European Journal of Plant Pathology **105:** 623–627, 1999.

Short communication

Location of Prunus necrotic ringspot Ilarvirus within pollen grains of infected nectarine trees: evidence from RT-PCR, dot-blot and *in situ* hybridisation

Federico Aparicio, Maria A. Sánchez-Pina, Jesus A. Sánchez-Navarro and Vicente Pallás*
Departamento de Mejora y Patología Vegetal, CEBAS (CSIC), Apartado de Correos 4195, 30080 Murcia, Spain;
**Author for correspondence (Fax: 34-968-266613; E-mail: vpallas@natura.cebas.csic.es)*

Accepted 8 June 1999

Key words: anthers, ilarvirus, PNRSV, pollen transmission

Abstract

Prunus necrotic ringspot Ilarvirus (PNRSV) is a pollen and seed-borne ilarvirus affecting most *Prunus* spp. The location of the virus in infected nectarine pollen grains was investigated by molecular hybridisation, RT-PCR and *in situ* hybridisation. The first two approaches revealed an internal location of the virus. *In situ* hybridisation demonstrated the virus in the bicellular pollen grain, where it was present in the cytoplasm of the vegetative cell but not in the generative cell. This result seems to indicate that the sperm cells, formed by the mitosis of the generative cell, are not involved in virus transmission to seed. Other possible mechanisms are discussed.

Prunus necrotic ringspot Ilarvirus (PNRSV) is the causal agent of several diseases affecting most cultivated stone fruits, including cherry, sour cherry, almond, peach, apricot and plum. Ilarviruses have the same genome organisation, encoding functionally similar translation products, as those of bromovirus, cucumovirus and alfamovirus, which belong to the family *Bromoviridae* (Murphy et al., 1995). The virus is pollen and seed borne (Cole et al., 1982; Hamilton et al., 1984; Kelley and Cameron, 1986) which contributes to its rapid spread in stone fruit trees (Uyemoto et al., 1992; Mink, 1992).

Infected pollen has been shown to play a key role in both seed and plant-to-plant transmission of ilarviruses. Recent evidence suggests, however, that other biological factors such as the mediation of thrips or other flower-working arthropods are required for plant-to-plant transmission (Greber et al., 1991, 1992; Mink, 1992; Johansen et al., 1994). PNRSV has been detected in seedlings grown from seed produced on healthy plants of several *Prunus* species after hand pollination with pollen from infected plants, which strongly suggested that fertilisation could be a method for virus transmission (reviewed by Mink, 1992). However, contradictory results have been obtained regarding the location of PNRSV on or in pollen grains from infected cherry trees. Cole et al. (1982) showed that PNRSV antigens were easily removed from intact pollen by washing, thus indicating that most if not all the virus was on the surface. Results obtained by Kelley and Cameron (1986) and Digiaro et al. (1992) suggested that PNRSV was located both externally and internally. As far as we know, no results have been reported on the analysis of the male gametes to detect the internal presence of this virus. The aim of this work was to study the location of PNRSV on or in nectarine pollen grains by different approaches, including one (*in situ* hybridisation) that could clarify whether or not the virus invades the male gametes.

Sampling was carried out three times, in March 1996 and 1997 and February 1998, from 3, 4 and 4 different nectarine (*Prunus persica*) trees respectively. Of these 1, 2 and 1, respectively, were healthy and the other 7 infected. Closed flowers were collected and the pollen grains separated from anthers after air-drying. Leaves and fruits were also collected three weeks later from the same trees. Total RNA was extracted from 20 mg pollen and anthers and from 0.1 g leaves, fruits

and petals taken from infected and healthy nectarine samples (Sánchez-Navarro et al., 1998) and analysed for the presence of the virus by non-radioactive molecular hybridisation, as described by Pallás et al. (1998). Non-radioactive riboprobe was obtained by transcribing clone pPN4-890 that corresponded to a full-length cDNA of PNRSV RNA 4 (Sánchez-Navarro and Pallás, 1997).

Figure 1 shows that PNRSV, in addition to being present in leaves and fruits, was clearly present in all floral organs examined including intact pollen. To gain evidence about virus location, 20 mg of pollen was suspended in 1.5 ml of phosphate saline–tween polyvinylpyrrolodone buffer pH 7.4, vortexed for 1 min and centrifuged at 3000 rpm for 5 min. This procedure was repeated three times, followed by an additional washing with 1.5 ml 1% SDS to remove particles tightly bound to pollen grains (Digiaro et al., 1992). Aliquots from the four supernatants were phenol-extracted and the aqueous phase was ethanol-precipitated and resuspended in sterile water. Washed pollen was homogenised for total RNA extraction as described above. Supernatants, intact pollen and washed pollen were analysed for the presence of PNRSV by dot-blot hybridisation and RT-PCR (Figure 2a and b, respectively). Specific primers and PCR parameters were as previously described (Sánchez-Navarro et al., 1997, 1998).

