Enzyme-Linked Immunosorbent Assay Testing of Shoots Grown In Vitro and the Use of Immunocapture-Reverse Transcription-Polymerase Chain Reaction Improve the Detection of *Prunus necrotic ringspot virus* in Rose

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ABSTRACT

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We developed and evaluated two different methods to improve the detection of the most prevalent virus of rose in Europe, *Prunus necrotic ringspot virus* (PNRSV). Immunocapture-reverse transcription-polymerase chain reaction was estimated to be about 100 times more sensitive than double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and showed an equivalent specificity. Based on the observation that PNRSV multiplies actively in young growing tissues (axillary shoots

and cuttings), an in vitro culture method allowing rapid (about 15 days) and homogeneous development of dormant axillary buds with high virus titers was standardized. ELISA tests of these young shoots showed, in some cases, a 10^4 to 10^5 increase in sensitivity in comparison to adjacent leaf tissues from the rose mother plants. Between 21 and 98% (depending on the season) more samples were identified as positive by using ELISA on samples from shoot tips grown in vitro rather than on leaves collected directly from the PNRSV-infected mother plants. This simple method of growing shoot tips in vitro improved the confidence in the detection of PNRSV and eliminated problems in sampling appropriate tissues.

Additional keywords: season effect, woody plant.

Prunus necrotic ringspot virus (PNRSV), a member of the genus *Ilarvirus* in the family *Bromoviridae*, occurs worldwide and infects mainly woody plants in the genera *Prunus* and *Rosa* and also hops (12). Depending on the host plant, the dispersion of PNRSV is mainly ensured by pollen (16,32), seed (12), vegetative propagation (cutting and grafting), or cultural practices (slashing, training, and harvesting [26]). PNRSV is currently the most prevalent virus of rose in Europe (B. Moury and L. Cardin, *unpublished data*). Its economic impact in rose is highly variable (31,35) and difficult to evaluate (22). A European and Mediterranean certification scheme for rose pathogens including viruses was approved in 1997 (25). According to this scheme, candidate plants for the nuclear stock (used for genotype propagation) should remain under observation in isolation for at least 1 year, during which time they should be tested twice for PNRSV, *Apple mosaic virus* (ApMV), *Arabis mosaic virus* (AMV), and *Strawberry latent ringspot virus*, with an interval of several months between tests. One of the tests should be performed in spring, the most favorable season for virus detection. This is essentially due to the difficulties of detecting viruses in rose: viral symptoms vary greatly with the season and the cultivar, viral concentration is usually low and variable, and virus distribution in the plant is often heterogeneous (1). When applied, this lengthy scheme will be a severe constraint for rose propagators. A more efficient detection method could shorten the quarantine period. The

easiest and most widely used detection test for PNRSV is enzyme-linked immunosorbent assay (ELISA). To enhance the efficiency of detection of PNRSV in rose, two research approaches have been investigated: (i) increasing the detection sensitivity of a method compatible with large-scale testing, and (ii) determining which types of rose samples have the highest PNRSV concentration for ELISA. For the first objective, we evaluated immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) sensitivity and specificity in comparison to double-antibody sandwich (DAS)-ELISA, since IC-RT-PCR has often proven more sensitive (6). For the second objective, we compared different plant organs as well as single-node cuttings and in vitro shoots.

MATERIALS AND METHODS

Virus isolates. The PNRSV isolates used in this study were part of a virus collection kept at INRA-Antibes. Each isolate was maintained by chip budding on healthy rootstocks of *Rosa indica* var. *vulgaris* L. cv. CE10 propagated in vitro and *Prunus persica* (L.) Batsch. cv. GF305 seedlings. In order to avoid cross-contamination, the plants were kept in an insect-proof glasshouse. Moreover, peach and *R. indica* plants were not allowed to flower, ensuring that there was no pollen transmission of the virus. *Cucumis sativus* L. cv. Poinsett plants at the cotyledon stage were rub-inoculated using Wong and Horst's buffer no. 8 (34) plus 90 mg of active charcoal per ml and 90 mg of Carborundum per ml to provide a source of tissue for the purification of PNRSV. *Ilarvirus* isolates from *Prunus* sp. were used as specificity controls for ELISA and IC-RT-PCR (four PNRSV, two ApMV, and one *Prune dwarf virus* [PDV] isolate).

