CORVINUS UNIVERSITY OF BUDAPEST
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INTERACTION BETWEEN PHYTOPLASMA STRAINS OF DIFFERENT VIRULENCE AND ITS IMPACT ON THE DEVELOPEMENT OF CROSS PROTECTION

Thesis of Doctoral Dissertation

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1. PREVIOUS RESEARCHES AND MAIN OBJECTIVES

Today, phytoplasmal diseases cause an increasingly important economic problem both in herbaceous and ligneous ornamental plants and crops.

Although the pathogens are studied since the 1900's, only when novel molecular approaches became available at the end of the 20th century could an effective and detailed analysis of their life-cycle and taxonomic classification be performed. Whereas there is no effective control against the diseases caused by phytoplasmas, prevention should be emphasized. In Hungary, currently ESFY (European Stone Fruit Yellows) and, since 2013, FD (Flavescence dorée) - affecting apricot and grapevine - cause the most serious damages respectively. In Hungarian apricot orchards, the ratio of apricot dieback can reach up to 70-80%. In the neighbouring countries, AP (Apple Proliferation), affecting apple trees, is the disease with the greatest economical importance. This disorder has slighter importance in Hungary, but because of the close geographic proximity and rapid spread of the vectors it may become a serious plant health problem.

With the surveys presented below our goal was to study the plant-pathogen interaction and the interactions between phytoplasma strains and to characterize phytoplasma strains with different virulence. These finding may help setting up a biological plant protection method against phytoplasmas.

Our main goals were the following:

1. to prove the putative inhibitory effect of '*Candidatus* Phytoplasma mali' strain 1/93 under greenhouse circumstances on periwinkle and tobacco test plants.

2. to test the effect of *'C*a. P. mali' 1/93 avirulent strain in cross protection modus against the *'Ca.* P. mali' AT virulent strain of the same species against *'Candidatu*s Phytoplasma prunorum' GSFY and *'Candidatu*s Phytoplasma pyri' PD1 virulent strains, which belong to the same taxonomic group, and against *'Candidatu*s Phytoplasma solani' STOL and *'Candidatu*s Phytoplasma asteris' AAY1 virulent strains, which belong to other taxonomic groups.

3. to detect the simultaneous presence of multiple different phytoplasma strains in naturally infected apple trees, and to investigatite the correlations between symptom expression and the consistence of phytoplasma populations *in planta* with molecular methods.

4. to characterize the '*Ca*. P. mali' 1/93 accession based on the *hflB* and *imp* genes.

2. MATERIALS AND METHODS

The phytoplasma groups and strains used in the surveys were maintained in periwinkle (*Catharanthus roseus* L.), tobacco (*Nicotiana occidentalis* H.-M. Wheeler) and apple (*Malus domestica* Borkh. 'Golden Delicious') test plants. Some of them were kept in the greenhouse of the Plant Protection Institute in Budapest and others were maintained in a climatized greenhouse in the Julius Kühn Institute in Dossenheim, Germany. *'Ca.* P. mali' 1/93 avirulent, 17/93 mild virulent, AT virulent strains (16SrX group), '*Ca.* P. prunorum' GSFY virulent strain (16SrX group), '*Ca.* P. pyri' PD1 virulent strain (16SrX group), '*Ca.* P. solani' STOL virulent (16SrXII group) and '*Ca.* P. asteris' AAY1 virulent strain (16SrI group) were used in the experiments.

The effect of the different phytoplasma strains on periwinkle plants was evaluated by measuring the stem diameter of 10-10 shoots of every plant in every 2 months from the 6th month post-infection. Symptoms were visualized at the 6th month post-infection.

Apple trees, older than 10 years of age naturally infected by '*Ca*. P. mali' strains and artifically infected trees maintained in an unheated greenhouse were subjected to studies about multiple infections. In addition, the relationship between multiple infection and the lack of symptoms was also studied.

DNA was extracted according to Doyle and Doyle (1990). A 528 bp ATP00464 fragment of the *hflB* gene and a fragment of the *imp* gene of phytoplasmas affected in multiple infection were used as a template for PCR. AP specific fHflB3_1/rHflB_3 (Schneider and Seemüller, 2009) primers derived from the *hflB* gene and f318B_seq/r318B_seq (Kube et al., 2008) primers derived from the *imp* gene were picked and used for the amplification.