As shown in Figure 2a, most if not all, the hybridisation signal was observed when pollen grains were ground after repetitive washes (row 6 in Figure 2a), indicating an internal location for the virus. This result was confirmed when a more sensitive technique, RT-PCR, was used (Figure 2b). An 891 nt. specific product corresponding to PNRSV RNA 4 was mainly obtained in intact and washed pollen grains (lanes 1 and 4, respectively) but not or very weakly in the phenol-extracted and ethanol-precipitated supernatant fluids from buffer washings of infected pollen (lanes 2 and 3).

To gain definitive evidence in support of the internal location of PNRSV in pollen grains and to know the distribution of the virus within them, *in situ* hybridisation (ISH) studies by light microscopy were carried out. Infected and uninfected anthers, at different developmental stages, were separated from the rest of the open and closed flower organs and fixed in Karnovsky fixative (Karnovsky, 1965), post-fixed with osmium tetroxide and embedded in Spurr resin. Semithin (1 µm) sections were cut and collected on TESPA (3-aminopropyltriethoxy-silane) (Sigma) coated slides. Hybridisation was performed at 55 °C overnight with the same riboprobe used in the dot-blot analysis in the presence of 50% formamide. A proteinase K (1 µg/ml) treatment was performed, at 37 °C for 10 min, before hybridisation. In addition, an RNase (20 µg/ml) treatment for 10 min followed by stringent

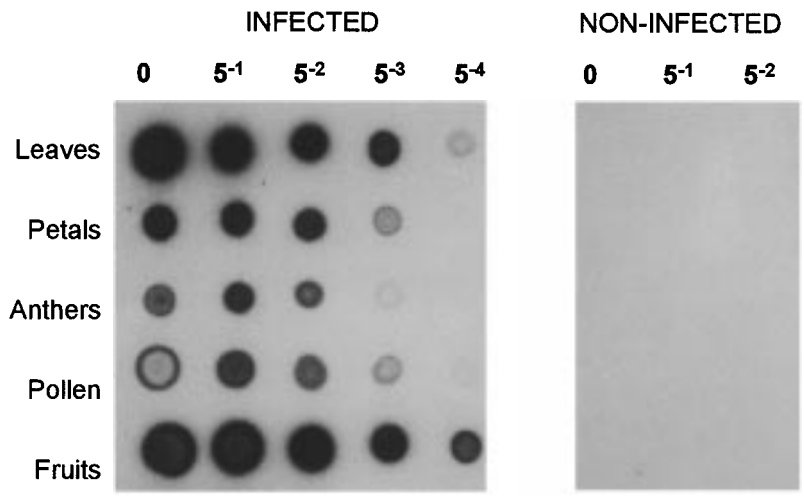

Figure 1. Detection of PNRSV in different organs of nectarine trees by non-radioactive dot-blot hybridisation. Total RNA was extracted from 20 mg pollen and anthers and from 0.1 g leaves, fruits and petals taken from infected and healthy nectarine trees and applied to nylon membranes and hybridised with the riboprobe (see text). The numbers correspond to the different dilutions used. As shown, the different organs analysed contained high amounts of PNRSV, which was detected in all the samples up to the 5^{-3} dilution. It was more abundant in leaves and fruits, where it could still be detected at 5^{-4}. Samples from uninfected trees showed no signal.

