Agricultura 5: 43 - 46 (2008)

Transmission of '*Candidatus phytoplasma mali*' by natural formation of root bridges in M9 apple rootstock

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An experiment was carried out to estimate the transmission of '*Candidatus* phytoplasma mali' (CPM) in M9 apple rootstocks by formation of root bridges. Healthy (CPM-free) and infected rootstocks were planted together in plastic containers and maintained for four years under isolated insect-proof conditions. After four years rootstocks were carefully extracted from growing substrate and rinsed by water jets. The presence or absence of CPM in rootstocks at the beginning and at the end of the experiment was confirmed by PCR molecular tests. We monitored the occurrence of root bridges among roots of healthy and infected trees. The formation of tiny root bridges was observed in 4 out of 50 analysed containers (8%); the transmission of phytoplasma from the roots of infected to the roots of healthy rootstocks was detected by molecular means in 12 out of 50 containers (24%). In the orchard trial we analysed the frequency of occurrence of root bridges between neighbouring trees by applying herbicide glyphosate. It was applied to the middle trees of 150 selected tree triplets. The herbicide damage in neighbouring trees of selected tree triplets was observed in 5 tree triplets (3.3%). The experiment confirmed the transmission of CPM by natural root bridges in M9 apple rootstock.

Key words: apple proliferation, '*Candidatus phytoplasma mali*', apple, disease transmission

INTRODUCTION

Apple proliferation disease (AP) caused by the phytoplasma which has recently been assigned as '*Candidatus* phytoplasma mali CPM' (Seemüller and Schneider 2004) is a widespread disease of apple in Europe. It can not be chemically controlled. A lot of efforts were made to research the disease spread mechanisms with the aim to develop strategies for the prevention of phytoplasma dissemination (i.e. by planting material or by vectors). According to the existing knowledge two psillids (*Cacopsylla melanoneura* and *C. costalis* (= *C. picta*) are the main vectors of CPM in apple plantations of Central Europe (Vindimian et al. 2003, Tedeshi et al. 2003, Tedeshi and Alma 2004). The main strategies applied for the prevention of disease spread are therefore the production of phytoplasma-free propagation and planting materials and consistent control of vectors.

In orchards with high frequency of AP infected trees, we can often observe that the disease appear in patches. Several neighbouring infected trees are followed one after another in a row. The apple tree roots sometimes naturally "graft" (i.e. produce root anastomoses) which could allow transmission of plant pathogens. This fact supports the assumption that CPM may also be transmitted via natural fusion of root systems of neighbouring trees. No scientifically confirmed CPM vectors living in soil and feeding on apple root system are known at a present time. In modern apple plantations, trees are planted in very dense stands (0.6-0.8 m within row and 2.7-3 m between rows). In such plantations roots of neighbouring trees are forced to interweave very closely in a limited soil volume. They are also pressed together due to tractor and trailer wheels pressure. In addition, roots are damaged by root cutting devices that are applied for regulation of tree growth instead of chemical growth retardants. In such conditions a considerable increase in frequency of formation of root bridges among roots of neighbouring trees can be expected.

In the past, many researchers discussed the root transmission as a possible way of disease transmission, but only recently the root transmission has attracted the researchers' attention again. In Italy, some trials were carried out and researchers were able to demonstrate the indirect transmission by the formation of root bridges (Vindimian et al. 2002, Ciccotti et al. 2006, 2007). The removal of infected trees revealed the existence of root bridges between neighbouring trees, and histological observations confirmed the tissue connection. The first direct proof was given by Ciccotti et al. (2007), who showed a 16% root bridge transmission in Golden Delicious seedlings.

The research on root-to-root transmission is still ongoing in Germany and other countries. The aim of our research was to evaluate experimentally the possibility of transmission of CPM in M9 apple rootstock, which is the most frequently used rootstock, by the formation of root bridges, and to compare the results with previous data.

