MOLECULAR DIAGNOSIS OF *Candidatus phytoplasma solani* INFECTION IN SOME TOMATO GENOTYPES AT THE DIFFERENT ONTOGENY STAGES

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Abstract. This paper presents the results of the molecular diagnosis of *Candidatus phytoplasma solani* infection in plants of three tomato varieties, Elvira, Desteptarea, Cerasus, created at the Institute of Genetics, Physiology and Plant Protection of the Moldavian Academy of Science. The dynamics of the appearance of stolbur infection was displayed in the process of plant development in the field conditions. Using molecular detection (nested PCR analysis) allowed to establish that phytoplasma infection was not present in the tomato plants in the field at the early stages of plant development. The first signs of the infection were registered at the beginning of the fruit ripening. About a half of plants were infected with *Ca. p. solani* when fruits ripened on I-III racemes. The most abundant phytoplasma infection was registered at the late ontogeny stages of the tomato: mature fruits on III, IV plant racemes. Clear differences in resistance were found between the three studied varieties to phytoplasma infection. The variety Elvira demonstrated significant sensitivity to this kind of infection in comparison with the varieties Desteptarea and Cerasus. The most resistant to *Ca. p. solani* variety was Cerasus - an abundant infection in its plants was registered only at late ontogeny stages, more specifically, at the end of the vegetative period. The obtained results confirm the usefulness of molecular diagnostic techniques for breeders in the creation of tomato varieties resistant to phytoplasma infection.

Keywords: molecular diagnosis, tomato, *Candidatus phytoplasma solani*, ontogeny stages, resistance.

Rezumat. Diagnosticul molecular al infecției *Candidatus phytoplasma solani* la unele genotipuri de tomate în diferite etape ale ontogenezei. În articol sunt prezentate rezultatele diagnosticului molecular al *Candidatus phytoplasma solani* la trei soiuri de tomate: Elvira, Desteptarea, Cerasus, create în Institutul de Genetică, Fiziologie şi Protecţie a Plantelor al Academiei de Ştiinţe a Moldovei. Dinamica apariţiei infecţiei fitoplasmice a fost analizată în timpul dezvoltării plantelor în condiţii de câmp. Utilizarea metodelor moleculare (analize nested-PCR) ne-a permis să stabilim că infecţia fitoplasmică nu este prezentă în etapele timpurii de dezvoltare a plantelor, dar s-a manifestat la începutul coacerii fructelor. Aproximativ jumătate din plante au fost infectate cu *Ca. p. solani* la etapa de coacere a fructelor la nivelul ramificaţiilor I-III. Cea mai abundentă infecţie fitoplasmică a fost înregistată în etapele tardive de dezvoltare a tomatelor: fructe coapte la nivelul ramificaţiilor III-IV. Diferenţa majoră a fost înregistrată cu privire la rezistenţa soiurilor de tomate la infecţie fitoplasmică. Soiul Elvira a prezentat o sensibilitate semnificativă la infecţie dată în comparaţie cu soiurile Deşteptarea şi Cerasus. Cel mai rezistent soi faţă de *Ca. p solani* a fost Cerasus, la care infecţia abundentă a fost determinată doar în etape tardive de dezvoltare, în special la sfârşitul perioadei de vegetaţie. Rezultatele obţinute confirmă importanţa utilizării metodelor moleculare de către amelioratori în crearea soiurilor de tomate rezistente la infecţia fitoplasmică.

Cuvinte cheie: diagnosticul molecular, tomate, *Candidatus phytoplasma solani*, etape de ontogeneză, rezistenţă.

INTRODUCTION

Phytoplasmas are worldwide pathogens colonizing plant phloem and transmitted by insects of the order *Hemiptera*, especially of the families Psyllidae, Cicadellidae and Cixiidae (BERTACCINI & DUDUC, 2009; HOGENHOUT et al., 2008; WEINTRAUB & BEANLAND, 2006). The genus *Candidatus phytoplasma* belongs to the class Mollicutes, family Acholeplasmataceae (LEE et al., 2000). Specific properties of phytoplasma such as a lack of cell wall make it impossible to isolate them in a pure culture (QUAGLINO et al., 2013; IRPCM, 2004). Due to these properties, it is difficult to identify the parasite.