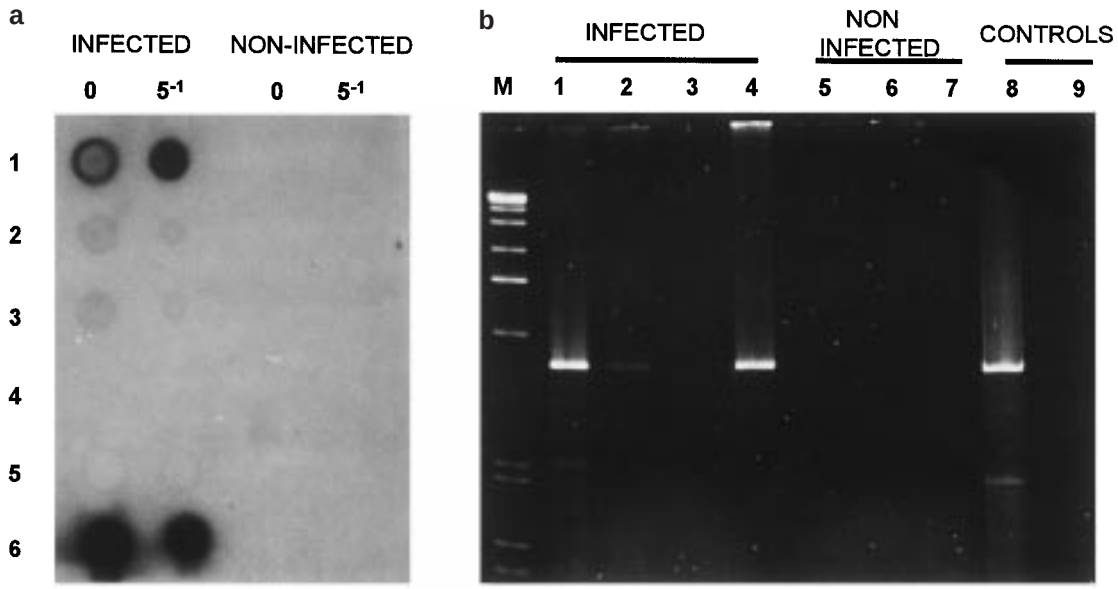

Figure 2. Presence of PNRSV inside pollen grains from nectarine trees. Non-radioactive dot-blot hybridisation (a) and RT-PCR (b) of intact and washed pollen. In (a) the numbers correspond to (1) unwashed pollen; (2) first PBS supernatant; (3) second PBS supernatant; (4) third PBS supernatant; (5) SDS supernatant and (6) washed pollen. In (b) the different lanes are: (M) 1 kb ladder; (1 and 5) unwashed pollen; (2 and 6) first PBS supernatant; (3) 1% SDS supernatant of infected pollen; (4 and 7) total RNA from washed pollen; (8) positive control of PNRSV-PCR (total RNA from PNRSV-infected cucumber) and (9) negative control of PNRSV-PCR (total RNA from uninfected cucumber). Note that rows 1, 2, 5 and 6 in (a) correspond to lanes 1–4 in (b) respectively. The amplified product corresponds to the 891 nt. of PNRSV RNA 4 (lanes 1 and 4).

washes in the presence of dithiothreitol (DTT) (Sigma) were made at room temperature after the hybridisation to eliminate unspecific labelling. For detection, gold-labelled sheep anti-digoxigenin (BioCell) diluted 1/25 in 1% BSA/PBS was applied for 1 h. After the silver enhancement treatment (8 min) (BioCell), the slides were washed several times in distilled water before drying and mounting with Merckoglass. Preparations were observed by a combination of epipolarization, which identified the labelling as bright spots, and phase contrast microscopy to observe the cellular structure, using a DMRB Mikroskop (Leitz). Colour pictures were taken with 800 asa film. The ISH was performed as described by Sánchez-Pina et al. (1998). As can be clearly observed from Figure 3a, most of the hybridisation signal, in the form of bright spots, was present within the pollen grains and in the cytoplasm of anther cells. No specific signal was observed when uninfected anthers were used (Figure 3c) or when the riboprobe was not added to the hybridisation solution (not shown). ISH experiments were repeated 10 times on the different samples taken, both healthy and infected, and the results were always consistent.

Three infected and two healthy trees had flowers with anthers containing bicellular pollen grains as observed by electron microscopy (not shown). Detailed examination of the bicellular pollen grains (Figure 3b) clearly demonstrated that the PNRSV RNA was cytoplasmic, and not present in the nuclei. More importantly, PNRSV was not present, at least in detectable amounts, in the generative cell of the bicellular pollen grain (Figure 3b, arrow-head). The cytoplasmic localisation of PNRSV RNA is consistent with the results obtained for alfalfa mosaic virus (AMV), a virus closely related to ilarviruses, although in that case it was suggested that virus distribution within the cell is both strain- and host-specific (Pesic et al., 1988).

Taken all our data together, it seems that PNRSV cannot be transmitted directly from the male gametes in nectarine, since the sperm cells are formed by mitosis of the generative cell (reviewed in Mascarenhas, 1989), which, as shown here, does not contain PNRSV components. However, the possibility that sperm cells are infected during or after mitosis, before fertilisation, cannot be ruled out. In addition, it is also possible that PNRSV could be transmitted by the vegetative

