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Interaction of root-knot nematodes (RKN) and the bacterium *Agrobacterium tumefaciens* in roots of *Prunus cerasifera*: evidence of the protective effect of the *Ma* RKN resistance genes against expression of crown gall symptoms

Maria-Jose Rubio-Cabetas*, Jean-Claude Minot, Roger Voisin and Daniel Esmenjaud**
*Nematology Group, 'Plant Health and Environment' Unit, Institut National de la Recherche Agronomique, 123 Bd Francis Meilland, 06600 Antibes France; *Present address: Aristotelian University of Thessaloniki, Department of Genetics and Plant Breeding, School of Agriculture, 54006 Thessaloniki, Greece; **Author for correspondence (E-mail: esmenjau@antibes.inra.fr)*

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Abstract

Agrobacterium tumefaciens (AT) is the causal agent of crown gall, a major problem in the family *Rosaceae* and particularly for *Prunus* spp. Crown gall symptoms result from the bacterial infection of the cells damaged mechanically at the collar or by root parasitic nematodes. Myrobalan plum (*P. cerasifera*) is susceptible to AT and is not a host for the root-knot nematode (RKN), *M. hapla*. Some clones of this plum carry single *Ma* resistance genes that control *M. arenaria*, *M. incognita* and *M. javanica*. The four above mentioned RKN and Myrobalan progenies segregating for *Ma* were used in experiments aimed at obtaining a better knowledge of the interaction between AT and RKN in relation to the RKN resistance genes. *Prunus* rooted cuttings, naturally infected with the bacterium were repotted, grown and inoculated individually with RKN. In a first experiment, *Prunus* plants were (i) either inoculated with 10,000 juveniles (J2s) of *M. arenaria* to provide a short inoculum pressure (SIP) or (ii) inoculated by association with one *M. arenaria*-galled tomato root system that produced a high and durable inoculum pressure of the same nematode species. Four months after RKN inoculation, plants were rated for nematode and bacterial root galling symptoms. RKN and AT galls were more numerous and more homogenous under DIP than under SIP. Nevertheless, for both inoculum regimes, AT galls were present in the RKN-susceptible clones (= carrying none of the *Ma* genes) and absent in the RKN-resistant clones. Subsequent experiments, conducted under DIP with *M. arenaria, M. incognita*, *M. javanica* and *M. hapla*, also showed, for the three first species, the presence of AT galls only in RKN-susceptible clones whereas *Prunus* plants inoculated with *M. hapla* and nematode-free controls were free of AT galls. Consequently RKN act as a wound agent in the AT infection process of Myrobalan plum only when the plant develops a compatible reaction (i.e. when it lacks the *Ma* resistance genes). Considering that J2s do penetrate the roots of resistant plants, the absence of crown gall symptoms on this material even under durable inoculum pressure strengthens the hypothesis that this nematode stage has a very weak effect on plant cells during the infection process. This is the first evidence of the protective effect of a RKN resistance gene against the expression of root crown gall consecutive to RKN infection. The protective effect of *Ma* and presumably of other RKN resistance genes against AT is a strong argument for their introgression into *Prunus* and other *Rosaceae* or bacterium-susceptible crops.

Introduction

The bacterium *Agrobacterium tumefaciens* (Smith and Townsend) Conn. has a wide host range and is particularly common in fruit trees (Garett, 1987) and ornamentals (Poncet et al., 1996) of the *Rosaceae* family. A total of 643 host plants have been recorded that belong to 93 botanical families (De Cleene and De Ley, 1976). With root-knot nematodes (RKN), this bacterium is an important disease of ligneous crops that are propagated vegetatively and is a real concern in nurseries. The typical symptoms of 'crown' galls are the consequence of bacterial penetration into the plant at sites of wounded tissue mainly at the collar or root level. Bacterial penetration is commonly found at wounded stem sections due to preparation of cuttings, cultivation practices or at nematode penetration sites near the root tip. The bacterium is distributed throughout Mediterranean climates and its incidence is favoured by hot and humid conditions (Faivre-Amiot, 1982). No resistance genes to crown gall have been described, but differences in tolerance against *A. tumefaciens* (AT) have been observed specially for *Prunus* species (Pierronnet and Salesses, 1996) and roses (Reynders-Aloisi et al., 1998).

