MORPHOLOGICAL AND HISTOLOGICAL CHANGES IN TULIP BULBS DURING INFECTION BY *Fusarium oxysporum* f.sp. *tulipae*

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**Abstract**

Morphological and histological studies in tulip bulbs ‘Apeldoorn’ infected by *Fusarium oxysporum* f.sp. *tulipae* were made. The strongest gum production in inoculated bulbs was observed up to late July, followed by a gradual disappearance of this process. Occurrence of gummosis and its expansion within the parenchyma of a bulb scale caused changes such as: thickening of cell walls in the vicinity of gum formation area, frequent injuries and degeneration of cell walls, cell plasmolysis, gradual degradation of starch grains and enlargement of intercellular spaces. The pathogen penetration through epidermis into the parenchyma of scales and histological changes accompanied the process are described.

**Key words:** tulip bulbs, *Fusarium oxysporum* f.sp. *tulipae*, infection, gums, histological studies

**Introduction**

Infection of tulip bulbs by *Fusarium oxysporum* Schlecht. f.sp. *tulipae* Apt. brings an intensive production of ethylene and formation of gums (Bergman 1965, Kamerbeek et al. 1971). As proved, under *in vitro* conditions this pathogen can produce much more (even by several thousand times) ethylene than other formae speciales of *F. oxysporum* and *Fusarium* species (Swart and Kamerbeek 1976, 1977). A function of gums induced by pathogens in various plants (i.e. tulip, plum, cherry,
apricot, peach) in relation to both the pathogenesis process and pathogen development, has not yet been explained. In literature there is a hypothesis that this kind of gum formation could reflect a defensive response of a plant to pathogenic infection. It is believed that gums have a function in limiting the spread of fungal and bacterial pathogens by isolating the infected tissues (Boothby 1983). Substantial gum production was also observed in healthy, uninfected tulip bulbs treated with ethylene or ethephon (2-chloroethanophosphonic acid). In chemical terms, tulip gums are mainly polysaccharides composed of xylose, arabinose and uronic acid (Saniewski et al. 2000).

The aim of this study was an analysis of morphological and histological changes occurring at various development stages in tulip bulbs infected by *F. oxysporum* f.sp. *tulipae*.

**Material and methods**

**Inoculation**

The study was carried out on ‘Apeldoorn’ tulip bulbs at the following development stages: before and after flowering, before lifting, during flower bud formation (June–September), in uncooled bulbs after flower bud formation, in cooled bulbs and in those obtained from forced bulbs. After superficially disinfection with 50% ethanol, bulbs were inoculated under laboratory conditions by placing 20 μl spore suspension (10⁶ spores per 1 cm³) or 5 mm potato dextrose agar discs overgrown with a seven-day-old mycelium of *F. oxysporum* f.sp. *tulipae*. Inoculation was performed both on damaged and undamaged epidermis. Inoculated bulbs were placed in trays lined with filter paper and aluminium foil which, to increase humidity, were enclosed in polyethylene bags.

At various stages of tulip development, 40 bulbs (10 × 4 replicates) were inoculated with mycelium of *F. oxysporum* f.sp. *tulipae* in two series three days apart. Additionally at some stages: before, during and after flowering, and before lifting, bulbs were inoculated at weekly intervals. However, during flower bud formation (June–September) as well as in uncooled bulbs after flower bud formation (until mid December) inoculation was performed every two, three or four weeks. Uninoculated bulbs were treated as the control.

**Disease estimation**

Observations conducted during a 14-day incubation of bulbs inoculated with *F. oxysporum* f.sp. *tulipae* focused on the development of fusariosis, gum formation induced by pathogenic infection and microscopic analysis of changes occurring in the tissues of scales.

Fusariosis development was recorded upon the measurements of infection spots during bulb incubation. Weights of produced gums by tulip bulbs infected
with *F. oxysporum* f.sp. *tulipae* on intact and damaged bulbs were also measured. Microscopic analyses of pathogen penetration into the host tissues and cytological changes connected with infection were performed with the use of a light microscope (NU-1 and Jenaval Zeiss), scanning electron microscope (JEOL JSM-S1) and a transmission electron microscope (JEOL JEM-120 EX).

A light microscope (LM) was used for determination of mycelium hyphae in gums. Samples of gums were crushed on the microscopic slides and examined without stain and next with addition 1% toluidyne blue dissolved in 1% borax. For anatomical microscopic sections scale pieces (20 × 15 mm) were fixed with CrAF agent (chronic acid, acetic acid, formalin), dehydrated in ethanol and dipped in paraffin, then cut across and stained with safranine and light green (Gerlach 1972).

