SHORT COMMUNICATION

POLLEN TRANSMISSION OF *PEACH LATENT MOSAIC VIROID*

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SUMMARY

Peach latent mosaic viroid (PLMVd), the causal agent of peach latent mosaic disease, is transmissible by grafting and pruning tools and, occasionally, by aphids. There is no information on other possible ways of transmission. The high incidence of field infections and the rate of natural spread (5-10% of newly infected plants per year), prompted us to investigate the role, if any, that pollen, seed and root contact could play in PLMVd dissemination. The viroid was detected by molecular assays in pollen, seeds and roots of infected trees. Five out of 18 plants, pollinated with pollen from infected trees, became positive for PLMVd. Seeds from PLMVd-infected trees were also viroid-positive, but their seedlings were apparently viroid-free. PLMVd was not detected in the roots of healthy seedlings grown for six years in a container together with infected seedlings, indicating that no transmission had occurred via roots. Thus PLMVd is pollen-transmitted but is not transmitted through seeds nor roots of infected peach plants.

Key words: PLMVd, transmission, pollen, seed, seedlings, soil, root anastomosis.

Peach latent mosaic is one of the most widespread and damaging diseases of peach trees. Its causal agent, *Peach latent mosaic viroid* (PLMVd), described by Hernadez and Flores (1992), has a worldwide distribution and occurs in most commercial peach varieties (Shamloul *et al*., 1995; Ragozzino *et al*., 2003) where it can cause heavy losses (Faggioli *et al*., 2003). The main symptoms on peach trees include: irregularly shaped, discoloured and deformed fruits with cracked sutures and enlarged pits, bud necrosis, delay in bud-burst, flowering and fruit ripening, decline and reduced longevity of the plants. Some strains also induce clear-cut foliar leaf discolouration (e.g. calico, Malfitano *et al*., 2003) or yellowing.

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PLMVd was experimentally transmitted at a low percentage by the aphid *Myzus persicae* (Flores *et al*., 1992), but natural spread by this vector has never been documented. It was also experimentally transmitted with blades dipped in purified viroid preparations (Flores *et al*., 1990) or surface-contaminated by slashing infected plants (Hadidi *et al*., 1997). No information is available on additional ways of transmission.

Although spread of PLMVd is thought to be mainly through infected propagation material, a 5-10% yearly increase in PLMVd-infected plants in commercial orchards has been recorded (Desvignes, 1986). However, the reason underlying this natural spread is unknown.

Transmission through infected seed or pollen has been reported for several viroids, i.e. *Potato spindle tuber viroid* (Hunter *et al.,* 1969; Singh, 1970; Kryczynski *et al*., 1988; Singh *et al*., 1992), *Coleus blumei viroid* (Singh *et al*., 1991), *Hop stunt viroid* cucumber pale fruit strain (Kryczynski *et al.,* 1988), grapevine viroids (Wah and Symons, 1997) and *Avocado sunblotch viroid* (Wallace and Drake, 1962; Allen *et al*., 1981). This prompted us to investigate the presence of PLMVd in the pollen, seeds and roots of infected peach trees, to determine whether transmission from plant to plant could occur through pollination, root anastomosis or through seeds. This study lasted six consecutive years.

The pollen-transmission trial was carried on using as donors nine peach trees of three cultivars (3 plants/cultivar) infected with an Italian isolate of PLMVd, that were used to pollinate 18 virus-free 5-year-old peach trees of 6 cultivars (3 plants per cultivar) (Table 1).

Pollination of healthy peach plants with infected pollen was done as follows: flowers of healthy peach trees were emasculated three days before anthesis, then pollinated with pollen from flowers of infected plants. All possible precautions were taken to prevent accidental mechanical infections. In fact, pollinated trees were grown for six years in a insect-proof screenhouse to keep out aphids and external pollen. Every summer, leaves and fruits were checked for the presence of PLMVd by RT-PCR and tissue printing hybridization (Loreti *et al*., 1999). Pruning tools were sterilized with 5% sodium hypochlorite.

Pollen collected from pollinated trees was analyzed

Table 1. Results of pollen transmission of PLMVd from infected to healthy peach cultivars.

Infected donor	Recipient pollinated cultivar	Infected/pollinated plants (No.)
Royal Diamond	Tasty Free	0/3
Nectaross	Iris Rosso	2/3
Nectaross	Velvet C	1/3
Royal Diamond	Red Skin	0/3
GF 677	Andross	0/3
Nectaross	Vivian C	2/3

for the presence of PLMVd by RT-PCR and spot-blot hybridization. To ascertain whether the viroid contaminated the surface or was inside pollen grains a modified protocol by Aparicio *et al.* (1999) was used: 0.2 g of pollen grains were suspended in 900 µl of 0.2 M Tris-HCl pH 8.2, 17.5 µl of 5M NaCl, 8 µl of 10% Triton X-100 and 2 µl of 2-mercaptoethanol and the mixture was centrifuged at 3,000 *g* for 5 min. This step was repeated 3 times and the pellets (washed pollen) were saved. In each step, supernatants were mixed with 0.5 ml of water-saturated phenol pH 7.0, 100 µl of 5% SDS and 100 µl of 0.1 mM EDTA pH 7.0 and centrifuged at 9,000 *g* for 20 min.

