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Translocation of apple proliferation phytoplasma via natural root grafts – a case study

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Abstract Apple proliferation (AP), a phytoplasma-induced disease of apple trees, was proven to be transmitted through infected grafting material and sap-sucking insects. To date there are little firm data on disease propagation in the field via natural root grafts. This question was thus addressed in the present case study by investigating trees of a 24-year old commercial apple orchard ('Red Chief' on MM 111), where the existence of root connections was discovered accidentally. After having displayed specific AP symptoms, nine trees were cut down and the stubs were infiltrated or brushed with glyphosate. Herbicide injury, however, remained not only restricted to the treated stubs, but also spread to approximately 50 neighbouring trees. Surprisingly, none of the pollinators ('Granny Smith' on M 9) growing interjacently and alternating between herbicide-damaged main crop trees was affected. Respective to the position of the nine AP-infected and glyphosate-treated cut stumps, four sections in the orchard were defined, from which a total of 122 trees was sampled and analysed using qualitative real-time PCR for detection of AP phytoplasma. The pathogen was found in 71.4% of 'Red Chief' trees with severe herbicide damage and 18.8% of trees with partial herbicide damage. None of the 31 investigated pollinators was AP-infected. Our data indicate that root connections seem to play a role for the spread of AP phytoplasma at least in older orchards and between trees on vigorous rootstocks.

Keywords '*Candidatus* Phytoplasma mali' · Glyphosate · Pathogen transmission

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Apple proliferation (AP) is currently one of the most serious apple tree diseases in Europe. It is caused by the AP phytoplasma ('*Candidatus* Phytoplasma mali'; Seemüller and Schneider 2004), which induces symptoms such as proliferation of auxiliary shoots (witches' brooms), reduced flowering, phyllody, enlarged stipules, leaf rosettes, chlorosis, yellowing and early leaf reddening (Kartte and Seemüller 1988). Symptoms of economic relevance are small fruit size, low fruit quality and overall yield decreases. Although the disease has been known for more than 50 years (Rui 1950), there is still no therapy available to cure infected trees. Thus, recommendations for disease containment imply planting of healthy material, uprooting of infected plants and vector control.

Transmission of AP phytoplasma can occur through grafting of infected propagation material (Kartte and Seemüller 1988) and sap-sucking insect vectors (Frisinghelli et al. 2000; Tedeschi and Alma 2004). Apple plants usually display an irregular distribution of the pathogen (Seemüller et al. 1984; Schaper and Seemüller 1984). While it can be completely absent from the above-ground organs of

an infected tree, the phytoplasma remains permanently present in the rootstock (Carraro et al. 2004). Due to this fact, questions about natural root contacts as a possible path for AP phytoplasma transmission continue to arise (Vindimian et al. 2002).

The present case study was undertaken in a commercial apple orchard of 4,500 m^2 close to Neumarkt/Egna in the Autonomous Province of Bozen/Bolzano (South Tyrol), northern Italy. The orchard comprises about 700 'Red Chief' trees on rootstock MM 111, which were planted in 1982 at a density of 2×4 m. Some of the rows partly contain pollinators ('Granny Smith' on M 9 rootstocks planted in 1998 and 1999) or main crop trees, which were inserted interjacently at 1-m spacing. The farmer observed visual symptoms of AP on 'Red Chief' trees for the first time in autumn 2005. The nine affected trees were cut down to about 0.5 m above ground level in January 2006 and treated with herbicide to destroy the roots in preparation for subsequent uprooting. In order to apply higher portions of herbicide to some of the stubs, holes were carved out or drilled into the cut wood surface. Finally, (a) three stubs with large cruciform holes were infiltrated with approximately 250 ml of pure TOUCHDOWN® (34% glyphosate; Syngenta, Wilmington, USA; Fig. 1a), (b) up to 100 ml was introduced into the drill holes of four stubs (Fig. 1b), and (c) two stubs without holes were treated by brushing only the cut surface with the same herbicide.

Fig. 1 Apple tree stubs with carved cruciform holes **a** infiltrated with approximately 250 ml of pure TOUCHDOWN®, while into stubs with drill holes **b** up to 100 ml of the herbicide were poured in order to destroy the roots before uprooting

At sprouting time it became evident that the herbicide impact was not only restricted to the treated stubs, but also the nearby trees were affected. In general, the higher the quantity of TOUCHDOWN® applied to a stub, the more adjacent trees with severe herbicide damage were observed (Fig. 2). While the damage spread as far as to the ninth neighbouring tree in the rows in which stubs were infiltrated with approximately 250 ml of herbicide (Section 1 in Fig. 2), no effect was detected around the stubs being treated by brushing only (Section 4 in Fig. 2). Moreover, these two cut stumps developed new shoots out from the base.