A scanning electron microscope (SEM) was used to analyse changes of surface scale affected by *F. oxysporum* f.sp. *tulipae* with gummosis induced around and beyond the infected tissue. The fragments (20 × 15 mm) of scales after fixation with CrAF agent and dehydratation in series of ethanol solutions were CO₂ critical point dried and then coated with a gold-palladium by sputtering (Hayat 1976).

A transmission electron microscope (TEM) served to examine particular the distribution of pathogen hyphae within the host tissues and changes in their fine structure. Samples (2 × 2 mm) of the inoculated, damaged tissues and of the tissue beyond this area, were obtained for further examination after 24, 48, 72 h and seven days of incubation. Material was fixed in 5% glutaraldehyde on cacodylic buffer with pH 7.2 for 4 h at room temperature, then in 1% osmum tetroxide on the same buffer for 2 h at 4°C, to be afterwards dehydrated in ethanol and propylene oxide, and finally immersed in Epone 812 (Mercer and Birbeck 1970). The ultra-thin sections were prepared with use Richerd-Jung ultramicrotome, and contrasted with uranyl acetate and lead citrate (Reynolds 1963).

**Results**

In tulip bulbs infected by *F. oxysporum* f.sp. *tulipae* can produce considerable quantities of ethylene, enough to cause gummosis in diseased and healthy bulbs stored in the same conditions (Phot. 1).

This study revealed that *F. oxysporum* f.sp. *tulipae* can infect tulip bulbs at all stage of their development. Gummosis induced by this pathogen first appeared in small bulbs collected during forcing, then during flowering outdoors, directly after lifting as well as during and after flower bud formation, up to the end of October. The strongest gum production in artificially inoculated bulbs was observed up to late July, followed by a gradual disappearance of this process. In early November, uncooled bulbs with progressing fusariosis showed only traces of gum induction, which finally ceased, in mid December. Extruded gums were visible on the surface of scale three–four days after inoculation with *F. oxysporum* f.sp. *tulipae*.

In the case of cooled bulbs, there was no gum formation in response to the pathogen (Phot. 2).
However, when flower buds, leaves and stem were already formed, inoculation of bulbs scale through its undamaged epidermis was often unsuccessful, or if performed, was difficult and prolonged, followed by a slower pathogenesis process, in comparison to the epidermis inoculated when slightly mechanically damaged (Phot. 3, 4).

It seems interesting that during flower bud formation (second half of July) in tulip bulbs inoculated with the pathogen both through damaged and undamaged epidermis, fusariosis development and gum induction were observed. In the case of undamaged epidermis the disease progress was evidently slow (Table 1). Probably natural injuries to this tissue caused by gums coming out on the surface allow the infection of a bulb scale. However, after flower bud formation, both in uncooled bulbs and those cooled not producing gums, the pathogen ability for penetration of undamaged epidermis was limited.

Light microscopic analysis of cross-sections of gums induced in tulip bulbs after inoculation with *F. oxysporum* f.sp. *tulipae* showed the presence of pathogen hyphae and spores in gums neighbouring with the infected scale (Phot. 5). Pathogen induced gums in tulip bulbs first revealed red coloured structures, and some time later, the entire gums turned pinkish-red with mycelium growing within (Phot. 6). Pathogen presence was also detected in gums induced in bulb areas not adjacent to the infected part of a scale. Mycelium colonies were growing out from gum fragments (Phot. 7). Results obtained again indicate that gums induced in tulip bulbs by *F. oxysporum* f.sp. *tulipae* are not a limiting factor for development of this fungus.
Phot. 2. Symptoms of ‘Apeldoorn’ infection with *Fusarium oxysporum* f.sp. *tulipae* and gum formation at various stages of bulb development (photo by A. Saniewska)

Phot. 2. a. Gummosis in daughter bulbs during forcing

Phot. 2. b. Gummosis at the end of flowering under field conditions
Phot. 2. c. Gummosis before lifting

Phot. 2. d. Gummosis during flower bud formation (June–September)
Phot. 2. e. Trace amount of gums in uncooled bulbs after flower bud formation (early November)

Phot. 2. f. Lack of gum induction in uncooled bulbs (mid December)
Microscopic analysis showed that gum production around the infected area in a scale started as soon as 48 h after inoculation with spores. Gum was also found within intercellular spaces distant (uninfected) to the infected tissue and in traces around areas, which were mechanically damaged. On the scale surface, gummosis

