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Published in:
Plant Science

DOI:
10.1016/0168-9452(92)90032-H

Publication date:
1992

Plant Science, 81 (1992) 127–134
Elsevier Scientific Publishers Ireland Ltd.

Tomato susceptibility to *Alternaria* stem canker: Parameters involved in host-specific toxin-induced leaf necrosis

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(Received July 29th, 1991; revision received September 30th, 1991; accepted September 30th, 1991)

AAL-toxin causes severe necrosis in leaves of susceptible tomato cultivars at nanomolar concentrations. In resistant tomato cultivars harbouring the semi-dominant *Alternaria* stem canker resistance locus necrosis is also observed, however at much higher toxin concentrations. In both lines the percentage of the leaf area exhibiting necrosis is dependent on toxin concentration and on length of toxin exposure. However, at the same toxin concentration, periods of toxin exposure resulting in similar necrosis are much longer for the resistant than for the susceptible tomato. It was demonstrated that toxin uptake in the leaves does not imply toxin uptake in the cells since a discrepancy was observed between death of protoplasts, isolated from leaves cut for protoplast isolation immediately after incubation on AAL-toxin and necrosis in leaves when further incubated on water. However, when after exposure to AAL-toxin leaves were further incubated on water for 24 h before they were cut for protoplast isolation, a correlation was found between leaf necrosis and death of protoplasts. This suggests that further transport is needed in leaves after toxin uptake, bringing toxin to all the cells, that cannot occur in leaves cut for protoplast isolation. Light plays an important role in AAL-toxin induced necrosis and it was shown that length of light exposure controls necrosis development like toxin concentration and length of toxin exposure. The product of these 3 parameters can provide a good hint to predict the extent of leaf necrosis. The effect of light might be restricted to differentiated leaf tissue, since it was not observed in callus tissue.

Key words: Alternaria alternata f.sp. *lycopersici*; host specific toxin; tomato; leaf necrosis development; resistance mechanism

Introduction

Alternaria alternata f.sp. *lycopersici* causes *Alternaria* stem canker in some cultivars of tomato. The disease is characterized by formation of dark brown cankers on stems and necrosis of leaf tissue between the veins [1]. The fungus produces a toxin, called AAL-toxin, that plays a major role in pathogenesis and is responsible for development of leaf necrosis [2,3]. AAL-toxin exhibits the same host-specificity as the fungus: tomato genotypes susceptible to the fungus are sensitive to AAL-toxin and tomato genotypes resistant to the fungus and non-host species are insensitive to AAL-toxin [2,4].

Resistance to the fungus and insensitivity to AAL-toxin in tomato are conferred by the *Alternaria* stem canker locus, the *Asc*-locus [5], that is mapped on chromosome 3, position 93, and was shown to be the same in 3 non-related tomato lines [6]. Insensitivity to AAL-toxin is semi-dominant and can be overcome by high toxin concentrations [5]. Protoplasts of the susceptible tomato were far more sensitive to AAL-toxin than protoplasts of the resistant tomato [7], indicating that (1) a cellular target for AAL-toxin is present and (2) the *Asc*-locus is expressed at the cellular level. It has been suggested that ACTase, an enzyme involved in pyrimidine synthesis, might be the cellular target for AAL-toxin [8]. However, in electron

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0168-9452/92/$05.00

microscopic studies an effect on mitochondria was observed [9]. These two observations are not consistent since tomato ACTase is suggested to be located in the nucleus [10].

Since indications on the cellular target site are limited, the mechanism of resistance is difficult to predict. Resistance, however, might reside at the level of the plasma membrane, or within the cell. For other host-specific toxin-plant cell interactions it has been demonstrated or suggested that specificity is mediated by plant cell plasma membranes or mitochondrial membranes, e.g., for host-specific toxin produced by *Helminthosporium victoriae* a plasma membrane receptor has been suggested [11,12], for host-specific toxins produced by *Helminthosporium maydis* and *Phyllostica maydis*, respectively, URF 13, a mitochondrial membrane protein has been shown to confer sensitivity, that is absent in insensitive cytoplasms [13–15]. Other cellular mechanisms of insensitivity might imply insensitivity of a toxin target site; overproduction of a cellular target or detoxification of toxic molecules, based on resistance mechanisms described for aspecific toxins and herbicides with enzymes as target sites [16].