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PNRSV purification. After multiplication in 'Poinsett' cucumbers, three PNRSV isolates from rose were purified according to a modification of the method of Crosslin and Mink (9). Cucumber tissues (5 to 10 g) were ground with a mortar and pestle on ice with 25 ml of ice-cold 30 mM potassium phosphate buffer, pH 8.0, containing 10 mM sodium diethyldithiocarbamate and 10 mM sodium thioglycollate. The homogenate was centrifuged at $50,000 \times g$ for 15 min. The supernatant was collected, pooled, adjusted to pH 4.5 with 5% (vol/vol) acetic acid, and again centrifuged at $50,000 \times g$ for 15 min. Pellets were discarded and viral nucleoproteins pelleted by centrifugation at $75,000 \times g$ for 4.5 h. The pellets were then resuspended in 100 μ l of 30 mM potassium phosphate buffer, pH 8.0, and left overnight at 4°C. The suspended material was placed into microcentrifuge tubes and centrifuged for 5 min at $8,800 \times g$. The supernatant was used for serological assays after dilution in ELISA sample buffer. Virus concentrations were estimated from the absorbance at 260 nm (A_{260}), assuming an extinction coefficient of 5 (20).

ELISA tests. DAS-ELISA was conducted with anti-PNRSV immunoglobulin G (IgG) and commercially available alkaline phosphatase-conjugated PNRSV IgG (Bioreba, Reinach, Switzerland). Polystyrene plates (Corning Costar Corp., Cambridge, MA) were coated for 2.5 h at 37°C with 150 μ l per well of IgG diluted at 1 μ g/ml in 50 mM carbonate buffer (pH 9.6). The plates were then washed three times with phosphate-buffered saline-Tween (PBS-T: 10 mM phosphate buffer, pH 7.2, containing 0.8% [wt/vol] NaCl, 0.02% [wt/vol] KCl, and 0.05% [vol/vol] Tween-20). Plant samples were ground in nine volumes (wt/vol) of PBS-T containing 2% (wt/vol) polyvinyl pyrrolidone (molecular weight 25,000) and 20 mM sodium diethyldithiocarbamate (sample buffer), centrifuged 15 min at $5,000 \times g$ at 4°C to remove plant debris, and 150 μ l of the supernatant was added to each well. The plates were incubated overnight at 4°C. Plates were then washed and incubated for 2.5 h at 37°C with 150 μ l of conjugated IgG per well at a 10^{-3} dilution in PBS-T containing 1% (wt/vol) bovine serum albumin and then washed again. An aliquot of 150 μ l of *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine, pH 9.8, was then added to each well at room temperature. The absorbance was read at 405 nm in a microtiter plate reader (Multiskan RC; Labsystems, Helsinki, Finland). Absorbance values were read after 2 h of incubation. Samples were considered positive when their absorbance value was at least two times greater than the mean absorbance value of five healthy controls.

To evaluate the specificity of reaction, the detection of PNRSV by ELISA and IC-RT-PCR was compared qualitatively for a collection of 37 PNRSV isolates from rose maintained in *R. indica* cv. CE10. Absolute sensitivities of both methods were estimated with a range of concentrations of purified virus in buffer during three experiments of three isolates each. To evaluate their relative sensitivities in experimental conditions, 10-fold successive dilutions of PNRSV-infected leaf extracts with healthy plant extracts were used for ELISA and IC-RT-PCR detection. Three independent experiments were conducted, each of them with 'GF305' peach leaves and 'CE10' *R. indica* leaf extracts.

IC-RT-PCR. The PCR primers used in this study were the 20-mer Ilar1 (5'-TTCTAGCAGGTCTTCATCGA-3') and the 17-mer Ilar2 (5'-CAACCGAGAGGTTGGCA-3') (7). They correspond to the 3' end of the coat protein gene (RNA 3) and have been defined as consensus sequences allowing the amplification of both the PNRSV and ApMV genomes.

