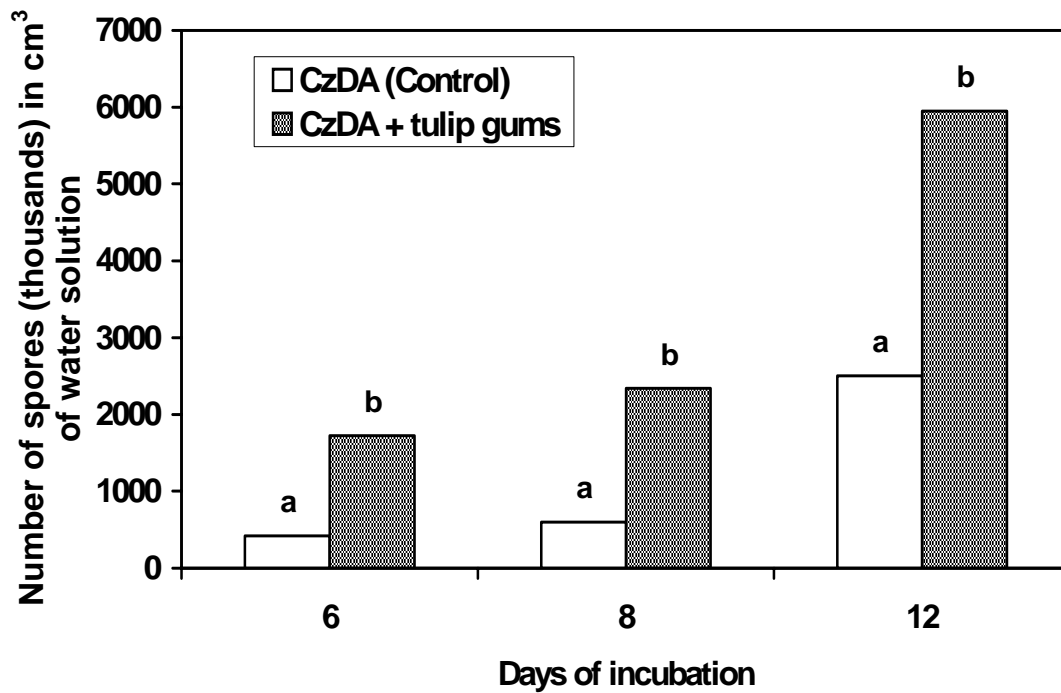


Fig. 3. The effect of tulip gums on sporulation of *Fusarium oxysporum* f. sp. *tulipae* on CzDA. In days of incubation, means followed by the same letter are not significantly different at P=0.05 according to Duncan's test; values are calculated separately for each used media.