To gain more insight into functioning of the *Asc*-locus and location of the recognition event(s), that determines a subsequent susceptible or resistant reaction of the plant to the pathogen, development of AAL-toxin induced necrosis was studied in leaves, that can be observed in both the susceptible and resistant lines. Development of necrosis in resistant lines, although occurring at much higher toxin concentrations, suggests that a target site is present in these lines as well [4,5]. Some factors have already been described to play a role in necrosis development. Necrosis, starting in the veins, develops in susceptible host plants dependent on age of leaves: going from top to bottom in a plant a decrease in sensitivity to AAL-toxin was observed [7]. Light was shown to play an important stimulating role in development of leaf necrosis induced by AAL-toxin [7,17]. However, an effect of light is not observed at the protoplast level, since protoplasts incubated in toxic medium died as soon in light as in dark and at the same toxin concentrations [7]. An explanation might be that the effect of light is related to a certain level of tissue organization; differences in sensitivity to AAL-toxin of different plant tissues have been described [4,18]. On basis of these observations, we further studied the influence of toxin concentration, period of toxin exposure and period of light exposure on leaf necrosis development. Moreover, the role of differentiated leaf tissue is further assessed in relation to these parameters.

Materials and Methods

Plant material

As plant material we used F9 near-isogenic *Asc*-lines of *Lycopersicon esculentum* Mill [5], derived from cultivars Ace 55 VFN (homozygous dominant; resistant) and Earlypak 7 (homozygous recessive; susceptible) by continuous selfing of heterozygotes. Near-isogenic lines were kindly provided by D. Gilchrist, University of California, Davis, USA. Plant material for leaf bioassays was grown in a greenhouse at 25°C, 60% relative humidity. Aseptical plant material was grown in MS medium [19] supplemented with 1% sucrose under a 16-h daylength light cycle under 3 000 lux warm white and grolux fluorescent lighting, 25°C and 60% relative humidity. Purification and characterization of AAL-toxin was performed as described previously [4].

Leaf bioassays

Leaflets of 4-week-old greenhouse grown plants were excised and placed in plastic petri dishes on 9-cm discs of filter paper saturated with 3 ml of AAL-toxin dissolved in H_2O [2]. Petri dishes were sealed with Nescofilm and incubated in a growth chamber at 25°C in light at 3 000 lux supplied by one Grolux F36W/GRO lamp together with one Grolux standard F36 W/133 cool white lamp per shelf (Sylvania, F.R.G.) or in dark: packed into 2 layers of aluminium foil. Bioassays were carried out in 2-fold: 2 petri dishes with 2 leaves per petri dish. Symptoms were expressed in percentage of the leaf surface that exhibited necrosis. Results were scored after 48 h.

For aseptically grown plants, 10 leaflets per petri dish were used and symptoms were expressed in percentage of the leaf surface that exhibited necrosis. Bioassays with leaves from aseptically

grown plants were scored after 72 h since necrosis develops more slowly in those leaves than in leaves from greenhouse grown plants.

Since sensitivity of leaves to AAL-toxin might not be constant due to variations in growth conditions in the greenhouse (light, temperature, season) experiments that had to be compared quantitatively were performed at the same time.

Protoplast assays

After 3–4 weeks growth, leaflets of aseptically grown plants were harvested and protoplasts isolated as described by Koornneef et al. [20]. Protoplasts were plated in 1/2 VKM medium [21] in 96 well minititer plates (Greiner) with 125 μl per well in a concentration of 2×10^5 per ml. Viability of protoplasts was assessed after 10 days by counting of minicalli after fluorescein diacetate staining [22] using a Nikon Diaphot-TMD inverted microscope.

Callus assays

Callus was induced on MS salts [19], 3% sucrose, 2 mg/l naphtalene acetic acid, 1 mg/l 6-benzylamino purine on leaf discs from plants grown aseptically. To determine the effect of AAL-toxin on callus growth in light and dark, 16 calli with a total weight of 0.5 g were placed on agar plates 5-fold. Plates were incubated in a growth chamber in light or in dark (see above). After 3 weeks callus weight on each plate was determined.