Root-knot nematodes (*Meloidogyne* spp.) are common pests of stone fruits (*Prunus* spp.) grown in Mediterranean areas. The main species that attack *Prunus* rootstocks are *M. arenaria* in the most northern regions and *M. incognita* and *M. javanica* in the warmer regions of the South. Due to the ban of nematicides, search for alternative control methods for these pests is in progress and plant resistance is an increasing matter of interest. Different resistance genes have been found for peach, almond and plum (Esmenjaud et al., 1997) which have various spectra of activity. The more promising source is Myrobalan plum, *P. cerasifera*, which has a wide and high-level resistance conferred by the *Ma* genes. This putative family of single dominant genes acts against all tested major and minor RKN species (Lecouls et al., 1997; Rubio-Cabetas et al., 1999) and their expression is not affected by high temperatures or a high inoculum pressure (Esmenjaud et al., 1996a).

Association between the bacterium *A. tumefaciens* and RKN have been reported on almond (Orion and Zutra, 1971), peach (Dhanvantari et al., 1975), grape (Aubert et al., 1983) and raspberry (Griffin et al., 1968). Nematode attack and consecutive wounds allow the penetration of the bacterium into the plant tissues, the integration of the bacterial T-DNA from the Ti plasmid into the plant genome of adjacent cells and the development of typical crown gall symptoms. We have observed in our genetic work on RKN resistance in Myrobalan plum, conducted from 1990 to 1995, an increasing frequency of crown gall on the roots of RKN-susceptible plant material. This observation was used in the present study, which started in 1995 and is based on naturally bacterium-infected plant material, genetically characterized for RKN resistance genes. As all Myrobalan plums are susceptible to crown gall (Pierronnet et al., 1996), our objective was to demonstrate, in presence of RKN as potential wound agent, the differential expression of the bacterial galls in relation to the resistance status (presence or absence of the *Ma* genes of Myrobalan plum clones). The origin of the plant infection by the bacterium is not the matter of this study.

Material and methods

Plant material

The clones of *P. cerasifera* (Myrobalan plum) used in the study belong to a diallel cross (G1 material) completed with appropriate backcrosses (G2 material) established at INRA Villenave d'Ornon (Bordeaux, France) and used previously for genetic study of the RKN resistance (Esmenjaud et al., 1996b). Their interaction with RKN nematodes is defined on a resistant/susceptible terminology. As *Ma* genes confer a high level of resistance, RKN do not reproduce in resistant clones (= carrying a *Ma* gene) which are completely free of nematode galls. In susceptible clones, nematode reproduction and subsequent galling range from low to high levels, depending on the inoculum pressure provided by the evaluation test as described by Esmenjaud et al. (1996a). Because of the high correlation between nematode numbers and galling (Esmenjaud et al., 1992), the classification of the clones can be reliably based on RKN symptoms. Susceptible/resistant clones are therefore differentiated by the presence/absence of RKN galls. The two types of RKN inoculation and the method for RKN resistance rating are described further under 'Interaction between RKN and the bacterium'.

The genotype of all tested clones is presented in Table 1. Molecular studies (Lecouls et al., 1999) have identified markers for the *Ma1* gene and stated that

Ma1 and *Ma2* are closely linked or allelic. In each cross, clones were sampled at random among available material. Crosses involving P.2175 (heterozygous for *Ma1*) and susceptible parents segregate in the G1 progenies whereas crosses involving P.1079 (homozygous for *Ma2*) segregate when backcrossed with any of the susceptible parents (recessive for both genes) (Esmenjaud et al., 1996b).

Nematode species

The following RKN isolates, one each of the predominant RKN species were used: *M. arenaria* 'Monteux' from Monteux, Vaucluse, France, *M. incognita* 'Calissanne' from Calissanne, Bouches-du-Rhone, France, *M. javanica* 'Higuera' from Cabrils, Cataluna, Spain, and *M. hapla* 'Canada', originally introduced from Canada. All isolates were reared from a single egg mass and maintained on tomato (*Lycopersicon esculentum* Mill.) cv. St Pierre. The specific identity of the isolates was verified each year before inoculation via their isoesterase phenotype (Janati et al., 1982).