Nucleic acids present in the aqueous phase obtained after centrifugation were recovered by ethanol precipitation and resuspended in 500 µl of sterile distilled water (Faggioli *et al.*, 2001). Washed pollen was triturated in liquid nitrogen and nucleic acids were extracted as described by Faggioli *et al.* (2001)**.** Nucleic acids from supernatants and washed pollen were analyzed for the presence of PLMVd by RT-PCR using specific primers that amplify the full-length genome, and by dot-blot hybridization, following the protocol of Loreti *et al.* (1999).

Results obtained either by hybridisation or in RT-PCR, showed that PLMVd occurred in 100% of analysed pollen samples collected from nine peach trees used as donors. Specifically, positive signals from hybridisation and RT-PCR, were obtained both in the supernatants and in washed pollen of PLMVd-infected donor trees, indicating the viroid was located both externally and an internally.

At the end of the sixth year, molecular hybridization and RT-PCR analysis of leaf and fruit extracts from the 18 trees pollinated with pollen from infected donors showed the presence of PLMVd in five of them, i.e. two of cv Iris Rosso, two of cv Vivian and one of cv Velvet, all pollinated by cv Nectaross (Table 1). Results were confirmed by polyacrylamide gel electrophoresis.

The PLMVd seed transmission trial was done as follows: 1000 seeds were collected from PLMVd-infected plants of 10 cultivars (100 seeds per cultivar, Table 2). Teguments of 50 seeds from each cultivar were removed and tested separately from the peeled kernels by RT-PCR or dot-blot hybridization using nucleic acid extracts. The remaining 50 seeds were stratified in sand and kept in the cold (4°C) for 3-4 weeks prior to germination in pots maintained in an insect-proof glasshouse under controlled conditions (24°C). Three-month-old seedlings, were then transferred and kept in an insect-proof screenhouse for six years and each summer their leaves were analyzed for PLMVd presence as previously described. Pruning was with tools disinfected with 5% sodium hypochlorite.

As shown in Table 2, PLMVd was detected in the teguments of 242 of 500 (*ca*. 49%) seeds tested and in 224 of 500 (*ca.* 45%) peeled kernels. In particular, tegument infection of tested cultivars ranged from 36% (cv Red Top) to 60% (cv Rossa di San Carlo) whereas the percentage of peeled kernel infection ranged from 36% (cv Red Top) to 58% (cv Rossa di San Carlo).

Seedlings from seeds of PLMVd-infected plants were always negative in both RT-PCR and hybridization tests made every summer in the 6-year trial, confirming the

Table 2. Results of RT-PCR and hybridization for PLMVd detection in peach seeds or seedlings originated from PLMVd-infected plants.

Tested cultivar	Infected / tested teguments (No.)	Infected/ tested peeled kernels (No.)	Infected/ tested seedlings (No.)
Maria Laura	25/50	22/50	0/50
Fairtime	23/50	20/50	0/50
Guglielmina	26/50	25/50	0/50
Red Top	18/50	18/50	0/50
Rossa di San Carlo	30/50	29/50	0/50
Rome Star	25/50	25/50	0/50
Diamaond Pricess	24/50	23/50	0/50
Fayette Rossa	21/50	22/50	0/50
Stark Red Gold	27/50	17/50	0/50
Maria Aurelia	23/50	23/50	0/50

previously reported lack of PLMVd transmission through seed (Desvignes, 1986; Flores *et al.*, 1992; Howell *et al.*, 1998).

For the root anastomosis experiment, 16 healthy GF 305 seedlings and four similar seedlings infected with an Italian isolate of PLMVd were planted together in a 2 x 2 m container that was kept in an insect-proof screenhouse for six years. Pruning was done as previously described.

PLMVd was detected in the roots of infected plants but not in the leaves of healthy plants, indicating that transmission through roots did not occur. At the end of the trial, infected seedlings were pollarded and treated with a systemic herbicide (Gliphosate) (Vindimiam *et al.*, 2002). Gliphosate-treated seedlings died whereas the untreated, originally healthy seedlings remained alive. This was taken as an indication that no herbicide translocation had taken place between infected and healthy seedlings, suggesting absence of root anastomoses.

Our results show that PLMVd is pollen-borne, as indicated by its detection both inside and outside pollen grains of viroid-infected plants, and is transferred to pollinated plants and to seeds. PLMVd is not transmitted from contaminated seeds to seedlings, probably because it does not enter the embryo. Finally, PLMVd can infect roots but these may not play a role in PLMVd dissemination since root anastomoses apparently did not occur, at least under the conditions of our trial.

This is the first experimental evidence that PLMVd-contaminated pollen can infect pollinated plants, thus can play a role in PLMVd transmission in the orchards.

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