Glyphosate is a systemic herbicide, which is translocated within the phloem and is effective only when applied directly to the plant (Franz et al. 1997). As soon as it comes into contact with soil, it becomes unavailable for uptake by plant roots, because of its strong affinity to soil particles (Giesy et al. 2000). Since in the present case study glyphosate was not sprayed, but was poured directly into the stubs, the possibility of aerial herbicide drift can be excluded and root connections remain the only plausible explanation for its propagation to adjacent trees. Particularly interesting is that root grafts were formed only among trees on rootstock MM 111, but in no case between the rootstocks MM 111 and M 9. This is evident by the observation that none of the interjacent pollinators showed any symptoms of herbicide damage (see Fig. 2 and red arrows in Fig. 3).

Natural root grafts are a well known phenomenon most notably occurring in forest and tropical trees (Graham and Bormann 1966). The formation of root connections between woody plants of the same species was shown to be favoured by higher stand densities, larger diameters and increasing age of the trees (Fraser et al. 2005). Where trees are joined through grafts, interchange of water, nutrients, minerals, hormones, herbicides as well as pathogens is possible (Graham and Bormann 1966; Epstein 1978; He et al. 2000). As evidenced by our data and the study of Vindimian et al. (2002), vascular root connections between trees in an apple orchard can emerge, across which a systemic herbicide might disperse. Since the AP phytoplasma is limited to the phloem sieve tubes, we further investigated whether the pathogen could also have spread from the infected cut down trees to those trees with which they were in apparent root contact.