The variability of the phytoplasma strains were analyzed by SSCP analysis, which is applicable for detecting small variances in nucleotide sequence of two phytoplasma strains. The denaturated PCR products were separated on polyacrylamide gel. PCR products showing complex SSCP profile were ligated into pGEM-T Easy vector system (Promega, Madison, WI) and were used to transform *Escherichia coli* XL1 Blue cells (Stratagene, La Jolla, CA). Colony PCR was employed to amplify the inserts of recombinant plasmids using the PCR primers. The PCR products were used in the SSCP analysis. For sequencing the PCR products, the recombinant plasmid DNA showing different SSCP profiles was extracted using a miniprep kit (Qiagen, Hilden, Germany). Sequence alignment with each other and with other sequences in NCBI database was performed by the Clustal X2 program (Thompson et al., 1994) and phylogenetic analysis were conducted in the MEGA4 program (Tamura et al., 2007).

Due to former observations on accession 1/93, a suppressive action of this strain was taken into consideration and tested against the same species, '*Ca*. P. mali' AT, against another species in the same taxonomic group, '*Ca*. P. prunorum' GSFY and '*Ca*. P. pyri' PD1 and against virulent strains belonging to other taxonomic groups: '*Ca*. P. solani' STOL and '*Ca*. P. asteris' AAY1 in periwinkle and in tobacco test plants in a number of biological replicates.

Both preliminary (cross protection modus, immunization) and simultaneous infection were applied. When simultanously infected, the two strains (avirulent and virulent as well) were grafted on the plant at the same time. When immunized, the plants were grafted first with the avirulent strain than post-infected with the virulent strain after 2.5, 3, or 4 months in the case of AT and 3 months in the case of GSFY, PD1, STOL, AAY1 strains. Samples were taken regularly from the graft site or far from the graft site. DNA was isolated from the leaf midribs with Doyle and Doyle DNA extraction method (Doyle and Doyle, 1990).

The phytoplasma infection was rated by Real-Time PCR. For the identification of the 1/93 avirulent and AT virulent strains of '*Ca*. P. mali', a set of AP specific primers (fhflB-RT/rhflB-RT1) and Cy5- and FAM-labeled probes were used. The GSFY and PD1 virulent strains were detected by the fECA-RT/rECA-RT and fPD_RT/rPD_RT specific primers, respectively, designed by ourselves. The presence of the STOL and AAY1 virulent strain was detected by PCR using the stolbur specific fSTOL/rSTOL and the AY specific fAY/rAY primer pairs, respectively.

3. RESULTS

The inoculated control plants differed considerably in their symptoms, which manifested in the vigor and the stem diameter of the diseased plants. Our results indicate that the suppressor strain 1/93 can inhibit the multiplication of a related but virulent strain of '*Ca*. P. prunorum'. The suppressive action was evident and long-term (still measurable after two years of observation period). According to our results, avirulent 1/93 strain can inhibit the propagation of a virulent strain of another taxon, '*Ca*. P. prunorum'. Cross protection activity among related but different phytoplasma species is reported here for the first time.

We confirmed through many repetitions that cross protection effect is active when the plant is immunized by the avirulent strain and the infection by the virulent strain happens later. The efficacy of the inhibition was not decisively influenced by the time period passed between the two infections. The decrease in the amount of the virulent strain is related to the time period between immunization and post-infection.

We also subjected the root samples from infected plants in the cross-protection modusto our surveys. The phytoplasma stayed over the detection level for the longest in root samples.

We analyzed the importance of the grafting site, and found that it influences only the initial period of the inhibition.

The inhibition by strain 1/93 was effective when the plant was infected by the two phytoplasma strains in the cross protection modus. In the case of simultaneous infection, the virulent strain dominated, although the presence of the avirulent strain was also detectable.

The inhibiton by the strain 1/93 is supposed to be effective against the virulent PD strain, which belongs to the same taxonomical group, but more experiments are required for clarification.

The results obtained in the cross protection modus with 1/93 against AAY1 and STOL strains showed that suppressor 1/93 strain was detected in all randomly collected stem samples, but the presence of virulent AAY1 and STOL strains (belonging to distantly related taxonomic groups) could be also detected. Thereby these results support the presumption that the cross protection is effective among closely but not distantly related phytoplasma strains.