The IC-RT-PCR protocol is as follows. Sterile polypropylene (Sarstedt, Molsheim, France) or polycarbonate (Appligène Oncor, Illkirch, France) microtubes (0.5 ml) were coated with the same anti-PNRSV IgG (150 μ l) as for ELISA and at the same dilution for 3 h at 37°C. The tubes were then washed once with PBS-T. A 150- μ l aliquot of plant extract supernatants obtained as for ELISA was added, and the tubes were incubated either overnight at 4°C or for 3 h at 37°C. The microtubes were washed one to three times with PBS-T, briefly centrifuged, and the remaining droplets were

removed. Microtubes were preheated for 15 min at 65°C before adding 50 μ l of the RT-PCR mix: 10 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 50 mM KCl, 0.3% (vol/vol) Triton X-100, 250 μ M each dNTP, 1 μ M each of the two primers, 2 U of AMV reverse transcriptase (Amersham, Les Ulis, France), and 1 U of *Taq* DNA polymerase (Appligène Oncor). After vortexing and spinning down the droplets, the reaction mix was overlaid with approximately 50 μ l of mineral oil. The cycling scheme used, performed on a Robocycler 40 apparatus (Stratagene, La Jolla, CA), was 1 h at 46°C (reverse transcription); 2 min at 92°C; followed by 40 cycles of amplification of 1 min at 92°C, 1 min at 52°C, and 90 s at 72°C. At the end of the program, the microtubes were held for 10 min at 72°C. Twenty-microliter aliquots of the amplification reactions were analyzed by 1.5% (wt/vol) agarose gel electrophoresis in 0.5 $\times$ Tris-borate-EDTA buffer (28).

ELISA detection of PNRSV in rose organs. PNRSV-infected *R. hybrida* cvs. Pekcougel (=Anna), Meicouf (=Léonidas), Korlimit (=Dallas), and Nirporbi (=Laguna) were obtained from rose growers in southeastern France (Anna and Léonidas), Portugal (Dallas), and Spain (Laguna) and grown in glasshouses according to usual horticultural practices. Three PNRSV-infected plants of each cultivar were tested by ELISA in August 1998 for the presence of virus in various organs: basal and apical leaves and bark from four floral stems per plant, roots, petals, and stamens. Petals and stamens could not be sampled on every stem because of heterogeneous flowering and anthesis among plants and stems. In subsequent experiments, Anna plants were used as a model for unfavorable conditions for PNRSV detection (low absorbance values and heterogeneous distribution of the virus) and Léonidas plants were chosen as positive controls (favorable detection with high virus titers).

In an experiment conducted in May 1999, floral stems of PNRSV-infected Anna and Léonidas plants were decapitated at anthesis to allow the development of one to five axillary buds. When the axillary shoots were 2 to 5 cm long, they were tested by ELISA and compared with leaflets from the corresponding axillary leaves. A total of 39 shoots from Anna and 10 shoots from Léonidas was tested.

ELISA test of cuttings. Six to eight single-node cuttings (approximately 5 cm long) of healthy and PNRSV-infected Anna and Léonidas plants were excised in the medial zone of floral stems at anthesis. Basal ends of the cuttings were dipped into 0.5% (wt/wt) β -indole butyric acid. To protect them against fungal infection, they were treated with 800 mg of pyrimethanil (SCALA; AgrEvo, Berlin) per liter. Cuttings were cultivated in perlite in a heated glasshouse (minimal temperature 25°C) at 80% relative humidity. After 15 days, the rooted cuttings were transferred to a culture room at 22°C with a 16-h photoperiod. For each cutting, leaflets from the axillary leaf were tested by ELISA when cuttings were removed from the mother plant. Young shoots grown from the axillary buds of each cutting were tested by ELISA when they were 2 to 5 cm long.