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MATERIALS AND METHODS

Growing of rootstocks in containers

Healthy (CPM-free) and CPM infected rootstocks M9-EMLA were selected and planted into plastic 10-L containers. Infected rootstocks showing proliferation symptoms were obtained from heavily infected trees. The rootstock outgrowths (suckers) were dug out of soil and cut off the main root system of trees. The donor trees were tested several times for presence of CPM using PCR based molecular tests. The healthy one-year old virus-free certified EMLA M9 rootstocks were obtained in a rootstocks nursery. The CPM-free status of those rootstocks at the beginning of the experiment was also proven by our own testing. Both root and leaf tissues were tested. Two infected and one healthy rootstock were planted in each container. 50 containers were included in the experiment. Before planting, rootstocks were carefully cleaned and soaked in the highly concentrated solution of systemic insecticides to control any present insects, mites and nematodes. The growing substrate (Neuhaus Humin Substrat N8) was heat-sterilised. The containers with rootstocks were maintained for four years in an experimental screen house in the inset-proof conditions. In order to avoid any insect vector presence we regularly treated plants and soil with different systemic insecticides.

Herbicide trial

To prove indirectly the existence of root bridges among neighbouring apple trees in natural conditions we carried out a herbicide trial similar to that presented by Vindimian and Branz (2000). Researchers treated apple trees with herbicide glyphosate and noticed damage also on nontreated adjacent trees. They concluded that damage on adjacent trees appeared because of transmission of glyphosate via root bridges.

Our trial was carried out in the 12-year old apple plantation of the Experimental Station of the Faculty of Agriculture Maribor (43° 34' N, 15° 38' E). In a 6 ha big plantation of different cultivars (Goleden Delicious, Jonagold, Elstar, etc.), several hundreds of AP infected trees showed clearly visible AP symptoms.

We selected 150 tree triplets (3 infected neighbouring trees in a row). Trees were grown on M9 rootstock and were planted at a distance of 0.8-1 m within the row and 2.8-3 m between rows. Glyphosate based herbicide (Boom Efect; 48% of glyphosate) was applied by two different methods. In case of 70 triplets 0.1 l of Boom Efect preparation was applied by spraying to each tree in the middle of selected triplets at the beginning of October. The middle trees of triplets were sprayed very carefully at low pressure by cnapsack sprayer with shield to prevent any drifting of spray to two adjacent trees. To other 80 additionally selected triplets, 0.1 l of glyphosate preparation (Boom Effect) per tree in the middle of triplets was applied in the spring (April). Selected middle trees of triplets were sawn aside 20 cm above ground. Glyphosate preparation was poured into holes made by chisel into tree stumps. Two trees adjacent to the treated middle tree were afterwards monitored for presence of herbicide damage for one growing season.

Molecular detection of phytoplasma in rootstocks

The rootstocks were grown in containers for four years. Afterwards the soil was removed from the roots by rinsing them with water jet. The rinsing was carried out very carefully with low water pressure, to avoid any tearing of roots or eventual root fusions (bridges). The root system of all plants was checked very accurately for the presence of root bridges. For each container the presence or absence of root bridges was recorded. After separation of rootstocks, samples of root and leaf tissues were taken to a laboratory for testing the presence of CPM.

The procedures for laboratory testing were used as described by Brzin et al. (2003). PCR of 35 cycles was performed in 40 µl reaction volume using the AP group specific pair of primers fO1/rO1 (Lorenz et al. 1995, Seemüller et al. 1996) and 2 µl of DNA as template. Negative samples were further tested in the nested PCR assays, where amplification products obtained after 35 cycles in 40 µl reaction volume with the universal primer pair P1/P7 (Seemüller et al. 1996) were diluted 1:100 in water and re-amplified with AP group specific primers fO1/rO1 as described above. All sets of reactions included DNA samples from healthy plants and controls lacking template DNA as well as positive controls. Samples (15 µl) of PCR product were resolved by 1 % agarose gel electrophoresis and visualized by staining with ethidium bromide ($2x10^{-4}mgml^{-1}$) and UV illumination.