Results

Period of toxin exposure

To gain insight into toxin uptake in leaves, the percentage necrosis was determined resulting from different periods of toxin exposure and different toxin concentrations for leaves of susceptible and resistant tomato lines. Leaves were incubated for a limited period on AAL-toxin solution and after toxin exposure leaves were washed and transferred to new petri dishes without toxin and further incubated for totally 48 h in light. Results are shown for the homozygous recessive genotype in Fig. 1A and for the homozygous dominant genotype in Fig. 1B. For both, the homozygous recessive and

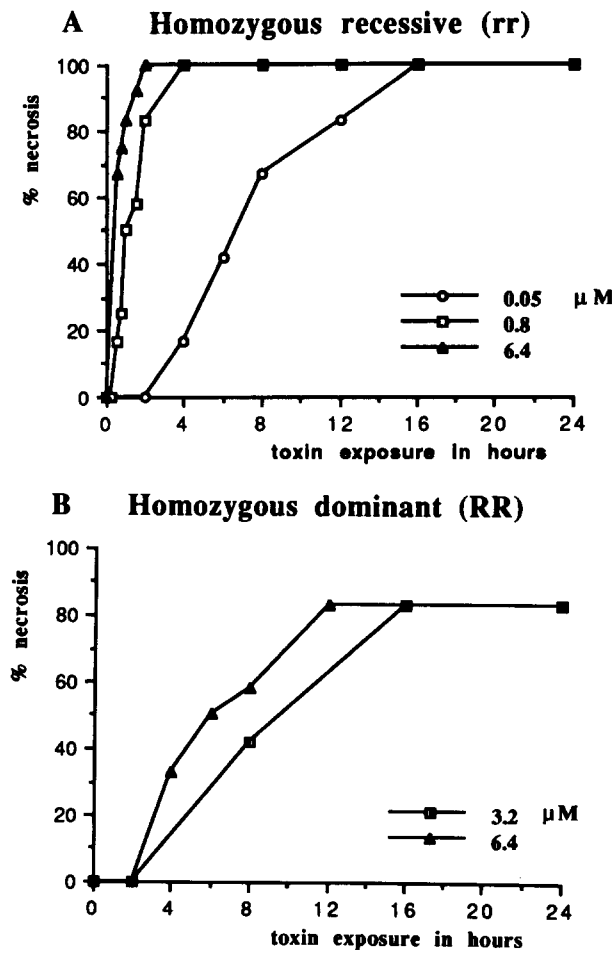

Fig. 1. Determination of percentages of the leaf area exhibiting necrosis as a result of period of toxin exposure and toxin concentration in leaf bioassays. Leaves were incubated for a limited period on toxin (X-axis) and further incubated on water. For the homozygous recessive genotype this was performed at 0.05, 0.8 and 6.4 μM of AAL-toxin (A); for the homozygous dominant genotype at 3.2 and 6.4 μM of AAL-toxin (B). Results were scored after 48 h incubation in light.

homozygous dominant genotype it was demonstrated that the percentage necrosis resulting from a certain period of toxin exposure depends on toxin concentration. For similar necrosis the period of toxin exposure decreases with increasing toxin concentrations. However, genotypic differences were obvious: when the same concentration of 6.4 μM of AAL-toxin was applied, the period of toxin exposure resulting in 50% necrosis was much

shorter for the homozygous recessive (<0.5 h) than for the homozygous dominant tomato (6 h).

Differences in toxin concentration between the homozygous recessive and homozygous dominant genotype resulting in similar necrosis were more than 100 times: the curves of 50 nM for the homozygous recessive genotype and 6.4 μM for the homozygous dominant are approximately overlapping (Fig. 1), representing the differences in sensitivity described for these phenotypes [4].