Phot. 4. Fusariosis development on tulip bulbs cooled at 5°C for nine weeks, in relation to a mode of damage of scale epidermis: a – with a cork-borer (ø 5 mm), b – with a cork-borer (ø 2 mm), c – punctured three times with a preparation needle, d – punctured once with a preparation needle, e – undamaged epidermis (photo by A. Saniewska)
can be easily detected, being visible as oval-shaped, very smooth areas with unclear contours of epidermal cells (Phot. 8). In a scale, gums are placed under the surface of its outer epidermis, which, in consequence, could be detached from the parenchyma tissue. With the infection progress, gums within intercellular spaces gradually expanded. Around those spaces there were frequent parenchyma cells with walls destroyed, accompanied by the decomposition of amyloplasts containing starch grains (Phot. 9).

Occurrence of gummosis and its expansion within the parenchyma of a bulb scale brought about changes such as: thickening of cell walls in the vicinity of gum formation area, frequent injuries and degeneration of cell walls, cell plasmolysis, gradual degradation of starch grains and enlargement of intercellular spaces. The latter was related to a loss of wall continuity frequently observed in parenchyma cells. Degradation of starch grains within the damaged tissue was accompanied with gum expansion.

Surface of the outer epidermis was overgrown with mycelium hyphae, which penetrated into the parenchyma tissue through the adjacent walls of neighbouring cells. It was revealed by the red stained areas with visible hyphae and lack of continuity in epidermal cell walls as a result of pathogen penetration (Phot. 10). Stomata on a gum surface were often covered with a cuticle-like, membranous and torn structure or with a fibrillar substance blocking stomal pores. On the inner side of a scale there were pathogen hyphae outgrowing from the stomata (Phot. 11). In a gum vicinity, both on the inner and outer side of a scale, mycelium was covered with fine-grained or membranous structures capable of changing the shape of hyphae by flattening them. Among the parenchyma cells filled with starch grains, pathogen hyphae were arranged around the cell walls. This arrangement was often similar to the shape of parenchyma cells, apparently as an effect of

### Table 1

Development of *Fusarium oxysporum* f.sp. *tulipae* (= *Fot*) and production of gums in tulip bulbs ‘Apeldoorn’ during flower bud formation (second half of July); n = 5 bulbs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight of gums produced by bulbs 7 days after incubation (mg)</th>
<th>Length of necrosis 12 days after incubation (mm)</th>
<th>Depth of necrosis 12 days after incubation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damaged epidermis and parenchyma of scale at upper part of bulb + <em>Fot</em></td>
<td>953.8 c</td>
<td>20.1 b</td>
<td>3.4 a</td>
</tr>
<tr>
<td>Intact epidermis and parenchyma of scale at upper part of bulb + <em>Fot</em></td>
<td>664.5 b</td>
<td>9.8 a</td>
<td>2.8 a</td>
</tr>
<tr>
<td>Damaged epidermis and parenchyma of scale at lower part of bulb + <em>Fot</em></td>
<td>606.3 b</td>
<td>19.6 b</td>
<td>9.6 b</td>
</tr>
<tr>
<td>Intact epidermis and parenchyma of scale at lower part of bulb + <em>Fot</em></td>
<td>420.6 a</td>
<td>10.4 a</td>
<td>3.2 a</td>
</tr>
</tbody>
</table>

Means in columns followed by the same letters are not significantly different at 5% level.
intercellular growth of mycelium within the tissue. Pathogen penetration affected the cell walls by causing characteristic waves and folds, and even specific “bays” or “loops” (Phot. 12) which often resembled intercellular spaces with fragments of fungus hyphae within. It appears that in a defensive response to inactivate the fungus, the host tissue tries to enclose the pathogen within the cell wall, which as a cellullosic structure, is difficulty for penetration by mycelium hyphae.

Phot. 5. Mycelium hyphae and microconidia of *Fusarium oxysporum* f.sp. *tulipae* (frequently with red structures) in gums surrounding infected area (bars – 25 \( \mu \)m).