Candidatus phytoplasma solani (common name stolbur phytoplasma) infects a large variety of plants - more than 300 species. In recent years, there was an increase of *Ca. p. solani* infection frequency in a number of agriculturally valuable crops (tomato, grapevine, wheat, corn, strawberry, potato, lavender and others), indicating a progressive spreading of the pathogen (EFSA, 2014; CAGLAR et al., 2010). Phytoplasma infection causes considerable losses of crops (70-100%) in cultures with high economic interest, dropping productivity and quality of the agricultural production significantly (GARCIA et al., 2005). However, disease control is possible. An early identification of this infection in plants is very important for a successful fight against this pathogen. Some difficulties with accurately detecting of *Ca. p. solani* in infected plants have to do with specific and non-specific symptoms of the disease that manifest in late stages of the infection and are similar to some viral and fungal infections. The impossibility of phytoplasma cultivation *in vitro* also makes its identification more difficult (IRPCM, 2004; NISHIGAWA et al., 2001). Taking into account the difficulties above, it is necessary to use alternative methods for detecting and characterizing phytoplasma. Unfortunately, serological methods and electron microscopy are not always effective and require considerable expenditures. Thus, the most accurate method of phytoplasma identification remains PCR technique (LEE et al., 1994; 2000; CLAIR et al., 2003).

Plants differ in their sensitivity to phytoplasma infection. Some plants exhibit spontaneous remission of symptoms, also known as recovery (MUSETTI, 2008). The differences in sensitivity have various causes including the presence and dominance of hypovirulent strains of the pathogens, the presence of phytoplasma antagonists, the activity of particular substances (H_2O_2) or plant secondary metabolites such as ROS, and the induction of systemic acquired resistance (ROMANAZZI et al., 2009). Environmental conditions such as temperature influence the insect vector

activity and the phytoplasma multiplication in their bodies (MURRAL et al., 1996). The breeding process directed towards the creation of new plant genotypes (varieties), which are more resistant to the phytoplasma infection and its negative consequences for the crops quality and productivity seems plausible and profitable.

The aim of the study was a molecular diagnosis (namely, nested PCR analysis) of *Ca. p. solani* infection in tomato plants having different genotypes and at different stages of development.

MATERIAL AND METHODS

The molecular diagnosis of *Ca. P. solani* infection was carried out in tomato plants cultivated in the field conditions. Two groups of plants were evaluated: the first group consisted of the plants sowed in the field as seedlings from the greenhouse (varieties Elvira and Desteptarea); plants from the second group were sowed as seeds directly in the field (varieties Elvira and Cerasus). All three studied varieties, Elvira, Desteptarea and Cerasus, were created in the Institute of Genetics, Physiology and Plant Protection (IGPPP) of the Moldavian Academy of Sciences.

Plants were numbered at the beginning of the experiment (i.e. at the beginning of flowering). Molecular analysis was made at different ontogeny stages for each marked plant.

The DNA for the molecular detection of phytoplasma was extracted from the basal part of the leaf at the stage of flowering raceme I and from the fruit peduncle at later stages using alkaline express-method (GUO et al., 2003).

Molecular diagnosis of the infection was carried out by nested-PCR analysis using pairs of primers specific to *Ca. P. solani*: cpn421 F / R (round I) and cpn200 F / R (round II). These pairs were created by us based on the chaperonin gene sequence (ZAMORZAEVA et al., 2016). The amplification was carried out according to the following program: I - 94°C 5 '; II - 94°C 30 ", 58°C 30", 72°C 30 " × 30 (round I) or × 35 (round II); III - 72°C 10 '; IV - 4°C ∞.

The results were registered in UV light after an electrophoresis of the amplification products stained with ethidium bromide in 1.5% agarose gel (buffer 1 × TBE). The size of the amplicon was measured by comparing it with the marker of DNA fragment lengths "O'Gene 100 bp DNA Ladder Ruler Plus" (Fermentas).

The statistical analysis of the obtained data was performed according to Fisher's criterion applied for qualitative traits in limited random sampling.

RESULTS AND DISCUSSION

In the first set of experiments, where tomatoes were planted in the field as seedlings from the greenhouse, phytoplasma infection appeared earlier and spread faster than in the case of directly sowing seeds in the soil (the second set of experiments). That is why the duration of experiments in the two groups was different.

Tomato plants from the first group were evaluated for the presence/absence of stolbur infection from the stage "flowering raceme I" (flowering I) to the stage "mature fruits on racemes I, II" (mature fruits I, II). The results of nested PCR analysis and the stages of the development of studied tomato plants are presented in Fig. 1.