Agrobacterium tumefaciens *strain*

The bacterial strain of *A. tumefaciens* was originally isolated from root crown galls of *P. cerasifera* naturally infected with RKN. The virulence of the strain was first verified using biological and biochemical tests (Pionnat et al., 1996). Since then the bacterial identification has been confirmed every year by PCR analysis based on the amplification of the plasmid Vir region (Pionnat, 1997). The strain referenced as CFBP 1932 (Collection Française de Bactéries Phytopathogènes, Angers, France) is a bacterium of the nopalin type and belongs to Biovar 1 (Poncet, pers. com.).

Interaction between RKN and the bacterium

The Myrobalan-plum material was propagated at INRA Villenave d'Ornon (Bordeaux, France) from softwood cuttings sampled on adult trees. These trees represent one of the putative sources of AT. Homogeneous cuttings (25 cm long, 5 mm diameter) were harvested in May or June, rooted in sandy beds, another putative source of AT, and grown individually into alveolated plates in the nursery until the following late autumn to allow development of rooted plants. The cuttings were then transferred in December to INRA Antibes (France). Cuttings of all clones were then repotted individually in early spring into a 5-l container filled with a sandy substrate (60% sand; 25% loam; 15% silt). All containers were placed on benches in a greenhouse and grown until harvest for rating. Greenhouse temperatures ranged from 20 to 30 °C.

RKN inoculation

Two types of RKN inoculation were used. The first type, termed 'short inoculum pressure' (SIP), was based on the direct inoculation of second-stage juveniles (J2s) of the nematode at a given date. The second type, termed 'durable inoculum pressure' (DIP), was based on the presence of one RKN-galled root system from tomato with each of the *Prunus* plants.

SIP was applied as follows: in mid-May each container was inoculated with 10,000 24–72-h-old J2s of one of the RKN species. The J2s were deposited into two holes, 2 cm deep and 2 cm from the stem. J2s were obtained in a mist chamber from tomato roots previously inoculated with the same RKN species.

DIP was applied as follows: in mid-March, at the same time when *Prunus* cuttings were repotted into 5-l containers, tomato plantlets grown in the same greenhouse in 250-ml plastic containers were inoculated with 500 24–72-h-old J2s of one of the RKN species and deposited as described for SIP. In mid-May top parts of tomato plants were cut and removed and one whole galled root system with soil was carefully transferred into each *Prunus* container.

Evaluation of Prunus *material*

Whatever the inoculum pressure used, containers inoculated with the same *Meloidogyne* species were arranged in a completely randomized block design on a greenhouse bench. Groups of containers corresponding to different RKN species were isolated from each other with transparent splash screens. For each clone–RKN species combination and each type of inoculum pressure, six replicates were tested. Four months after RKN inoculation, *Prunus* plants were harvested. The root system of each plant was carefully washed and rated for RKN and crown gall root symptoms. RKN rating was based on a gall index rating according to the 0–5 scale (Barker, 1985) (0 = no gall; 1 = 1–10% of root system galled; 2 = 11–30%; 3 = 31–70% ; 4 = 71–90% ; 5 > 90%). Nematodes were not extracted and counted because a previous study (Esmenjaud et al., 1992) had clearly shown that for a given RKN species gall index is highly significantly correlated with the

Table 1. Phenotype and genotype for RKN resistance of the Myrobalan plum material used in the study

Parental and hybrid material	Phenotype	Genotype[1]
P.2175	R[2]	*Ma1 ma1, ma2 ma2*
P.1079	R	*ma1 ma1, Ma2 Ma2*
P.2646	S[2]	*ma1 ma1, ma2 ma2*
P.16.5	S	*ma1 ma1, ma2 ma2*
P.2175 × P.16.5		
Clones 23, 25, 28, 31	R	*Ma1 ma1, ma2 ma2*
Clones 18, 19, 20, 22	S	*ma1 ma1, ma2 ma2*
P.2175 × P.2646		
Clones 15, 20	R	*Ma1 ma1, ma2 ma2*
Clones 17, 24	S	*ma1 ma1, ma2 ma2*
(P.2646 × P.1079)9[3]	R	*ma1 ma1, Ma2 ma2*
P.2175 × P.2646[4]	segregates for *Ma1*	
– 64 clones	R	*Ma1 ma1, ma2 ma2*
– 55 clones	S	*ma1 ma1, ma2 ma2*
P.2646 × (P.2646 × P.1079)9	segregates for *Ma2*	
– 8 clones	R	*ma1 ma1, Ma2 ma2*
– 18 clones	S	*ma1 ma1, ma2 ma2*

[1]All genes expressed in a dominant fashion; *Ma1* and *Ma2* closely linked or allelic.
[2]R = resistant; S = susceptible.
[3]Individual (= clone) number 9 of the cross between the female P.2646 and the male P.1079.
[4]Reciprocal crosses P.2175 × P.2646 and P.2646 × P.2175 are added.