The slides not stained (photo by B. Dyki)
Phot. 6. Gums formed in a bulb scale near an area infected with *Fusarium oxysporum* f.sp. *tulipae* turning pinkish-red (photo by A. Saniewska)

Phot. 7. Mycelium colonies of *Fusarium oxysporum* f.sp. *tulipae* outgrowing from tulip gums, on potato dextrose agar (PDA) culture medium; for a mycological analysis gums in a bulb scale were sampled from their induction areas not adjacent to fusariosis symptoms on a scale (photo by A. Saniewska)
Phot. 8. Gum formation visible on a scale as oval-shaped areas with a distinctly smooth surface and unclear contours of epidermal cells seen under SEM (bars – 100 µm) (photo by B. Dyki)
Phot. 9. Frequent areas of gum formation (dark stained) visible in bulb scale parenchyma; seen under a light microscope 96 h after inoculation with 5 mm agar discs overgrown with mycelium of *Fusarium oxysporum* f.sp. *tulipae* (bar – 100 μm) (photo by B. Dyki)

Phot. 10. Penetration of *Fusarium oxysporum* f.sp. *tulipae* mycelium through the scale epidermis, then between the parenchyma cells, inducing locally a red colouration (bar – 50 μm) (photo by B. Dyki)
Phot. 11. Stomata on a gum surface covered with a membraneous, torn structure blocking the stomal pore (a); mycelium hyphae growing from stomata on the inner side of a bulb scale (b) seen under SEM (bars – 50 µm) (photo by B. Dyki)
Discussion

One of the important questions is why the response of tulip bulbs cv. ‘Apeldoorn’ by gum formation after infection with *Fusarium oxysporum* f.sp. *tulipae* or ethylene treatment disappears gradually after lifting. Probably the metabolic changes during flower bud formation include some senescence processes of scales. Another open question is why only some tulip cultivars showed a strong gummosis response to ethylene treatment (for instance ‘Apeldoorn’, ‘Enterprise’), whereas bulbs of other cultivars (e.g. ‘Red Champion’, ‘White Sail’) hardly showed any response to ethylene – lack of gummosis (Kamerbeek et al. 1971). It might depend on the differences in the sensitivity of tulip tissues to ethylene.

Although *Fusarium oxysporum* f.sp. *tulipae* is a soil pathogen attacking tulip bulbs, all other organs of tulip shoot can also be infected with no specific preference (Saniewska and Wach 2003). However, gummosis occurs exclusively in infected bulbs and such symptoms have not been found around infected areas on other tulip organs.

It is understood that tuliposides A and B found in tulip bulbs scales are precursors of tulipalines which have antibiotic properties and probably play a major part in inducing the resistance against infections with soil pathogens, mainly *F. oxysporum* f.sp. *tulipae*. Tuliposides A and B were also found in tulip leaves – slight
amount in their epidermis and considerably more in the mesophyll (Rutter et al. 1977). However, the epidermis is regarded as a main barrier for pathogen penetration and infection process, so the development of *F. oxysporum* f.sp. *tulipae* both on damaged and undamaged tulip leaves does not seem to be directly connected with the resistance barrier attributed to tuliposides A and B. Some other tuliposides were found in leaves and stems of *Tulipa sylvestris* Linn. and *T. turkestanica* Regel. (Christensen 1999).

As previously reported (Saniewska 2002 a, b) there was a strong stimulative effect on mycelium growth and sporulation of *F. oxysporum* f.sp. *tulipae* when tulip gums induced by this pathogen were added into some culture media such as potato dextrose agar (PDA, Merck), malt extract agar (MEA, Difco) and mineral, solid Czapek Dox agar (CzDA, Difco), while the control culture was not supplemented with gums.

*Fusarium oxysporum* f.sp. *tulipae* grown on liquid Czapek Dox broth (CzDB) medium containing sucrose and supplemented with tulip gums clearly secreted β-1,4-glucosidase, α-1,4-galactosidase, β-xylosidase and polygalacturonase (Saniewska et al. in press). However, on CzDB containing sucrose *F. oxysporum* f.sp. *tulipae* did not secrete these enzymes to the medium. Moreover, grown on mineral CzDB caused secretion of β-1,4-glucosidase, α-1,4-galactosidase, and polygalacturonase and the supplementation of the medium with tulip gums greatly increased secretion of these enzymes and additionally of β-xylosidase (Saniewska et al. in press).

It seems interesting that gums induced in tulip bulbs by *F. oxysporum* f.sp. *tulipae*, apart of this pathogen, also show a stimulative effect on the growth and development of *F. oxysporum* Schlecht. f.sp. *callistephi* (Beach.) Snyd. et Hans. and *F. oxysporum* Schlecht. f.sp. *narcissi* Snyd. et Hans. (Saniewska 2002 c). For these two formae speciales, which are nonpathogenic to tulip, gum induction process in host tissues has never been revealed. In the case of *F. oxysporum* f.sp. *tulipae*, there was a strong stimulation of linear growth of mycelium on PDA, CzDA and MEA culture media supplemented with gums. Although such an effect was not observed for *F. oxysporum* formae speciales nonpathogenic to tulip, in those conditions they developed more substantial mycelium hyphae, both in the medium and air, when compared to the control cultures free of gums.