Correlation between necrosis in leaves and death of protoplasts

To investigate whether toxin uptake in leaves implies toxin uptake in the cells, the relationship between necrosis in leaves and death of protoplasts was studied. Leaves from aseptically grown plants of the homozygous recessive genotype were incubated for 2 h at increasing concentrations of AAL-toxin. Subsequently, part of the leaves was immediately cut for protoplast isolation, part of the leaves was further incubated without toxin for 24 h before they were cut for protoplast isolation and part was further incubated without toxin for 72 h to score necrosis development. Controls consisted of leaves and protoplasts treated in the same way, however without toxin. Results are shown in Fig. 2. Leaves, incubated for 2 h at 1.25 μM AAL-toxin and further incubated without toxin showed 50% necrosis after 72 h, at higher toxin concentrations 100% necrosis was observed. Death of protoplasts, isolated from leaves cut directly after 2 h toxin exposure also increased with increasing toxin concentrations, compared to protoplasts isolated from untreated leaves. However, a discrepancy was observed between leaf necrosis and protoplast death. From leaves showing 100% necrosis after 72 h (when incubated on 2.5 and 5 μM AAL-toxin; Fig. 2), 92% and 58% surviving protoplasts were isolated, respectively. After incubation on 10 μM of AAL-toxin only 8% protoplast survival was observed. Apparently, AAL-toxin when applied in concentrations of 1.25–5 μM did not enter most of the target cells within the period of toxin exposure, at least not in concentrations high enough to cause cell death. However, a good correlation between necrosis in leaves and protoplast death was

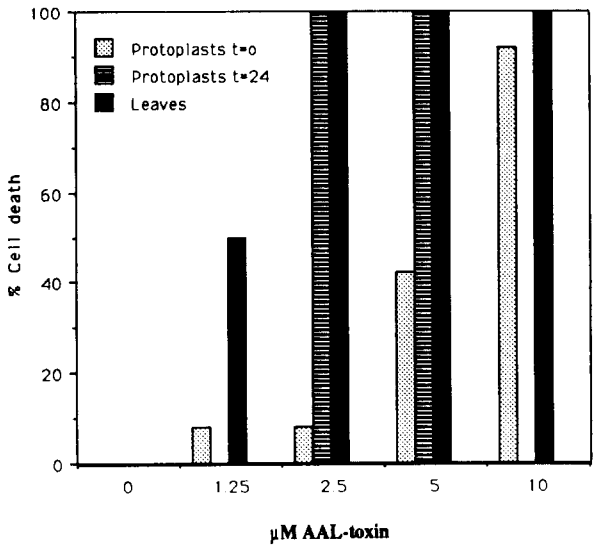

Fig. 2. Correlation between necrosis in leaves of the homozygous recessive genotype and death of protoplasts isolated from those leaves after 2 h toxin exposure on increasing concentrations of AAL-toxin (X-axis). Leaves were cut for protoplast isolation directly after toxin exposure on 1.25, 2.5, 5 and 10 μM of AAL-toxin (protoplasts $t = 0$) or after toxin exposure on 2.5 and 5 μM of AAL-toxin and further incubation on water for 24 h (protoplasts $t = 24$). Controls consisted of leaves and protoplasts isolated from leaves incubated in the same way, however without toxin. Survival of protoplasts was determined by counting of minicalli in 7 wells (originally 1.75×10^5 protoplasts) after 10 days and data were converted to percentages cell death relative to the controls. For controls a plating efficiency of 0.2% was calculated.

observed when leaves were further incubated on water for 24 h before they were cut for protoplast isolation. Protoplasts isolated from leaves exposed to 2.5 and 5 μM AAL-toxin and further incubated for 24 h all died while control protoplasts isolated from leaves incubated on water were still alive. Plating efficiencies of control protoplasts isolated from leaves either or not further incubated were comparable (0.2%). These results suggest that transport in leaves after toxin exposure plays an important role in necrosis development.

Relationship between toxin concentration, toxin exposure time and light exposure time

Light has been reported to play an important role in necrosis development. The relationship be-