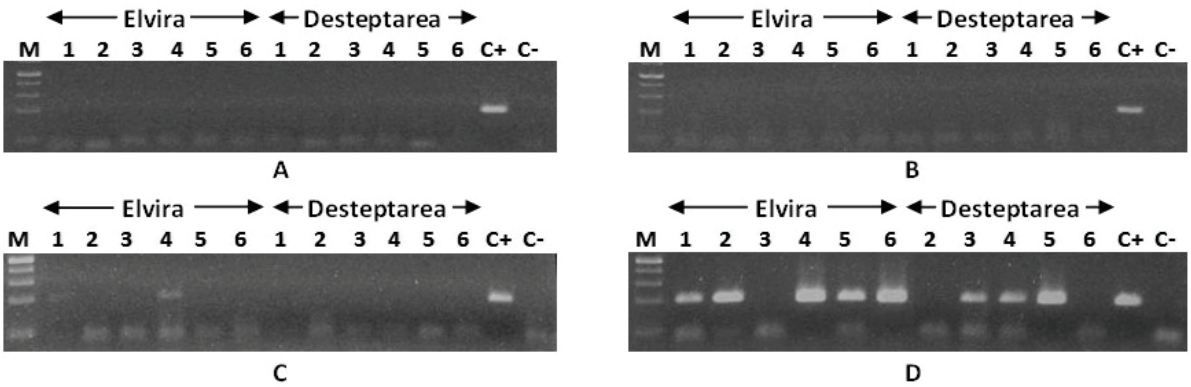

Figure 1. Results of nested PCR analysis detecting *Ca. P. solani* infection (expected fragment 200 b.p.) in tomato plants of the varieties Elvira and Deșteptarea (original).
A – stage of the flowering I (flowering raceme I); **B** – stage of the green fruits I (green fruits on the raceme I); **C** – stage of the ripening fruits I (ripening fruits on the raceme I); **D** – stage of the mature fruits I, II (mature fruits on the racemes I, II).
M – Marker of DNA fragment lengths "O'Gene 100 bp DNA Ladder Ruler Plus"; 1-6 – number of the plant of the respective variety; C+ – positive control of nested-PCR; C- – negative control of nested-PCR.

No plants infected with phytoplasma were found at early stages "flowering I" and "green fruits I" (Fig. 1 A, B). This fact can be explained by the lack of infected insect vectors in the beginning of July. Plant-to-plant transmission of stolbur infection by flying adults in Europe takes place in summer (EFSA, 2014) at different times for different areas and environmental conditions. Wet and relatively cool weather in Moldova in June and beginning of July 2016 influenced negatively to the appearance and activities of cicadas. Moreover, phytoplasma reproduction in the insect body ranges from 10 days to 12 weeks depending on the phytoplasma strain, insect species and further environmental factors such as temperature (MURRAL et al., 1996; HOGENHOUT et al., 2008). This period is named latent period.

After colonization by phytoplasma, the insect is a competent vector which is able to infect a new plant (WEINTRAUB, 2007). After a carrier insect feeds on a tomato plant, phytoplasma colonizes the plant and symptoms may appear in 6-45 days (BLANCARD, 2012).

The first tomato plants in the field infected with *Ca. P. solani* were registered at the stage "ripening fruits I" in the variety Elvira (Fig. 1 C). Their number increased significantly at the stage "mature fruits I, II" in both studied varieties (Fig. 1 D). However, a comparison of the appearance and spreading dynamics of stolbur infection in varieties Elvira and Desteptarea demonstrates a higher resistance (i.e. a later stage of appearance and a lower percentage of infected plants) of the variety Deşteptarea to this kind of infection (Fig. 2).

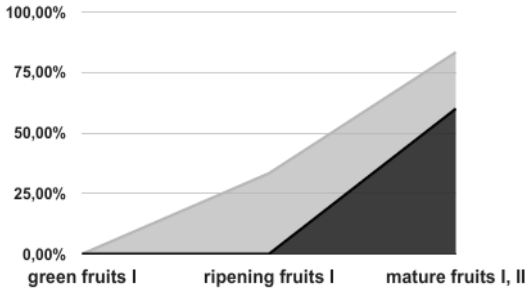

Figure 2. The increase in the percentage of infected with *Ca. P. solani* tomato plants of the varieties Elvira (grey) and Desteptarea (black) depending on the ontogenetic stage.