In vitro culture of excised axillary buds. Stem segments from healthy and PNRSV-infected Anna and Léonidas plants bearing axillary buds were surface-sterilized with 70% ethanol for a few seconds and then in 15% calcium hypochlorite for 20 min. The segments were washed three times in sterilized distilled water. The axillary buds were aseptically excised (with a 1-cm-long stem cross section or shield) and individually placed in tubes containing 10 ml of semisolid (0.7% agar) Murashige-Skoog medium (23) with the following modifications: *myo*-inositol at 100 mg/liter, glycine at 200 mg/liter, glutamine at 200 mg/liter, glucose at 40 g/liter instead of sucrose, N⁶ benzyl adenine at 1 mg/liter, gibberellic acid (GA3) at 0.5 mg/liter, 2.8 μ M indole-3-acetic acid, and without 1-naphthaleneacetic acid. The high cytokinin/auxin ratio allowed the rapid development of a shoot from axillary buds. Cultures were incubated in a climatic room (22°C, 16-h photoperiod, and photosynthetic active radiation at 11 W m⁻² at the canopy level). As for the cuttings, each sample was tested by ELISA both when removed

from the mother plant (leaflets from the axillary leaf) and after the growth of the axillary bud (when 2 to 3 cm long).

Absorbance values of ELISA tests of cuttings or in vitro shoots were compared with absorbance values of adjacent leaflets of the mother plants in order to evaluate the efficiency of detection in various organs. They were also determined at different dates to evaluate season influence. Six different tests were conducted from December 1998 to September 1999, as indicated in Table 1.

Statistical analyses. Analyses of ELISA results were conducted on the ratios (*R*) between the absorbance values of the samples and the mean absorbance value of at least five healthy controls. Healthy controls showed a mean absorbance value of 0.115 after 2 h of reaction for all experiments conducted. The standard deviation varied from 0.002 to 0.019, depending on the experiment. The coefficient of variation of absorbencies was about 10%. *R* values of ELISA tests were analyzed either qualitatively (percentages of positive and negative samples) or quantitatively by analysis of variance (ANOVA) using a type III sum of squares calculated by the GLM procedure of the SAS software (SAS Institute Inc., Cary, NC). Unless indicated, hypotheses for ANOVA were confirmed by the Shapiro and Wilk test (30) (residues show a normal distribution if the probability is above 0.05) and Bartlett's test (10) (variance of treatments is homogeneous if the probability is above 0.05). When ANOVA results showed significant effects (Fisher test probability below 0.05), means were compared with a Newman-Keuls test (10).

RESULTS

Optimization of IC-RT-PCR for PNRSV detection in rose.

We improved the efficiency of IC-RT-PCR by changing the number of washings before reverse transcription, comparing the effect of tube quality on PCR yield, and adjusting pretreatment temperatures and reverse transcription incubation time. Positive PCR reactions lead to the amplification of the expected 206-bp DNA band. The most crucial step in our IC-RT-PCR protocol was the number of washings before reverse transcription. At a 10^{-1} dilution factor (in buffer), a single washing is adequate for IC-RT-PCR amplification from cucumber cotyledon extracts (Fig. 1, sample 1), whereas two or three washings are necessary for peach leaf, rose petal, or rose leaf extracts (Fig. 1, samples 2 to 4, respectively). In further experiments, three washings were routinely performed.

The type of plastic tubes used in these tests (polypropylene or polycarbonate) had an effect on sensitivity. Polypropylene microtubes showed an approximately 10-fold higher sensitivity than did polycarbonate tubes (data not shown). Further experiments were conducted with polypropylene tubes.

We observed that preheating the tubes (65°C for 15 min) before reverse transcription increased the efficiency of IC-RT-PCR (data not shown). Similar to results obtained by Rosner et al. (27), we observed that AMV reverse transcriptase yielded a stronger PCR band when incubated at 46°C rather than 42°C.

We also obtained very erratic results with less than 30 min of reverse transcription. Better results were obtained with a 45-min

reverse transcription step, and particularly stronger bands were obtained with 1 h of incubation (data not shown).