The results obtained indicate that gums induced in tulip bulbs by *F. oxysporum* f.sp. *tulipae* are not a limiting factor for this pathogen, but on the contrary, they highly stimulate its development. It appears that polysaccharides of tulip gums may act as elicitors, which control some processes connected or responsible for mycelium growth and sporulation, or partly can be used as a substrate. 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).
Summary

Although *Fusarium oxysporum* f.sp. *tulipae* is a soil pathogen attacking tulip bulbs, all other organs can also be infected with no specific preference. However, gummosis occurs exclusively in infected bulbs and such symptoms have not been found around infected areas on other tulip organs. The strongest gum production in artificially inoculated bulbs was observed up to late July, followed by a gradual disappearance of this process. In early November, uncooled bulbs with progressing fusariosis showed only traces of gum induction which finally ceased in mid December. In the case of cooled bulbs, there was no gum formation in response to the pathogen. Microscopic analysis showed that gum production around the infected area in a scale started as soon as 48 h after inoculation with spores. Gums were also found within intercellular spaces distant (uninfected) to the infected tissue, moreover, and around areas, which were mechanically damaged. On the scale surface, gummosis can be easily detected, being visible as oval-shaped, very smooth areas with unclear contours of epidermal cells. In a scale, gums are placed under the surface of its outer epidermis that, in consequence, could be detached from the parenchyma tissue. With the infection progress, gums within intercellular spaces gradually expanded. Occurrence of gummosis and its expansion within the parenchyma of a bulb scale caused changes such as: thickening of cell walls in the vicinity of gum formation area, frequent injuries and degeneration of cell walls, cell plasmolysis, gradual degradation of starch grains and enlargement of intercellular spaces. The latter was related to a loss of wall continuity frequently observed in parenchyma cells. Degradation of starch grains within the damaged tissue was accompanied with gum expansion. Surface of the outer epidermis was overgrown with mycelium hyphae that penetrated into the parenchyma tissue through the adjacent cell walls.

Streszczenie

MORFOLOGICZNE I ANATOMICZNE ZMIANY W CEBULACH TULIPANA ZACHODZĄCE W CZASIE INFEKCJI WYWOŁANEJ PRZEZ *FUSARIIUM OXYSPORUM* F.SP. *TULIPAE*

*Fusarium oxysporum* f.sp. *tulipae*, patogen glebowy porażający cebule tulipanów, może infekować wszystkie organy tulipanu; patogen zatem nie wykazuje specyficzności względem organów rośliny, natomiast indukcja gum następuje tylko w zainfekowanych cebulach tulipanów. Najsilniejszą indukcję obserwowano w cebulach do końca lipca, a potem stopniowo tworzenie się gum zanikało. W pierwszych dniach listopada na cebulach nieprzechłodzonych obserwowano, wraz z rozwojem fuzariozy, indukcję gum w śladowych ilościach, a w połowie grudnia nie było jej już wcale. Nie stwierdzono indukcji gum w cebulach przechłodzonych po zakażeniu grzybią patogenu. Badania mikroskopowe wykazały, że tworzenie się gum nastę-
puje w łuskach cebuli wokół miejsc zainfekowanych już po 48 h od inokulacji łusek zarodnikami F. oxysporum f.sp. tulipae. Występowanie gum obserwowano również w przestrzeniach międzykomórkowych odległych od porażonych fragmentów łuski cebuli (wolnych od patogenu), a w śladowych ilościach – także wokół miejsc uszkodzonych mechanicznie. Obrazy z mikroskopu świetlnego pokazują w gumach indukowanych w cebuli tulipana struktury zabarwione na kolor czerwony, a po dłuższym czasie całe gumy przybierały różowoczerwone zabarwienie, wraz z rozrastającą się w nich grzybią patogenu. Gumy w łuskach cebuli występowały pod powierzchnią epidermy zewnętrznej, powodując jej odczynnianie się od komórek miękiszu łuski. Powstawaniu i nagromadzaniu się gum w miękiszu łusek towarzyszyły takie zmiany, jak: zgubienie ścian komórkowych w pobliżu miejsc tworzenia się gum, uszkodzenie i rozkład ścian, plazmoliza komórek, sukcesywna degradacja ziaren skrobi w tych komórkach i powiększanie się przestrzeni międzykomórkowych. Strzępki grzybiń F. oxysporum wnikały do tkanki miękiszowej gospodarza poprzez ściany komórkowe przylegających do siebie komórek.

Literature

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