In the second group of tomato plants in the study (varieties Elvira and Cerasus), the results at early stages of development were similar with the data presented above: no plants infected with *Ca. P. solani* were recorded at stages of flowering and green fruits on raceme I (Fig. 3, A, B). The first plants with the signs of phytoplasma infection were registered at stages "ripening fruits I" and "mature fruits I, II" (Fig. 3 C, D).

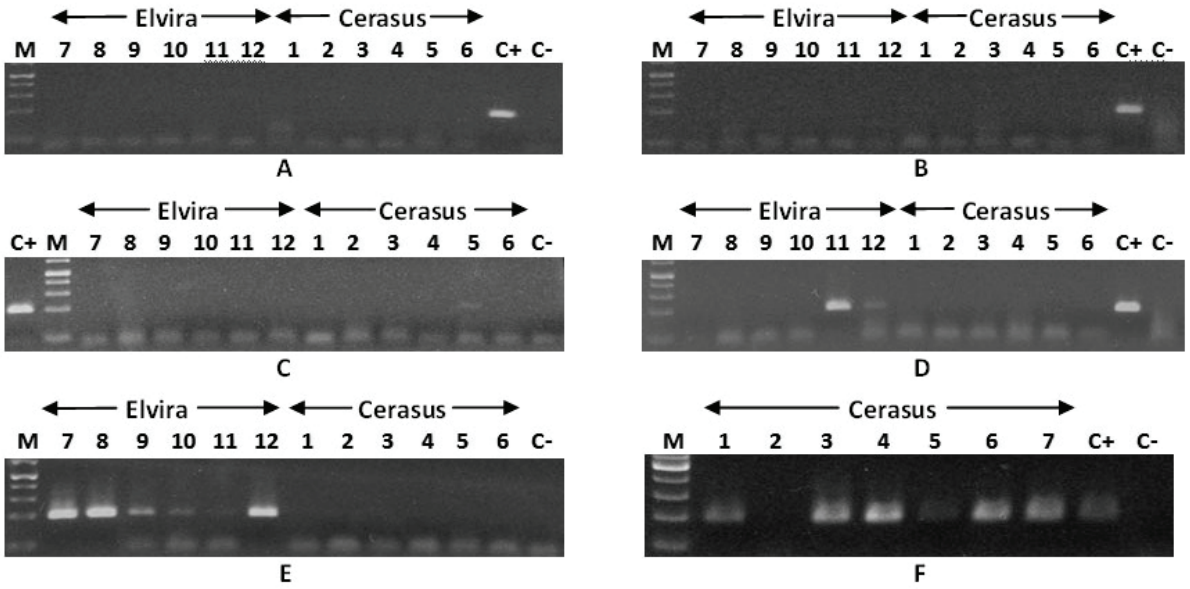

Figure 3. The results of nested PCR analysis detecting *Ca. P. solani* infection (expected fragment 200 b.p.) in tomato plants of the varieties Elvira and Cerasus (original).
A – stage of the flowering I (flowering raceme I); **B** – stage of the green fruits I (green fruits on the raceme I); **C** – stage of the ripening fruits I (ripening fruits on the raceme I); **D** – stage of the mature fruits I, II (mature fruits on the racemes I, II); **E** – stage of the mature fruits II, III (mature fruits on the racemes II, III); **F** – stage of the mature fruits III, IV (mature fruits on the racemes III, IV). M – Marker of DNA fragment lengths "O'Gene 100 bp DNA Ladder Ruler Plus"; 7-12 – number of the plant of the variety Elvira;1-6 (7 in F) – number of the plant of the variety Cerasus; C+ – positive control of nested-PCR; C- – negative control of nested-PCR.

A large number of tomato plants of the variety Elvira infected with *Ca. P. solani* were recorded at the ontogeny stage "mature fruits II, III" (Fig. 3 E). A significant number (percentage) of plants of the variety Cerasus infected with the same phytopathogen appeared later, at the stage "mature fruits III, IV" (Fig. 3 F).

Thus, the reaction of the Elvira and Cerasus varieties to the phytoplasma infection was different. This difference is evident in the Fig. 4 that presents the results of *Ca. P. solani* detection in plants of both studied varieties at different stages of development.