Comparison of specificity of ELISA and IC-RT-PCR for detection of PNRSV from rose. All of the 37 PNRSV isolates from rose could be detected by DAS-ELISA and IC-RT-PCR (data not shown). Four PNRSV isolates from *Prunus* sp. could also be detected by both methods, whereas it was impossible to detect two ApMV isolates or a PDV isolate by any of these methods. RT-PCR conducted on total nucleic acid extracts (4) of infected 'GF305' peach plants yielded the expected PCR bands for PNRSV and ApMV isolates but no PCR products for PDV (data not shown), which shows that the primers used are specific for PNRSV and ApMV (the two most frequent ilarviruses known to infect rose). Detection of ApMV by IC-RT-PCR was evaluated elsewhere (7).

Comparison of sensitivities of ELISA and IC-RT-PCR for PNRSV detection. A_{260}/A_{280} values of purified virus ranged from 1.42 to 1.51, which allowed calculation of the concentration of the virus and evaluation of the absolute sensitivities of ELISA and IC-RT-PCR using purified virus dilutions in buffer. The sensitivity limits of DAS-ELISA and IC-RT-PCR were approximately 3 ng/ml and between 3 and 0.3 pg/ml, respectively (data not shown). These results fit well with those obtained by Sanchez-Navarro et al. (29), who estimated sensitivity limits of ELISA and RT-PCR on nucleic acid extracts from infected nectarine plants to be 4 ng/ml and 1.28 pg/ml, respectively.

IC-RT-PCR was around 100 times more sensitive than was ELISA for peach and *R. indica* extracts (Fig. 2).

ELISA detection of PNRSV in rose organs. PNRSV could be detected by ELISA in every organ and cultivar tested. Few petal and stamen samples were examined due to heterogeneous flowering and anthesis. ELISA data of petals and stamens were very heterogeneous, varying from negative ELISA results to high absorbance values. Thus, results concerning these two organs were not further analyzed. Significant differences were observed between cultivars and between plant organs (ANOVA, $P < 0.001$). No significant interaction between cultivars and plant organs was detected. Absorbance values were higher for leaves than for bark or roots (Table 2). Differences between rose cultivars are shown in Table 2.

ELISA detection of PNRSV in young axillary shoots developed after the decapitation of the floral stems and the corresponding axillary leaf (on the floral stem) was efficient for Léonidas (data not shown). For Anna, 28% of the samples were found negative in both organ types. Fifteen percent of the samples were negative in axillary leaves and positive in the corresponding axillary shoot. ANOVA of the absorbance of the remaining 57% of the samples showed a significant difference ($P \approx 0.012$), with a higher response of young shoots (means of *R* values were 25.9 for young shoots and 20.9 for axillary leaves).

ELISA detection of PNRSV in cuttings and in vitro shoots.

We compared ELISA results of single-node cuttings and in vitro shoots with the ELISA results of the corresponding axillary leaf sampled on the mother plant (PNRSV-infected Anna and Léonidas plants). Six tests were conducted from December 1998 to Sep-

TABLE 1. Test dates and numbers of samples for enzyme-linked immunosorbent assay (ELISA) detection (excluding controls)^y

Test	ELISA on mother plants	ELISA ^z on cuttings	No. of cuttings	ELISA ^z on in vitro shoots	No. of in vitro shoots
1	8 December 1998	57, 86	48		0
2	3 February 1999	56, 64, 81	40		0
3	31 March 1999	28, 40	45		0
4	10 May 1999	30, 44, 66	76	17	51
5	15 July 1999		0	23	43
6	1 September 1999		0	15	42

^y Detection efficiency was compared in leaves of PNRSV-infected Anna mother plants and in cuttings and in vitro shoots derived from these plants.

^z Number of days after the test on mother plants.

Fig. 1. Influence of plant extracts and number of washings before reverse transcription on the detection of *Prunus necrotic ringspot virus* by immunocapture-reverse transcription-polymerase chain reaction. 1, Cucumber cotyledon; 2, peach leaf; 3, rose leaf; and 4, rose petal.

tember 1999. No statistical differences could be found for detection efficiency between cuttings and in vitro shoots in the fourth test conducted in May (data not shown). Results obtained for these two categories (cuttings and in vitro shoots) were grouped for further analyses.