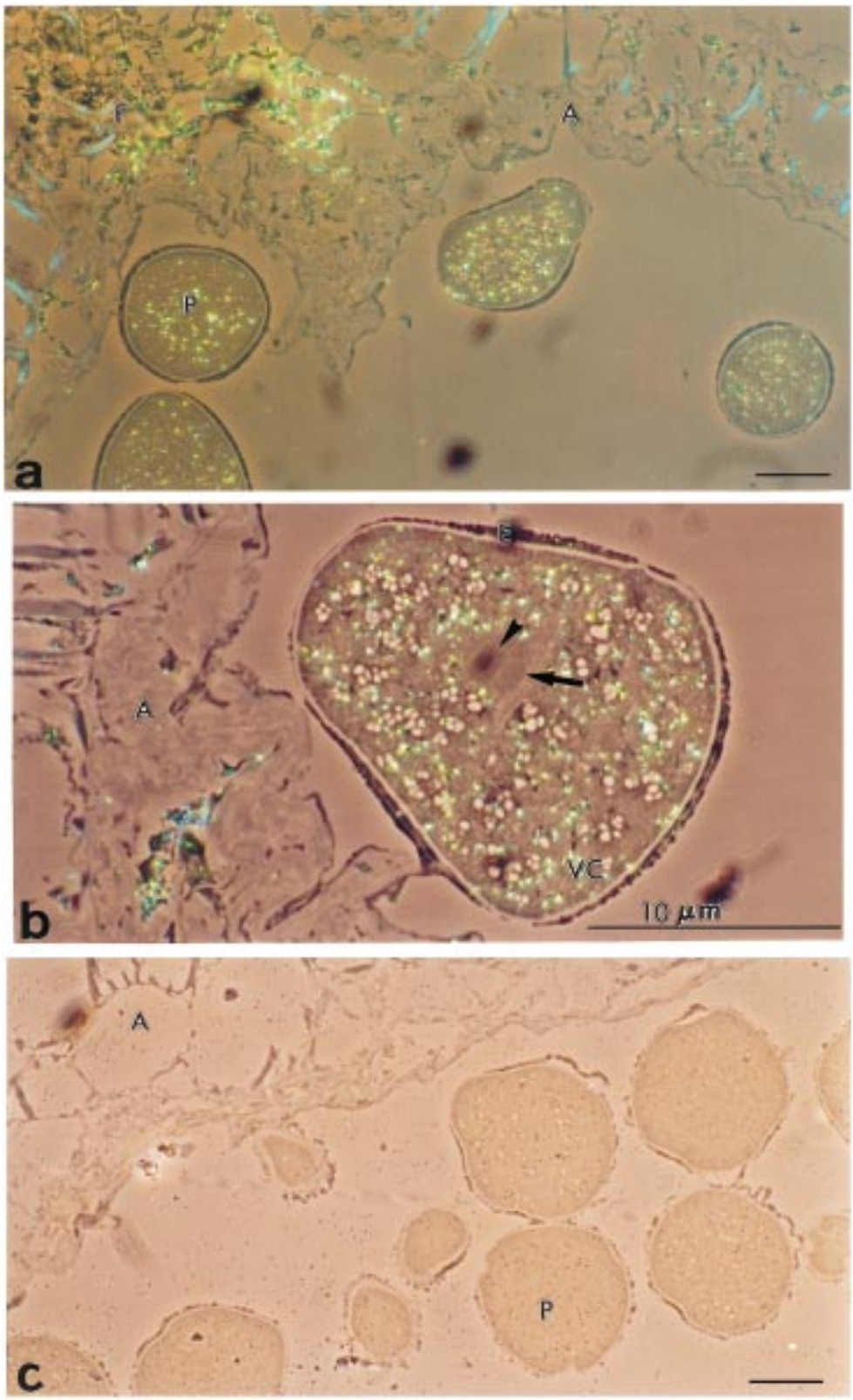

Figure 3. Light microscopy*in situ* hybridisation of PNRSV RNA in anthers from nectarine trees. a, b: Infected. c: Uninfected. Observations were made using a combination of epipolarisation and phase contrast. a, b: In all tissues (anther and pollen grains) the RNA localisation is cytoplasmic. Notice the absence of labelling inside the generative cell (arrowhead) of the bicellular pollen grain (b). Both nuclei, the vegetative (arrow) and the generative are label-free. The exine (E) appears also free of label suggesting that there is no virus present in the exine. c: No specific signal was detected when the ISH was performed on healthy nectarine anthers. E: exine; F: filament of the anther; A: anther; P: pollen grain; Arrow: vegetative nuclei; Arrow-head: generative cytoplasm. Bar 10 µm.

cytoplasm of the pollen, which contains high amounts of the virus. This vegetative cytoplasm is the origin of the pollen tube that grows into the pistil, carrying within it the male sperm cells, until it reaches the embryo sac, where double fertilisation takes place. Since the vegetative cytoplasm is full of PNRSV RNA, this can be delivered into the embryo sac by the pollen tube and incorporated into the newly formed seed. Recent evidence indicates that during fertilisation some male cytoplasm can be transferred into the egg cell (Russell, 1995), thus facilitating the transmission of cytoplasm-located viruses (reviewed by Maule and Wang, 1996).