RESULTS AND DISCUSSION

Our results support the first finding of Ciccotti et al. (2007) that, in the case of phytoplasma causing AP disease, root-to-root transmission is possible. They reported on visible anastomosis after two years experiment, therefore we are surprised that clearly visible root bridges did not develop within a period of four years, although rootstocks developed very dense knitted root systems. In their experiment they forced rots to fuse by inserting them into very narrow hoses (1 cm diameter). We just planted rootstocks in containers without inserting roots in any hose and because of that the experimental conditions at both experiments were not completely comparable.

We found only a few very loose fusions of roots at rootstocks developing in 4 containers (4/50 = 8% of all containers). PCR test results showed that CPM transmission took place in 12 containers (12/50 = 24% of all containers). In most containers where positive PCR results were obtained in previously healthy rootstocks, no clearly visible root bridges were observed (see Table 1). This result differs from finding of Ciccotti et al. (2007). We can therefore conclude that apple rootstocks can form very tiny root bridges that are not visible by naked eye. We assume that despite their small size those tinny root fusions can still enable CPM to pass from phloem tissue of one root to active phloem tissue of another root. There were also two containers where very tiny fusions were observed and CPM transmissions were not confirmed by PCR tests.

Table 1. Transmission rate of CPM achieved by growing infected and healthy apple M9 rootstocks together in containers for four years in insect-proof conditions and confirmed by PCR based tests

	Root fusion observed	Root fusion not observed	Sum
CPM transmission confirmed by PCR	2/50 = 4%	10/50 = 20 %	12/50 = 24 %
CPM transmission not confirmed by PCR	2/50 = 4%	36/50 = 72 %	38/50 = 76 %

In the herbicide field trial we indirectly tested the presence of root bridges by applying highly systemic, only phloem-transported glyphosate herbicide. This is easily transposable via root bridges between trees and can cause easily detectable damages. Among 150 tested tree triplets herbicide damage appeared only in 5 triplets (5/150 = 3.3%). This result shows that frequency of root bridges in this experimental orchard was quite low. In trials of Ciccotii et al. (2007) much higher root bridge formation rate was observed (in 84 of 268 trees = 31%). Additional proof for existence of root bridges was presented by Baric et al. (2007) who studied root transmission in 24-years old orchard developing on M111 rootstocks. They concluded that root-to-root transmission could play an important role in disease epidemiology in old apple plantations developed on vigorous rootstocks. They could not prove transmission to trees developed on M9 rootstocks which also grew between experimental glyphosate treated trees.

We assume that apple trees commonly do not form root bridges more frequently as it was observed in our trial. Some findings confirmed the spread of some apple viruses through natural root grafting (Atkinson 1956, Hunter et al. 1958, McCrum 1965, Dhingra 1972, Desvignes et al. 1999). They stated opinions that root bridge formation was not a rare phenomenon in some types of apple rootstocks. It is likely that root bridges do not develop frequently in the absence of huge external physical forces. The planting system and orchard management system influence significantly the frequency of root bridge formation. Suitable conditions are fulfilled in orchards with very narrow interrow spacing and where heavy tractors and trailers are used. Their wheals may press the layers of roots together enabling the formation of root bridges, after wounds are overgrown with callus.

We believe that in orchards with high CPM vector pressure the root-to-root transmission phenomenon is of a lesser importance. We assume that this type of transmission might be more important in apple rootstock nurseries with trench layering or stool bedding production systems. In such nurseries we can observe very dense row plantations of rootstocks, which develop for many years. Roots are damaged and pressed by machines applied for soil cultivation, earthing-up and cutting of rootstocks when they ripen. In such production systems many root bridges can develop. We noticed root bridges when we were collecting rootstocks out of stoolbed for our experiment. Despite very strict vector control and other prevention measures, the percentage of latently infected rootstocks can slowly increase year by year.

CONCLUSIONS

Our experiment proved the existence of root bridge formation among M9 apple rootstocks and the possibility of CPM transmission via root-to-root bridges. We also indirectly confirmed the formation of root bridges in orchard conditions. Our results show that CPM can be transmitted by natural root grafting in M9 apple rootstocks, what could be important for apple rootstock nurseries.