All the Léonidas samples were highly positive in ELISA in all the tests (data not shown). In contrast, from 0.0 to 65.6% of the leaves from PNRSV-infected Anna plants were ELISA positive, depending on the particular test (Table 3). Comparatively, a higher and almost steady proportion (from 86.8 to 97.7%) (Table 3) of the cuttings and in vitro shoots from these plants was ELISA positive. A low proportion of the stems from PNRSV-infected Anna plants were ELISA negative when axillary leaves and cuttings and in vitro shoots were analyzed for tests 1, 2, 4, and 5 (Table 3). The axillary leaf and in vitro shoot samples from two (test 4) and one (test 5) of these stems were tested by IC-RT-PCR in parallel and were negative. The proportion of samples positive in ELISA tests relative to positive Anna stems varied from 0.0 to 73.3% for axillary leaves and 91.2 to 100% for cuttings and in vitro shoots (Table 3). We observed heterogeneity in virus concentrations even among samples from a single infected stem, as four samples (among 315 cuttings and in vitro shoots, i.e., 1.2%) originating from four different ELISA-positive stems were negative. The gain in detection efficiency for ELISA tests of cuttings and in vitro shoots versus leaves of mother plants varied from 21.2 to 97.6% between tests (Table 3).

A quantitative analysis (ANOVA) of absorbance values of samples that were PNRSV positive both in axillary leaves and in cuttings and in vitro shoots revealed significantly ($P < 0.001$) higher values than absorbencies of axillary leaves of the mother plants in all tests (Table 4). The difference in ELISA values was most significant in winter and summer, but less significant in spring (Table 4). Seasonal variation of the absorbance values was estimated by ANOVA. In the particular case of cuttings and in vitro shoots, the hypothesis of normality of residues was not respected for ANOVA. ANOVA is known to be robust in the absence of normality (10), but the result should be considered more carefully. The absorbance of axillary leaves was prone to seasonal variation, whereas absorbance of cuttings and in vitro shoots showed lower variations among the tests (Table 4). No statistical difference was observed between

TABLE 2. Detection of *Prunus necrotic ringspot virus* by enzyme-linked immunosorbent assay in various organs and four rose cultivars

Rose organs	<i>R</i> values ^y	Rose cultivars	<i>R</i> values ^y
Apical leaves	11.6 a ^z	Léonidas	17.8 a ^z
Basal leaves	10.7 a	Dallas	10.2 b
Bark	8.9 b	Laguna	9.8 b
Roots	8.3 b	Anna	4.4 c

^y Ratio between absorbance values of samples measured at 2 h and the mean absorbance value of at least five healthy controls.

^z Means within each column followed by the same letter are not significantly different based on a Newman-Keuls test ($P < 0.05$).

TABLE 3. Comparison of enzyme-linked immunosorbent assay (ELISA) detection of *Prunus necrotic ringspot virus* (PNRSV) in leaves of Anna rose plants and in cuttings or in vitro shoots derived from these plants

Test	Positive ^y leaf samples in PNRSV-infected plants (%)	Positive cuttings and in vitro shoots from PNRSV-infected plants (%)	Positive stems from PNRSV-infected plants/total no. of stems tested	Positive leaf samples on ELISA-positive stems (%)	Positive cuttings and in vitro shoots from ELISA-positive stems (%)	Difference ^z in detection efficiency between both methods (%)
1	33.3	87.5	7/8	38.1	100	54.2
2	50.0	87.5	6/7	57.1	100	37.5
3	53.3	97.7	8/8	53.3	97.7	44.4
4	65.6	86.8	19/21	73.3	99.2	21.2
5	22.7	93.0	7/8	23.8	97.7	70.3
6	0.0	97.6	7/7	0.0	97.6	97.6

^y Samples were considered positive when their absorbance was at least two times greater than the mean absorbance of five healthy controls.