$\log_{10}(x+1)$ transformed numbers of each of the developmental stages in the roots. Root crown gall rating was based on four classes : 0 = no gall; 1 = 1–5 galls; 2 = 6–25 galls; 3 = over 25 galls (a 0.5-step increment was assigned when galling was intermediate between two classes). Mean RKN and AT gall ratings (6 replicates) per clone were compared between the DIP and SIP inoculation type, using a one-way analysis of variance. Means were separated using Newman-Keuls multiple range test at $P = 0.05$.

Planning of the experiments and distribution of Prunus *material*

Trials were carried out over a three-year period. During the first year, a comparison between the two types of inoculum pressure (SIP and DIP) was made with *M. arenaria* to find out whether this factor played a role in crown gall expression. This experiment was performed with eight clones of the cross P.2175 × P.16.5 (segregating for the *Ma1* gene into four resistant (R) and four susceptible (S) clones; Table 1) and four clones of the cross P.2175 × P.2646 (segregating for *Ma1* into two R and two S clones; Table 1). During the second and third years, all experiments were performed with a durable inoculum pressure. During the second year, trials aimed at studying the interaction among the three major RKN species, *M. arenaria*, *M. incognita* and *M. javanica*, which are all controlled by the *Ma* genes. The tests involved parents and a G1 cross segregating for *Ma1* gene (P.2175 × P.2646: 119 clones ranging into 44 hybrids P.2175 × P.2646 and 75 reciprocal hybrids P.2646 × P.2175). All 119 G1 clones (64 R and 55 S) were tested for *M. arenaria*. A subset of the 119 clones (96 clones splitting into 53 R and 43 S) were also tested for *M. javanica*. A subset of the 96 clones (76 clones splitting into 43 R and 33 S) were also tested for *M. incognita.* During the third year, the experiment was carried out using *M. hapla*, for which *P. cerasifera* is not a host (Esmenjaud et al., 1994), together with *M. arenaria* (as a reference species controlled by *Ma* genes). The experiment involved a G2 cross segregating for *Ma2* gene (P.2646 × (P.2646 × P.2175)9 : 26

clones; 8 R and 18 S (Table 1)). Each year, one set of nematode-free control plants of the tested clones was grown and rated at harvest in the same way as inoculated plants. Additionally, the roots of one set of plants were also injured with a knife (15 cm deep, five times per container) in the 2nd- and 3rd-year tests to provide the mechanically-injured *Prunus* controls.

Results

Effect of inoculum pressure

In the experiment that compared the effect of inoculum pressure, RKN and AT galling was generally more pronounced and more homogenous under DIP than under SIP (Table 2). Nevertheless, as expected, the six RKN-resistant and the six RKN-susceptible clones from the crosses P.2175 × P.2646 and P.2175 × P.16.5 could be clearly separated whatever the type of nematode inoculation. Crown gall rating showed that none of the six R clones tested under both SIP or DIP expressed bacterial symptoms. In contrast, all of the six S clones tested under both SIP or DIP expressed bacterial symptoms. These results relating to *M. arenaria* infection suggest that crown gall symptoms only develop in susceptible clones, regardless of the type of inoculation regime.

Effect of the RKN species controlled by the Ma1 *gene*

In the experiments conducted during the 2nd and 3rd years, crown gall infection was less marked than in the SIP/DIP comparison conducted during the 1st year

Table 2. Root-knot nematode (RKN) and root crown gall ratings on G1 hybrid clones segregating for the *Ma1* gene of *Prunus cerasifera* after inoculation by *Meloidogyne arenaria*, using either a short inoculum pressure (SIP) or a durable inoculum pressure (DIP). Each clone naturally infected with *Agrobacterium tumefaciens* (AT) was inoculated individually with the isolate of *M. arenaria*. Data are means of six replicates per clone

