ABSTRACT

*Increasing sustainable agricultural production has always been related with cultivation of crops that are better able to tolerate biotic stresses (pests, diseases and weeds) and abiotic stresses (drought, salinity, and temperature stress). During last decades breeding programs apply different biotechnology approaches to improve the performance and productivity of crops, incl. increased resistance to plant diseases caused by toxin produced fungal pathogens. Fungi from genus Alternaria produce a group of mycotoxins as alternariols, altertoxins, tenuasonic acid etc. Host specific toxins of Alternaria spp. plant pathogens play an important role in pathogenesis and they could be applied as selective agents in in vitro selection at the cellular level for disease resistance. Toxin compounds’ effect on development of necrotic lesions on tobacco and tomato leaves was observed. The correlation between plant susceptibility to the pathogen and sensitivity to the toxin substances is discussed in the light of possibilities for application in the crop breeding programmes.*

**Keywords:** *Alternaria*, resistance, toxins

**Introduction**

Plant pathogens are normally specialized to infect certain range of plant hosts and revealing the interactions between them and their hosts is a complicated question. Many factors affect the plant disease development - physiology of the pathogen and host plant, environmental conditions, biochemistry and genetics of the plant-pathogen interactions. Part of these interactions is the role of pathogen toxins in the disease development. Toxins are compounds that are produced by the pathogens and cause part or all of the symptoms of a disease. They are of various chemical types and include peptides, glycoproteins, polysaccharides, organic acids, fatty acids and derivatives, polyketides and terpenoids (13).

Microbial toxins are considered as possible pathogenesis or virulence factors for the producer pathogens and the special weapons of the plant pathogens overcome the inherent resistance strategies of host plants (5). In of some plant diseases phytotoxins may play a critical role in development of the disease symptoms (11). The role of a toxin as a disease determinant is proved by the occurrence of the toxin in an infected plant and the ability of the toxin alone to elicit at least part of the symptoms of the disease (4). The major visible symptoms caused by the toxins are chlorosis, necrosis, and wilting. Some of the toxins have general phytotoxic properties and are active toward a broad range of plant species.

Phytotoxins are divided in two big groups. The non-host-specific toxins contribute to the virulence or symptom development in the disease in which they occur, but are not primary determinants of host range (14). *Alternaria* species are known to produce many non-host-specific toxins as brefeldin A, tenuasonic acid, tentoxin and zinniol are produced from several. They exert their phytotoxic activities through different modes (12). Second group is consisted by host-specific toxins (HSTs) that affect only certain plant varieties or genotypes (7). The HSTs are playing role in determining the host range of specificity of plant pathogens and can act as agent of virulence of those pathogens acting in very low concentrations (10 pM to 1 μM) (14). HSTs can be very divers by chemical structure even when they are produced from pathogens belonging to one genus, as it is in species *Alternaria alternata*. Different pathotypes of this plant pathogen produce different toxins known as AK-, AF-, ACR-, ACT-, ACTG-, AM-, and AAL-toxins, which differ in their structure and host range (1,8).

In vitro selection was considered as a tool aiding classical
selection in breeding disease resistant cultivars based on several advantages as fast testing of large number of individuals on a small place, easier manipulation of mutants, somaclones and haploids with higher variability in the genome and precise evaluating of quantitative differences by avoiding the unfavorable weather conditions.

Materials and methods

Pathogen
Isolate of Alternaria alternata tobacco pathotype isolates No 5 and NB12 (Bulgarian origin) were applied in this study. The pathogen was maintained on potato-dextrose agar (PDA) slants in test tubes at 4° C. Single spores cultures were developed on oat meal agar in Petri dish for the experimental use. The toxic culture filtrate (CF) was collected after cultivation the mycelium in liquid Richard’s medium in 500 ml flasks, each containing 100 ml of the medium. Each flask was inoculated by 5 pieces (5x5 mm in size) of agar containing mycelium of fungus grown on PDA for two weeks. Cultivation of the fungus in the liquid medium was also performed at 25° C in darkness. After spores and mycelium of the fungus were removed by filtration of the CF through Millipore filter (0.22 µm) the sterile CG was stored at 4° C before used.

Plant material
Two Bulgarian tobacco (Nicotiana tabacum L.) cultivars - Krumovgrad 90 and Nevrokop 1146, and Nicotiana rustica, as well as tomato (Lycopersicon esculentum L.) cultivar Bela, were used in this study. All plants were grown under greenhouse conditions in pots and fertilized with the equal amount of fertilizer. Fully expanded leaves of approximately 40 cm high plants were used as a leaf material for detached leaf bioassays.

Inoculation test and toxin bioassay
Small mycelium pieces (5 mm in diam.) excited from 1 week old culture and drops of crude CF (10 µl each) were applied to the upper epidermis of leaves excited from mentioned above plan cultivars, and the leaf epidermis was slightly injured by a needle. The leaves were kept in petri dishes on moist filter paper at 25° C and light 16 hr day / 8 hr night. Diameter of spots was measured three days after inoculation.