REFERENCES

1. Atkinson JD. Unusual features of some New Zealand fruit tree viruses. Eur. J. Plant Pathol. 1956;62(2):39-42.
2. Baric S, Kerschbamer C, Vigl J, Dalla Via J. Translocation of apple proliferation phytoplasma via natural root grafts – a case study. Eur. J. Plant Pathol. 2007, (10.1007/s10658-007-9256-z) http://www.springerlink.com/content (7. 01. 2008).
3. Brzin J, Ermacora P, Osler R, Loi N, Ravnikar M, Petrovič N. Detection of apple proliferation phytoplasma by ELISA and PCR in growing and dormant apple tees. J. Plant Dis. and Prot. 2003;110(5):476-483.
4. Ciccotti AM, Bianchedi PL, Bragagna P, Deromedi M, Filippi M, Forno F, Mattedi L. Natural and experimental transmission of Candidatus Phytoplasma mali by root bridges. XXth International symposium on virus and virus-like diseases of temperate fruit crops fruit crops. Antalya, Turkey, May 22-26, 2006;125. (http://www.apfeltriebsucht.info/info_apfeltrieb.html) (7. 01. 2008).
5. Ciccotti AM, Bianchedi PL, Bragagna P, Deromedi M, Filippi M, Forno F, Mattedi L. Transmission of '*Candidatus* Phytoplasma mali' by root bridges under natural and experimental conditions. Bull. Insect. 2007;60(2):387-388.
6. Desvignes JC, Grasseau N, Boye R, Cornaggia D, Aparicio F, Flores R. Biological properties of apple scar skin viroid: Isolates, host range, different sensitivity of apple cultivars, eliminiation and natural transmission. Plant Dis. 1999;83:768-772.
7. Dhingra KK. Transmission of apple mosaic by natural root grafting. Ind. J. Hort. 1972;29:348-350.
8. Hunter JA, Chamberlain EE, Atkinson JD. Note on transmission of apple mosaic virus by natural root grafting. NZ J. Agric. Res. 1958;1:80-82.
9. Lorenz KHB, Schneider U, Ahrens E, Seemüller E. Detection of the Apple Proliferation and Pear Decline Phytoplasmas by PCR amplification of ribosomal and non ribosomal DNA. Phytopathol. 1995;85:771-776.
10. McCrum R.C. Spread of apple chlorotic leaf spot virus from tree to tree. Plant Dis. Rep. 1965;49:958-959.
11. Seemüller E, Kison H, Lorenz KHB, Schneider U, Marcone C, Smart CD, Kirkpatrick BC. Detection and Identification of fruit tree Phytoplasmas by PCR amplification

of ribosomal and nonribosomal DNA. In: Manceau C, Spak J (eds.): Abstract Book of Workshop of the nucleic acid-based technology; Advances in the detection of plant pathogens by polymerase chain reaction (Cost 823). Part two – PCR detection of Phytoplasma, Češke Budejovice, Czech Republic. 1996:56 – 66.

12. Seemüller E, Schneider B. 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and 'Candidatus phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int. J. Syst. Evol. Microb. 2004;54:1217-1226.
13. Tedeshi R, Visentin C, Alma A, Bosco D. Epidemiology of apple proliferation in north-western Italy: Evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura*. Ann. Appl. Biol., Assoc. Appl. Biol. 2003;142(3):285-290.
14. Tedeshi R, Alma A. Transmission of Apple Proliferation Phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). J. Econ. Entom. 2004;97(1):8-13.
15. Vindimian EM and Branz A. Untersuchungen zum Besenwuchs des Apfelbaumes. Obstbau – Weinbau. 2000;10:294-296.
16. Vindimian EM, Ciccotti A, Filippi M, Springhetti M, Deromedi M. Spread of Apple proliferation by root bridges. Petria. 2002;12(3):375.
17. Vindimian EM, Forno F, Mattedi L. Untersuchungen zur bedeutung der Blattsauger bei der Überträgung der Apfeltriebsucht. Obstbau. 2003;27(4):205-208.

Received: January 9, 2008
Accepted in final form: May 3, 2008