Type of cross	Clone identity	RKN under SIP		RKN under DIP	
		Rating[1]/ RKN	Rating[2]/ AT	Rating/ RKN	Rating/ AT
P.2175 × P.16.5	S[3] clones				
	23	2.1a[4]	2.0A[5]	3.5b	2.9B
	25	3.5a	2.2A	4.0a	2.9B
	28	3.9a	2.7A	4.5a	3.0A
	31	3.5a	2.5A	4.4b	3.0A
	R[3] clones				
	18	0	0	0	0
	19	0	0	0	0
	20	0	0	0	0
	22	0	0	0	0
P.2175 × P.2646	S clones				
	15	3.8a	2.8A	4.3a	3.0A
	20	3.2a	2.2A	4.1b	2.9B
	R clones				
	17	0	0	0	0
	24	0	0	0	0

[1]RKN gall ratings: 0 = no gall; 1 = 1–10% of root system galled; 2 = 11–30%; 3 = 31–70%; 4 = 71–90%; 5 = over 90%.
[2]Crown gall ratings: 0 = no gall; 1 = 1–5 galls; 2 = 6–25 galls; 3 = over 25 galls.
[3]R = resistant (see Table 1 for resistance genotype) and S = susceptible for *M. arenaria*.
[4]Rating/RKN: data within the same row followed by the same lowercase letter are not significantly different ($P = 0.05$) according to Newman-Keuls multiple range test.
[5]Rating/AT: data within the same row followed by the same uppercase letter are not significantly different ($P = 0.05$) according to Newman-Keuls multiple range test.

and data were therefore summarized on a presence-absence basis. A given clone was considered as positive when at least one characteristic gall of the crown gall type was observed on one of the six cuttings (= replicates). When the nematode species controlled by the *Ma1* gene, i.e. *M. arenaria*, *M. incognita* and *M. javanica* , were inoculated, most susceptible clones showed crown galls whereas none of the R clones (except one plant, in the case of *M. javanica*, which may have been injured accidentially) expressed bacterial symptoms (Table 3). The strong relationship between RKN susceptibility and crown gall incidence is evident for each RKN species considering that a high number of segregating clones have been tested: 119 for *M. arenaria*, 96 for *M. javanica*, 76 for *M. incognita*. Thus the 76 clones tested simultaneously for each of the three major species show that the development of crown gall is independant from the nematode species considered. The role of the nematodes in the infection process is confirmed by the complete absence of crown gall on nematode-free control plants. As expected, mechanically-injured control plants expressed bacterial symptoms but very few galls were produced.

Effect of M. hapla *(for which* P. cerasifera *is not a host)*

In a test with a G2 cross segregating for *Ma2* conducted with the control species *M. arenaria*, the clones carrying *Ma2* (as for *Ma1*) were not affected by *A. tumefaciens* whereas clones lacking the resistance gene expressed bacterial symptoms (Table 3). The same 26 clones, whatever their *Ma* genotype, did not express any bacterial symptoms when inoculated with *M. hapla*. As expected, crown gall symptoms were absent in the nematode-free controls and mechanically-injured control plants expressed bacterial symptoms (although very few galls were produced).

Discussion

Results showed that root gall symptoms caused by *A. tumefaciens* only occurred on RKN susceptible clones (i.e. carrying none of the *Ma* genes) in the presence of the nematode. In contrast, crown galls were not detected when plants are non hosts for the inoculated nematode species or when the plant carries a RKN gene that controls this nematode species. Consequently, the dominant alleles of the *Ma1* and *Ma2* genes protect Myrobalan plum material against root crown gall and the nematodes act as causative agents to render the roots more receptive to the penetration and the subsequent development of the bacterium and plant transformation by the Ti plasmid.

This association of bacterial and nematode symptoms has already been reported by several authors. All cases correspond to associations for which the plant species is a host for the RKN, e.g. almond and *M. javanica* (Orion and Zutra, 1971), raspberry and *M. hapla* (Griffin et al., 1968) or grapevine *Vitis vinifera* and *M. incognita* (Aubert et al., 1983). Here, we report in a particular plant species the first evidence of the protective effect of RKN resistance genes against the expression of the crown gall symptoms. It is highly probable that other RKN resistance genes act in the same way against *A. tumefaciens* and such a protective effect is a strong argument for their introgression into *Prunus* and other *Rosaceae* or bacterium-susceptible crops.