Acknowledgements

Since F.A. and M.A.S.-P. have equally contributed to this work, we request that the first two authors be considered as co-first authors. We thank P. Sánchez and P. Thomas for helping us with the English grammar of this manuscript and J. Martínez-Fresneda for her technical assistance. This research was partially supported by grant BIO96-0459 from the Spanish granting agency DGYCIT. F.A. and J.A.S.-N. were recipients of fellowships from the Ministerio de Educación y Ciencia from Spain.

References

Cole A, Mink GI and Regev S (1982) Location of Prunus necrotic ringspot virus on pollen grains from infected almond and cherry trees. Phytopathology 72: 1542–1545

Digiaro M, Di Terlizzi B and Savino V (1992) Ilarviruses in apricot and plum pollen. Acta Hortic 309: 93–98

Greber RS, Klose MJ, Milne JR and Teakle DS (1991) Transmission of prunus necrotic ringspot using plum pollen and thrips. Ann Appl Biol 118: 580–593

Greber RS, Teakle DS and Mink GI (1992) Thrips-facilitated transmission of prune dwarf and prunus necrotic ringspot viruses from cherry pollen to cucumber. Plant Dis 76: 1039–1041

Hamilton RI, Nichols C and Valentina B (1984) Survey of Prunus necrotic ringspot and other viruses contaminating the exine of pollen collected by bees. Can J Plant Pathol 6: 196–199

Johansen E, Edwards MC and Hampton RO (1994) Seed transmission of viruses: current perspectives. Ann Rev Phytopathol 32: 363–386

Karnovsky MJ (1965) A formaldehyde–glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27: 137A–138B

Kelley RD and Cameron HR (1986) Location of prune dwarf and prunus necrotic ringspot viruses associated with sweet cherry pollen and seed. Phytopathology 76: 317–322

Mascarenhas JP (1989) The male gametophyte of flowering plants. Plant Cell 1: 657–664

Maule AJ and Wang D (1996) Seed transmission of plant viruses: a lesson in biological complexity. Trends in Microbiol 4: 153–158

Mink GI (1992) Ilarvirus Vectors. Adv. Dis. Vector Res 9: 261–281

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA and Summers MD (1995) Virus Taxonomy. Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York (Archives of Virology [Suppl] 10)

Pallás V, Más P and Sánchez-Navarro JA (1998) Detection of plant RNA viruses by non-isotopic dot-blot hybridisation. In: Foster G and Taylor S (eds) Plant Virus Protocols: From Virus Isolation to Transgenic Resistance (pp 471–478). Humana Press, Totowa, New Jersey

Pesic Z, Hiruki C and Chen MH (1988) Detection of viral antigen by immunogold cytochemistry in ovules, pollen and anthers of alfalfa infected with alfalfa mosaic virus. Phytopathololology 78: 1027–1032

Russell SD (1995) The egg cell: development and role in fertilisation and early embriogenesis. Plant Cell 5: 1349–1359

Sánchez-Navarro JA and Pallás V (1997) Evolutionary relathionships in the ilarviruses: nucleotide sequence of prunus necrotic ringspot RNA 3. Arch Virol 142: 749–763

Sánchez-Navarro JA, Reusken CBEM, Bol JF and Pallás V (1997) Replication of alfalfa mosaic virus RNA 3 with movement and coat protein genes replaced by corresponding genes of Prunus necrotic ringspot Ilarvirus. J Gen Virol 78: 3171–3176

Sánchez-Navarro JA, Aparicio F, Rowhani A and Pallás V (1998) Comparative analysis of ELISA, non-radioactive molecular hybridisation and PCR for the detection of Prunus necrotic ringspot virus in herbaceous and *Prunus* hosts. Plant Pathol 47: 780–786

Sánchez-Pina MA, Más P and Pallás V (1998) Tubular structures formed by cherry leaf roll nepovirus (CLRV) in systemically infected *Nicotiana tabaccum* leaf cells: immunocytochemistry and *in situ* hybridization. In: Electron Microscopy 96, Vol. 3 (pp 187–188), edited and published by the Committe of European Societies of Microscopy, Brussels

Uyemoto JK, Asai WK and Luhn F (1992) Ilarviruses: evidence for rapid spread and effects on vegetative growth and fruit yields of peach trees. Plant Dis 76: 71–74