Results and Discussion
Fungal plant pathogens from genus Alternaria are known to produce toxin substances when grown in liquid medium so far (6, 12). In the inoculation experiments presented here the mycelium of Alternaria alternata tobacco pathotype infected tobacco leaves causing the typical for brown spot disease necrotic lesions surrounded by chlorotic hallo.

The level of infection on tobacco leaves was different when mycelium from single spore cultivation was applied (Table 1). Some of the single spore cultures (5-1, 5-2, 5-5) infected leaves of Nicotiana rustica, but the other single spore cultures did not developed infection (5-3, 5-4)(Figure 1a). Those five single spore cultures all originated from one A. alternata isolate No 5. The fact that only some of them were infectious, demonstrating that spores with different virulence could be produced within one isolate and the level of infection differed when those isolates were applied on the leaves of highly susceptible N. rustica. The mycelium of all five cultivations did not developed lesions on the leaves of both cultivars Krumovgrad 90 and Nevrokop 1146 that belong to species N. tabacum (data not shown). The more virulent isolate A. alternata NB12 successfully infected all tobaccos used in the experiment (Table 1), causing the typical symptoms of brow spot diseases (Figure 1b). The lesions appeared on the more susceptible N. rustica on the third day after inoculation, while on the cultivated tobacco cultivars the first lesions developed a week after inoculation. At the same time when this isolate was applied on tomato leaves, the mycelium was growing above the leave surface without damaging them (Figure 2a, b). When culture filtrate without mycelium but containing toxic substances produced by the fungi was applied on the leaf surface of the all used plant species typical lesions with chlorotic halo developed on the tobacco leaves, but not on tomato leaves (Table 2). Only small necrotic pinpoints were observed on the place of application, but they did not developed further in larger lesions, as it happened in case of the tobacco leaves (Figure 2a, c). Those small necrotic injuries are most probably an answer of tomato cells which is a non host for A. alternata plant pathotype to the non HST substances contained in the culture filtrate, while the developed large lesions on tobacco leaves are result of the action of HST, contained in the same culture filtrate. The host-specific toxins determine the host range of specificity of plant pathogens and can act as agent of virulence of those pathogens (14). The equal symptoms produced on tobacco leaves from fungal mycelium and from the toxic culture filtrate proved the role of the specific toxins produced by the fungus in the pathogenesis. This is a correlation shown in tobacco - Alternaria plant-pathogen interactions, but it is not always the case. There are
examples when resistance expressed from nonorganized tissue does not correlate and is just the opposite of the expressed resistance in vivo at whole plant level (2, 9). This toxin could selectively kill the susceptible plant cells and thus to be potentially used as selective agent in the in vitro selection schemes.

In the case of *Alternaria alternata* tobacco phtotype, when the toxin is not purified and his structure is yet not known, a partially purified toxins or culture filtrates of the pathogens can be used as selective agents. Comparing to a toxin alone, these the culture filtrates produced during growth of the fungus in liquid medium have an advantage, because they contain a set of toxic compounds that may be involved in the plant-pathogen interactions. Their toxic specificity can be detected on species, cultivars or lines plant level. In our previous investigations a partially purified AT-toxin of *Alternaria alternata* tobacco pathotype showed toxin specificity at cultivar level (10). It is allow using the culture filtrate of this fungus as selective agent. If a chosen toxin is used as a screening agent it must be involved in the disease development and must act directly at the cellular level (3, 15). The method was widely functional for years, but still not fully exploited. The other application of HST is in model plant-pathogen systems helping the reveal of some aspects of pathogenesis during infection of host plant by the plant pathogen *Alternaria alternata*.

**TABLE 1**

Infection of tobacco leaves by different isolates of *A. alternata* tobacco pathotype

<table>
<thead>
<tr>
<th>A. alternata single spores cultures</th>
<th>Tobacco species / cultivars</th>
<th>N. tabacum cv. Krumovgrad 90</th>
<th>N. tabacum cv. Nevrokop 1146</th>
<th>N. rusica</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5-2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5-5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NB12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+ lesion with chlorotic hallo developed  
- no lesion no chlorotic hallo either developed

**TABLE 2**

Response of tobacco and tomato to artificial inoculations with mycelium and culture filtrate of *A. alternata* tobacco pathotype

<table>
<thead>
<tr>
<th>A. alternata tobacco pathotype</th>
<th>Tobacco</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter and type of lesions</td>
<td>8,5</td>
<td>7</td>
</tr>
<tr>
<td>mycelium</td>
<td>necrotic lesions with chlorotic hallo</td>
<td>necrotic lesions with chlorotic hallo</td>
</tr>
<tr>
<td>culture filtrate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Developed brown spot lesions by different single spore isolates of *A. alternata* on leaves *N. rustica* (a), and *N. tabacum* (b).](image)
Fig. 2. Mycelium growth of A. alternata tobacco pathotype (a, b) and lesion produced by culture filtrate (c) on tomato leaves.

REFERENCES