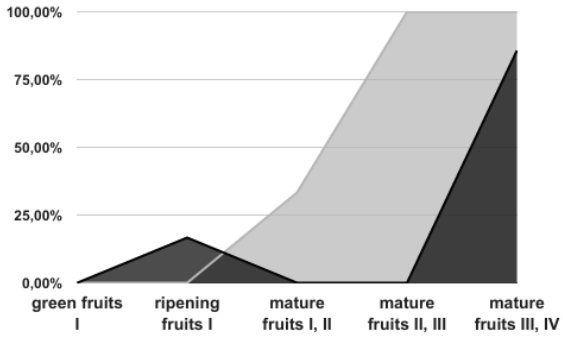

Figure 4. The increase in the percentage of infected with *Ca. P. solani* tomato plants of the varieties Elvira (grey) and Cerasus (black) depending on the ontogenetic stage.

The statistical analysis of the obtained data using Fisher's criterion confirms significant difference in phytoplasma infection of plants of Elvira and Cerasus varieties especially at the late stage of development "mature fruits II, III" ($P \leq 0.001$). In contrast finding only one infected plant of the variety Cerasus at the stage "ripening fruits I" is within the range of a statistical error. At the same time, we can suggest that later, in the process of subsequent development, this plant recovered and became healthy (compare plant 5 of the variety Cerasus in Fig. 3 C, D, E). The recovery phenomenon or spontaneous remission of symptoms of infected plant is well known for phytoplasma (MUSETTI, 2008).

Additional information about the dynamics of appearance of *Ca. P. solani* infection in tomato plants was obtained from the combined results of molecular diagnosis in both experimental groups (Table 1).

Table 1. Phytoplasma infection at different stages of development of tomato plants.

Ontogeny stage	% of the infected plants	Number of tomato plants		
		analyzed	infected	uninfected
Flowering I	0%	24	0	24
Green fruits I	0%	24	0	24
Ripening fruits I	12.5%	24	3	21
Mature fruits I, II	43.5%*	23	10	13
Mature fruits II, III	50.0%*	12	6	6
Mature fruits III, IV	92.3%**	13	12	1

Legend: * significant with $P \leq 0.01$
** significant with $P \leq 0.001$

One can see that the appearance of the first tomato plants infected with *Ca. P. solani* began in the field at the beginning of fruit ripening. At the stages of mature fruits on racemes I, II, III about a half of plants were infected ($P \leq 0.01$). A considerable increase of the number / percentage of plants infected with phytoplasma was registered at the latest stage of "mature fruits III, IV" (about 92% of infected plants, $P \leq 0.001$).

CONCLUSIONS

Molecular diagnosis established that *Ca. P. solani* infection was absent in tomato plants at the early stages of development (from the beginning of flowering till the green fruits on the first raceme). The first infected tomato plants were recorded from the beginning of fruits ripening. Abundant phytoplasma infection in the tomato field took place considerably later, when fruits were mature on racemes III and IV. At intermediate stages of fruits ripening on racemes II and III about a half of plants were infected.

Clear differences in the timing of the stolbur appearance and spreading were shown on tomato plants depending on the genotype. The variety most sensitive to the phytoplasma infection was Elvira. *Ca. P. solani* infection appeared earlier and spread faster in plants of the variety Elvira. The variety Desteptarea occupied an intermediate position between Elvira and Cerasus from the point of view of the résistance to the stolbur infection. The most resistant variety was Cerasus. Abundant *Ca. P. solani* infection in plants of the variety Cerasus was found when fruits were ripening on racemes III, IV. We suggest that a breeding process directed towards creating tomato plants resistant to phytoplasma infection (i.e. for which the infection spreads at the late ontogeny stages, like in the variety Cerasus) is very important for the maintenance of high productivity and good fruit quality. Molecular diagnosis is a useful technique for breeders to resolve such problems.

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REFERENCES

BERTACCINI A. & DUDUK B. 2009. Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathol. Mediterr.* Firenze University Press. Bologna. **48**: 355–378

BLANCARD D. 2012. *Tomato diseases. Identification, Biology and Control.* Second Edition. Edit. Manson Publishing Ltd. 688 pp.

CAGLAR B. K., ELBEAINO T., KUSEK M., PEHLIVAN D., FIDAN H., PORTAKALDALI M. 2010. Stolbur Phytoplasma Infections in Potato and Tomato Plants from Different Locations in Turkey. *Journal Turk. Phytopath.* Edit. Turkish Phytopatology Assaciation. Izmir. **39**: 1-8.

CLAIR D., LARRUE J., AUBERT G., GILLET J., CLOQUEMIN G., BOUCON-PADIEU E. 2003. A multiplex nested-PCR assay for sensitive and simultaneous detection and direct identification of phytoplasma in the Elm yellow group and Stolbur group and its use in survey of grapevine yellows in France. *Vitis*. Edit JKI (Julius Kühn Institute). Quedlinburg. **42**: 151-157.