The data also provide useful information for integration into the current knowledge of the behaviour of the nematode. In Myrobalan plum, Voisin et al. (1999) have shown that, during the first 48 hours following inoculation, *M. arenaria* J2 penetration into the roots does not differ between the susceptible and the resistant clones. The nematodes do not cause much root damage during this early step as infective J2s migrate intercellularly in the root tips (Gravato-Nobre et al., 1995; Wyss and Grundler, 1992) before they establish their permanent feeding within the root's vascular cylinder. The absence of crown gall symptoms in resistant material suggests that, despite J2 invasion, the bacterium infection is inhibited. The plant's response suggests a rapid necrosis, characteristic of the putative hypersensitive reaction, in presence of a RKN resistance gene although the histological studies to demonstrate this have not yet been completed. In susceptible clones it is not known when the nematode assists the bacterial penetration. It may happen either when the J2s establish their feeding site or more likely later, when the females cause root tissue disruption by increasing body size and by the extrusion of the gelatinous egg sac (Orion and Franck, 1990). Our study does not provide information on the dynamics of AT infection between the time of RKN inoculation and four months later when the final symptoms have been assessed. However, our results do illustrate that *M. hapla* does not parasitize *Prunus* and also confirm Myrobalan plum as having a non-host status for this RKN.

Table 3. Root symptoms caused by *Agrobacterium tumefaciens*[1] on parental clones and on G1 and G2 segregating hybrid clones (*Ma1* and *Ma2* genes) after inoculation with different root-knot nematode (RKN) species (*Meloidogyne* spp.). Each clone, naturally infected with *A. tumefaciens*, was inoculated individually with one isolate of *Meloidogyne*

Genes involved and type of crosses	Total numbers of clones in crosses	*M. arenaria*		*M. javanica*		*M. incognita*		*M. hapla*	Symptoms in	
		R[2]	S[2]	R	S	R	S		nematode-free control	mechanically-injured control
Parents										
P.2175 (*Ma1*) or P.1079 (*Ma2*)										
RKN resistance status		R		R		R		Non host	RKN: −[3]	RKN: −[3]
AT symptoms[1]		No		No		No		No	AT: −[3]	AT: +[3]
P.2646										
– RKN resistance status			S		S		S	Non host	RKN: −	RKN: −
– AT symptoms		Yes		Yes		Yes		No	AT: −	AT: +
Gene Ma1										
2175 × P.2646[4]	119[5]									
– Distribution of clones into the R and S classes		64	55	53	43	43	33	Non host	RKN: −	RKN: −
– Proportion of clones with AT symptoms		1/64	38/55	0/53	34/43	0/43	32/33	0/119	AT: −	AT: +
Gene Ma2										
P.2646 × (P.2646 × P.1079)[9]	26									
– Distribution of clones into the R and S classes		8	18					Non host	RKN: −	RKN: −
– Proportion of clones with AT symptoms		0/8	18/18					0/26	AT: −	AT: +

[1]AT symptoms are rated on a presence-absence basis. A clone is considered positive when at least one of its six replicates (cuttings) showed one or more AT symptoms. [2]R = resistant (see Table 1 for resistance genotype) and S = susceptible for the species of *Meloidogyne*. [3](RKN: −) and (AT: −) mean that the corresponding clone(s) do(es) not show RKN and AT symptoms, respectively; (AT : +) means that the corresponding clone(s) show(s) AT symptoms. [4]Reciprocal crosses P.2175 × P.2646 (44 clones) and P.2646 × P.2175 (75 clones) are added. [5]119 clones (splitting into 64 R and 55 S) have been evaluated for *M. arenaria*; 96 (53 R + 43 S) of the 119 clones have been evaluated for *M. javanica*; 76 (43 R + 33 S) of the 96 clones have been evaluated for *M. incognita*.

Even though we have demonstrated the effect of RKN in the expression of crown gall symptoms under natural infection by the bacterium, the origin of this natural infection is still unknown. A complementary study is in progress. Preliminary data indicate that the bacterium may not be of external origin (rooting substrate) but may be already present in the root tissues and might be introduced via the cuttings from the mother plant material. To date our attempts to develop such AT symptoms after artificial bacterial inoculations of the substrate coupled with inoculations with RKN or the root-lesion nematode *Pratylenchus vulnus*, have not been successful.

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