tween length of light exposure, toxin concentration and length of toxin exposure was investigated. Leaves of the homozygous recessive genotype were incubated in light at 3 different toxin concentrations of 0.05, 0.1 and 0.8 μM of AAL-toxin for different periods of toxin exposure. Combinations of toxin concentration and period of toxin exposure were made which in previous experiments (Fig. 1A) resulted in 100% necrosis when exposed to light for 48 h. After toxin exposure and transfer to new petri dishes without toxin, leaves were exposed to variable periods of light. Eleven combinations were made of toxin concentration, period of toxin exposure and period of light exposure . All combinations tested are presented in Fig. 3 with resulting percentages necrosis. Twenty four hours light exposure turned out to be a prerequisite for 100% necrosis under the applied conditions (Fig. 3 compare data on lines 5, 6, 10 and 11). At a light exposure of 24 h, toxin concentration and toxin exposure time determine the percentage necrosis in the leaves (Fig. 3 compare data on lines 1, 2 and 7). However, more than 24 h light exposure can compensate for decrease in toxin concentration and period of toxin exposure (Fig. 3 compare data on lines 1, 2, 3, 4, 8 and 9). When the product of AAL-toxin concentration, period of toxin exposure and period of light exposure was more than 19.2 under our conditions, a mean value of 96% leaf necrosis was observed, a product of 12.8 resulted in a mean value of 48% necrosis and a product of 9.6 in a mean value of 29% necrosis. Therefore, length of light exposure is a parameter influencing the percentage necrosis in leaves, like toxin concentration and length of toxin exposure.

AAL-toxin induced effects on calli grown either in light or in dark

To learn whether the effect of light on development of necrosis could be translated into an effect on growth of undifferentiated cells, calli of the homozygous recessive genotype were incubated either in light or in dark at increasing AAL-toxin concentrations. Effects of AAL-toxin on callus growth scored after 3 weeks are summarized in Table I. Relative callus growth decreased at increasing toxin concentrations after incubation in dark as well as in light. Differences were relatively small: callus growth decreased to 6% of the control in light and to 15% in dark at the highest tested toxin concentration of 12.5 μM suggesting that light is not an important parameter in AAL-toxin

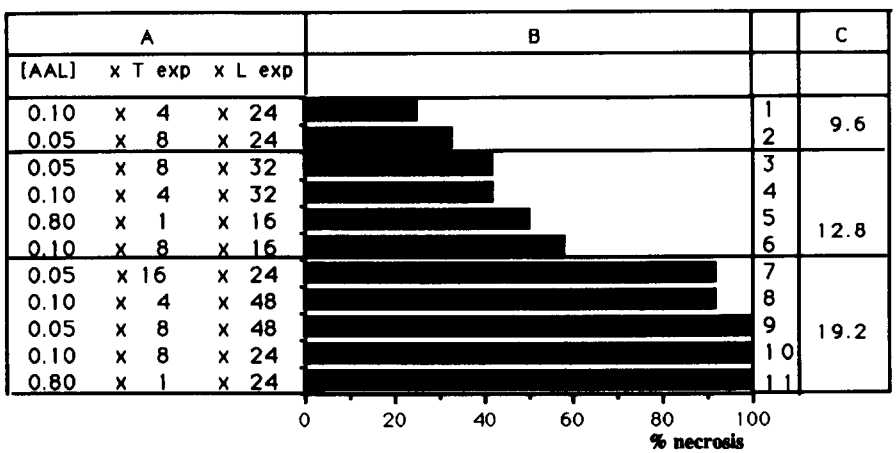

Fig. 3. Determination of the percentage leaf necrosis as a result of period of light exposure, AAL-toxin concentration and period of toxin exposure determined in leaf bioassays with leaves of the homozygous recessive tomato genotype. Leaf bioassays were performed under the conditions described in column A, containing values tested for the 3 parameters: AAL-toxin concentration in μM [AAL], period of toxin exposure in hours [T exp] and period of light exposure in hours [L exp]. Results were scored after 48 h and are presented in column B: bars represent the resulting percentages necrosis. Column C contains the product of the 3 parameters given in column B: AAL-toxin concentration, period of toxin exposure and period of light exposure (μM × h × h). Line numbers are referred to in the text.

Table I. Growth of calli of the homozygous recessive genotype incubated either in light or in dark at increasing AAL-toxin concentrations. Results were scored after 3 weeks and are presented as relative percentages callus growth.

Conc. AAL (μM)	Light (%)	Dark (%)
0	100 ± 9	100 ± 24
0.10	78 ± 8	70 ± 17
0.50	46 ± 9	52 ± 14
2.50	20 ± 5	27 ± 11
12.50	6 ± 5	15 ± 6

induced growth decrease as it is for leaf necrosis development. However, it is difficult to compare effects on callus growth and necrosis development in leaves since they are qualitatively different.