New results of the simultaneous presence of '*Ca*. P. mali' strains with different pathological and other biological traits and their possible interactions were reported. We revealed that the fluctuation in symptom appearence in apple trees is depends on the predominance of the infecting phytoplasma strains. The differences between the strains can be established at the level of the *hflB* gene sequences. Our results suggest that some phytoplasma accessions are composed of different

strains, which may vary in their virulence. These variations appear at the nucleotide level of the *hflB* and *imp* genes. We showed that strains of phytoplasma complex in apple possess high host plant preference.

New scientific results:

1. We proved that '*Candidatus* Phytoplasma mali' 1/93 strain exerts an antagonistic effect and can inhibit the multiplication of the virulent AT strain, belonging to the same species, in tobacco and periwinkle test plants and this inhibition is long-term.

2. Our results may suggest that '*Candidatus* Phytoplasma mali' 1/93 avirulent strain can inhibit the multiplication of a closely related but other virulent strain ('*Candidatus* Phytoplasma prunorum' GSFY). It is proven for the first time that cross protection can be involved in successful inhibition of the spread and multiplication of closely related but different phytoplasmas.

3. We could prove that cross protecion can be effective if the plant is pre-immunized by the avirulent strain and the post-infection happens later.

4. Our new and supplementary results about the pathogenicity impact of multiple infections suggest that symptom fluctuation is related to the predominance of the infectous strains. The differences between the strain virulence can be represented at the *hflB* gene level.

5. Our results suggest that multiple infections are caused by a phytoplasma strain complex. The virulence of the different strains may vary. These differences are also present at the level of the *imp* and *hflB* genes.

6. We established that the accession 1/93 is composed of two strains which are specific in tobacco and periwinkle, respectively.

4. CONCLUSIONS

The virulence of the different phytoplasma strains can be observed visually, based on the symptoms.

The symptom fluctuation is due to the multiple infections of different strains of '*Ca*. P. mali'. Strain complexes may exist which are composed both of virulent and avirulent strains. More than 50% of the studied 10-years-old apple trees proved to be infected by 2-5 different strains. Nevertheless, the ratio of the naturally multiple infected trees can be much higher due to the vector activity through many years. The symptom expression may be influenced by the multiplication conditions of virulent and avirulent strains, by their interactions and so by the shifts in populations. It is supposed that some compounds of the avirulent accession can inhibit the virulence factors. The variability of the strain complexes are present at the level of the *hflB* and *imp* genes.

The 1/93 strain complex has great host plant preference on tobacco and periwinkle. Probably both the avirulent and virulent strains are present in both test plants but due to their distinct fitness, the predominant strain is determinative. Strains which caused only mild symptoms in apple trees induced only mild symptoms also in periwinkle and tobacco plants.

Our results indicate that inhibition between phytoplasma strains is effective when the suppressor and challenger strains belong to the same taxonomic group. Our data show that inoculation in the cross-protection modus yields better results than co-inoculation and that the time between first and second inoculation may also play a role. This can be explained with two possible reasons: either the avirulent strain has more time to multiply in the phloem sieve tubes or during the multiplication it may trigger such a reaction in the plant that it will be protected against an infection by a virulent strain. It is still poorly understood if the plant acquires its immunity during the infection by the avirulent or the virulent strain.

Based on our results, not only the same phytoplasma species can exert inhibition against the multiplication of another strain but a distinct strain, too, which belongs to other taxonomic group.

We concluded that avirulent strains were not able to suppress strains belonging to other taxonomic groups (AAY1, STOL).

The experiences gained in these studies show that the roots are more sensible for phytoplasma infections than the stem. The survival rate of the virulent strain was higher in the root compared to the stem. Therefore it is conceivable that the antagonist strain inhibits multiplication of severe strains and their spread from the roots to the stem.

It is presumed that in the case of successfull suppession, the virulent strain gets degraded or loses

its virulence. Although the interactions between phytoplasma strains are explicated with different theories, the molecular background of the processes is still not clarified yet. Having regard to these facts, our results may lead us to achieve a feasable and practical protection method. But first it is required to clarify the processes behind the mechanism of cross protection in all details.

5. PUBLICATIONS

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