EFSA. 2014. Scientific Opinion on the pest categorisation of *Candidatus Phytoplasma solani*. *EFSA Journal*. **12**: 3924. (online). 2014. Publisher: European Food Safety Authority, Parma. Italy. www.efsa.europa.eu/efsajornal (Accessed April 6, 2016).

GARCIA A., VICENTE L. P., BLANCO E. 2005. Phytoplasmas and Plant Quarantine in Cuba. *Petria*. Edit. Istituto Sperimentale per la Patologia Vegetale. Roma. **15**: 225 pp.

GUO Y., CHENG Z.-M., WALLA J. A. 2003. Rapid PCR-based Detection of Phytoplasmas from Infected Plants. *Hort Science*. Edit. American Society for Horticultural Science, Alexandria, USA. **38**: 1134 - 1136.

HOGENHOUT S. A., OSHIMA K., AMMAR E. D., KAKIZAWA S., KINGDOM H. N., NAMBA S. 2008. Phytoplasmas: Bacteria that manipulate plants and insects. *Molecular Plant Pathology*. Edit. BSPP and John Wiley & Sons Ltd. London. **9**: 403-423.

IRPCM. 2004. Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group. 2004. '*Candidatus Phytoplasma*', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and and Evolutionary Microbiology.* Microbiology Society. London. **54**: 1243-1255.

LEE I. M., GUNDERSEN D. E., HAMMOND R. W., DAVIS R. E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology.* Edit. American Phytopathological Society. St. Paul. Minnesota. **84**: 559–66.

LEE I. M., DAVIS R. E., GUNDERSEN-RINDAL D. E. 2000. Phytoplasma: Phytopathogenic Mollicutes. *Annual Reviews of Microbiology*. Edit. Annual Rewiews, Palo-Alto. CA. **54**: 221–255.

MURRAL D. J., NAULT L. R., HOY C. W., MADDEN L. V., MILLER S. A. 1996. Effects of temperature and vector age on transmission of two Ohio strains of aster yellows phytoplasma by the aster leafhopper (Homoptera: *Cicadellidae*). *Journal of Economic Entomology*. Oxford University Press. Oxford. **89**: 1223-1232.

MUSETTI RITTA. 2008. Management of ecology of phytoplasma diseases of grapevine and some fruit crops. In: Ciancio & Murkerji (Eds.) *Integrated Manegment of Diseseas caused by Fungy, Phytoplasma and Bacteria*. Edit. Springer science and Business Media: 43 - 60.

NISHIGAWA H., MIYATA S., OSHIMA K., SAWAYANAGI T., KOMOTO A., KUBOYAMA T., MATSUDA I., TSUCHIZAKI T., NAMBA S. 2001. In planta expression of a protein encoded by the extrachromosomal DNA of a phytoplasma and related to geminivirus replication proteins. *Microbiology*. Microbiology Society. London. **147**: 507–513.

QUAGLINO F., ZHAO Y., CASATI P., BULGARI D., BIANCO P. A., WEI W., DAVIS R. E. 2013. '*Candidatus Phytoplasma solan*i', a novel taxon associated with stolbur- and bois noir-related diseases of plants. *Int J Syst Evol Microbiol.* Microbiology Society. London. **63**: 2879-2894.

ROMANAZZI G., MUSETTI RITTA, MARZACHÌ C., CASATI P. 2009. Induction of resistance in the control of phytoplasma diseases. *Petria*. Edit.Istituto Sperimentale per la Patologia Vegetale, Roma. **19**: 113-129.

WEINTRAUB P. G. & BEANLAND L. 2006. Insect vectors of phytoplasmas. *Annual Review of Entomology*. Edit. Entomological Society of America. Annapolis. **51**: 91–111.

WEINTRAUBP. P. G. 2007. Insect vectors of phytoplasmas and their control – an update. *Bulletin of Insectology*. Edit. Università di Bologna. Bologna. **60**: 169-173.

ZAMORZAEVA IRINA, BAHŞIEV AIGHIUNI, MIHNEA NADEJDA. 2016. Testarea primerilor creaţi pentru diagnosticul molecular al infecţiei fitoplasmice. *Buletinul Academiei de Ştiinţe a Moldovei. Ştiinţele Vieţii*. Edit. CSŞDT AŞM. Chişinău. **2**: 55-61.

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