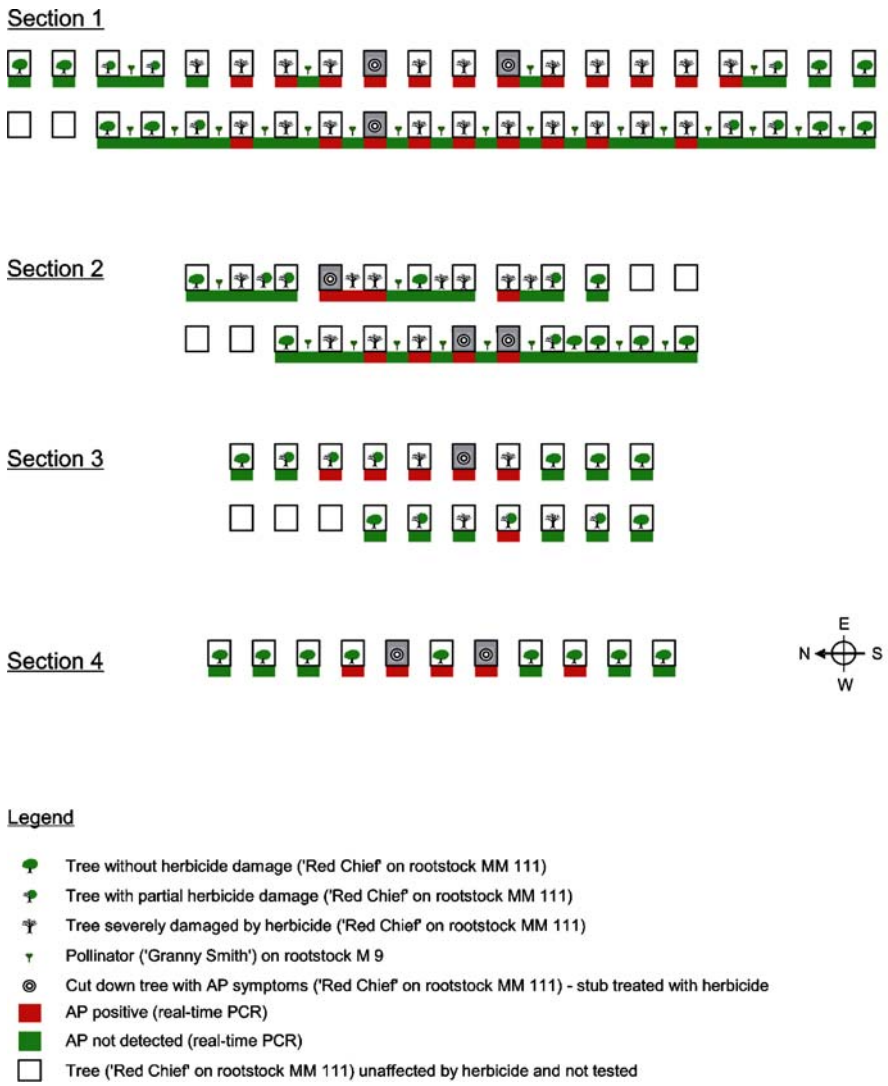

Fig. 2 Four different sections of a commercial orchard were included in the case study, according to the position of the nine AP-infected and glyphosate-treated cut stumps. Section 1 included a total of 56 trees adjacent to three cut stumps, to which approximately 250 ml of pure TOUCHDOWN® were applied. Section 2 comprised 32 trees in two rows in the vicinity of three stubs, treated with up to 100 ml of pure TOUCHDOWN®. Section 3 involved 16 trees in two rows in the neighbourhood of one stub treated with about 100 ml of TOUCHDOWN®. Section 4 included nine trees in a line with two stubs, of which the cut surface was brushed only with TOUCHDOWN®. Each box indicates a tree at a regular spacing of 2×4 m. Boxes containing a tree symbol indicate trees sampled for PCR analysis. Tree symbols in between boxes denote analysed trees ('Red Chief' or pollinators), which grew at 1-m distance. Colour bars below the boxes indicate the outcome of the real-time PCR analysis (*red*: AP positive; *green*: AP not detected)

For this purpose, in May/June 2006 root samples from a total of 122 trees were collected, comprising the nine 'Red Chief' cut stumps formerly showing symptoms of AP, 35 'Red Chief' trees with severe herbicide damage (defoliated trees), 16 'Red Chief' trees with partial herbicide damage (partial growth, deformed leaves), 31 'Red Chief' trees without apparent herbicide damage, and 31 pollinators. Sampling occurred in four different sections of the orchard according to the position of the nine AP-infected and glyphosate-treated cut stumps (see Fig. 2). On each side of the stubs, where herbicide damage extended further than the fourth neighbouring tree, the sampling was continued until parts of the row without any sign of glyphosate injury were reached (Fig. 2, Sections 1 and 2). In case the damage did not spread

Fig. 3 Severity of herbicide damage in Section 1 of the orchard, which spread from three cut stumps (*black arrows*), each infiltrated with 250 ml of pure TOUCHDOWN®. None of the pollinators (*red arrows*) showed any sign of herbicide damage

as far from the cut stump, at least four neighbouring trees at either side were sampled (Fig. 2, Sections 3 and 4). All the collected root samples, including those from herbicide-treated stubs and herbicide-damaged trees, were still vigorous and total nucleic acid was isolated according to the protocol described in Baric et al. (2006). Each DNA isolate was analysed in duplicate applying a highly sensitive qualitative TaqMan real-time PCR approach for specific detection of AP phytoplasma (Baric and Dalla Via 2004).

As expected, AP phytoplasma was detected in the roots of all nine stubs, which were cut down because of obvious AP symptoms. Moreover, the major part of the trees with severe glyphosate damage also tested positive for AP phytoplasma (25 out of 35 trees; Table 1), while the pathogen was found only in three out of 16 trees with partial herbicide injury, and in three out of 31 herbicide-unaffected trees (Table 1). Surprisingly, none of the 31 interjacent pollinators was infected with the AP phytoplasma although 20 of them were immediate neighbours of at least one infected plant or were even located alternating between a sequence of AP phytoplasma-positive main crop trees (Fig. 2, Sections 1 and 2). This observation cannot be explained by resistance of the pollinators, because cultivated apple varieties were generally found to be susceptible to this disease (Kartte and Seemüller 1988). Although in an open field study the action of insect vectors cannot be ruled out and the trees might have become infected years ago, it is difficult to imagine that such a regular and alternating distribution pattern of infected and uninfected trees could have been generated exclusively by insect transmission of the pathogen. So far, two psyllid species, *Cacopsylla picta* and *C. melanoneura*, are recognised as the vectors of '*Candidatus* Phytoplasma mali' (Frisinghelli et al. 2000; Tedeschi and Alma 2004). In addition, two publications suggest the leafhopper *Fieberiella florii* as a further vector of the disease (Krczal et al. 1989; Tedeschi and Alma 2006). While the latter species is not known from South Tyrol, *C. picta* and *C. melanoneura* were found on various apple cultivars in the region (including 'Granny Smith') and no indications about preferences for particular varieties seem to exist (Walch 2006). Therefore, we conclude that at least in older apple orchards root connections between trees appear to play a role for the propagation of AP. However, it is conceivable that the tendency for root graft formation might vary between different apple rootstocks. One would expect to find it more commonly between vigorous rootstocks with shallow penetration, such as MM 111, due to the higher probability of establishment of physical contact between roots of different trees. Further studies will be required to address the question of natural root grafting between different commercially used rootstocks. Additionally, in order to unequivocally prove transmission of the phytoplasma by root bridges, experiments under controlled insect-free conditions will have to be carried out.

Table 1 Summary of the outcome of the qualitative real-time PCR analysis for detection of AP phytoplasma

	n AP infected/*n* investigated	% AP infected
Felled trees with AP symptoms	9/9	100
Trees with severe herbicide damage	25/35	71.4
Trees with partial herbicide damage	3/16	18.8
Trees without herbicide damage	3/31	9.7
Pollinators	0/31	0

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