Discussion

In this study we tried to gain more insight into AAL-toxin action and functioning of the *asc*-locus by studying factors influencing necrosis development. For both the homozygous dominant and the homozygous recessive genotype, it was shown that development of leaf necrosis is dependent on toxin concentration and length of toxin exposure; periods of toxin exposure resulting in 50% necrosis decrease at increasing toxin concentrations (Fig. 1). However, in order to acquire the same amount of damage, periods of toxin exposure at the same toxin concentration were much longer in the resistant than in the susceptible tomato. Regulation of necrosis development in the resistant tomato is comparable to regulation of necrosis development in the susceptible tomato. Whether differences in sensitivity to AAL-toxin are mediated by differences in an intracellular target site or by a resistance mechanism located in the plasmamembrane remains to be determined.

It was demonstrated that toxin uptake in the leaves within the period of toxin exposure does not imply toxin uptake in the cells, since no correlation was observed between necrosis in leaves and protoplast death when leaves were cut immediately after toxin exposure. Only when leaves where further incubated on water for 24 h prior to cutting for protoplast isolation a correlation was observed between leaf necrosis and protoplast death. Leaf structures, therefore, seem to be involved in transport of toxin to all the cells that are no longer present in intact leaves cut for protoplast isolation. It can be concluded that transport plays an important role in the AAL-toxin-tomato leaf interaction.

In leaf bioassays, increase in toxin concentration, length of toxin exposure and length of light exposure all resulted in increase in necrosis development and it was shown that these 3 parameters could compensate each other quantitatively within a certain range and the product of the 3 parameters was shown to correspond to a certain percentage necrosis in the leaves. It can be envisaged that all 3 parameters help in concentrating toxin at the membrane or intracellular target site and that light achieves this by stimulating toxin transport. Further research should reveal whether and how AAL-toxin enters the cells for instance by using fluorescent or radioactively labelled AAL-toxin. Since the effect of AAL-toxin in callus hardly increased by light as in protoplasts [7] the stimulating effect of light might be related to specialized leaf tissue.

Stimulating effects of light on toxin action are easily explained when cellular targets of toxins are part of light-regulated processes. Tabtoxin, produced by *Pseudomonas tabaci* inhibits glutamine synthetase, which leads to accumulation of ammonia formed during photorespiration; both accumulation of ammonia and subsequent development of necrosis are light dependent [23]. However, the effect of light can also be very complex as can be examplified by the T-toxin-susceptible corn leaf interaction, where the effect of light has been extensively studied. Host-specific T-toxin binds to a 13 kD protein in membranes of susceptible corn mitochondria, resulting in a number of cellular responses [15]. An effect of light on symptom development in corn leaves was shown [24], that could also be observed in protoplasts [25]: protoplasts isolated from etiolated leaves were insensitive to T-toxin, while protoplasts from green leaves were highly sensitive. However, mitochondria isolated from both tissues were equally sensitive, suggesting that the effect of light is not mediated by the toxin target. When subsequently

exposed to light, light increased sensitivity of protoplasts from etiolated tissue, but decreased sensitivity of protoplasts from green tissue. Beside a light induced negative effect, also a positive effect of light was observed; photophosphorylation in protoplasts from green tissue might compensate for toxin induced reduction in ATP levels [25,26]. Therefore, the effects of light might be quite diverse resulting in an effect on development of disease symptoms.

We have to be aware that by use of leaf bioassays pathogenesis is studied in an artificial system. However, it might tell us that by influencing toxin transport light might effect the concentration of toxin within or around the cells and subsequent cell death. In this way light might determine occurrence of the disease: *Alternaria* stem canker has only been reported in California and Japan, countries with sunny climates [1,27]. It is probable that *Alternaria alternata* f.sp. *lycopersici* does not produce high amounts of toxin: in resistant tomato cultivars no necrosis is observed after infection with the pathogen, while resistance to the pathogen implies only relative insensitivity to AAL-toxin [5]. Why the pathogen has not developed into a higher toxin producer, making it a more virulent pathogen independent of climate conditions is an intriguing question, asking for more profound analysis of this plant-pathogen interaction.

Acknowledgements

This work was supported in part by a grant from Stichting Innovatiefonds Plantenveredeling (InPla).

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