^z (% positive cuttings and in vitro shoots from PNRSV-infected plants) – (% positive leaf samples in PNRSV-infected plants).

Fig. 2. Comparison of detection of *Prunus necrotic ringspot virus* (PNRSV) by enzyme-linked immunosorbent assay (ELISA). A, y-axis, Ratio between the absorbance value of the sample and that of healthy controls. Immuno-capture-reverse transcription-polymerase chain reaction (IC-RT-PCR) in B, 'GF305' peach and C, 'CE10' *Rosa indica* leaf extracts diluted in healthy plant extracts. The results presented are for one of three tests performed. IC-RT-PCR was about 100 times more sensitive than was ELISA. H = healthy control, M = molecular weight marker, figures correspond to the same dilution factor in ELISA and IC-RT-PCR ($-\log[\text{dilution}]$).

absorbance values of leaf samples taken from different positions along the stems or for absorbance values of cuttings and in vitro shoots (ANOVA, data not shown). It is difficult to precisely compare the increase in sensitivity in ELISA detection in cuttings and in vitro shoots versus that for leaves of the mother plants, since both tests cannot be conducted on the same sample. However, some leaves of the mother plants were ELISA negative (at a 10^{-1} dilution), whereas adjacent axillary buds developed from cuttings and in vitro shoots were ELISA positive up to a 10^{-5} to 10^{-6} dilution endpoint (range of 20 samples). This shows an increase in sensitivity of a 10^4 to 10^5 factor in some cases.

DISCUSSION

We developed and evaluated two different methods of improving the efficiency of detection of PNRSV in rose tissues. The first method, IC-RT-PCR, was dependent on adequate washing of the microtubes before reverse transcription, especially for rose leaf extracts. Inhibitors of reverse transcriptase or DNA polymerase present in these extracts may explain this (6). Preheating the tubes before reverse transcription and incubation at 46°C rather than 42°C for reverse transcription also increased the efficiency of IC-RT-PCR. We assume that the virus coat proteins become dissociated, access of reverse transcriptase to the viral RNA is improved, and secondary structures of RNA are modified when exposed to higher temperatures (27). IC-RT-PCR detected all 37 rose PNRSV isolates we tested and showed a 100-fold higher sensitivity than did ELISA for the same plant extracts. Usually, it is about 100 to 1,000 times more sensitive than ELISA for plant virus detection, with extreme values of a 2- and a 10^5 -fold increase of sensitivity (6). Sanchez-Navarro et al. (29) observed a 15,625-fold increase of sensitivity of RT-PCR in comparison to ELISA for PNRSV detection in nectarine leaves. However, they first extracted total nucleic acids of infected samples with phenol chloroform before performing RT-PCR, a method that is more difficult to apply for routine detection than is a single-tube IC-RT-PCR. Rosner et al. (27) developed a protocol for IC-RT-PCR detection of PNRSV in peach trees, but did not evaluate its specificity or sensitivity.

The second method we developed is based on choosing the appropriate tissue samples to obtain sufficient viral concentration for DAS-ELISA detection. We evaluated the detection method in cuttings and in vitro shoots for a particularly unfavorable situation: Anna rose plants infected with a low titer of PNRSV. Among the PNRSV isolates in our collection, approximately one third exhibit a low pathogenicity in rose, giving few or no symptoms, low virus concentration, and heterogeneous distribution in the plant (B. Moury and L. Cardin, unpublished data). We observed a much higher efficiency of ELISA for detection of PNRSV in actively growing tissues of these plants when compared with adjacent tissues (axillary leaves) of the mother plants. The advantage is both qualitative

TABLE 4. Comparison of enzyme-linked immunosorbent assay (ELISA) detection of *Prunus necrotic ringspot virus* (PNRSV)-positive samples of Anna rose plants in leaves of mother plants and in cuttings and in vitro shoots^w

Test	Leaves from mother plants	Cuttings and in vitro shoots
1	4.7 ^x c ^y	18.0 c ^y
2	7.8 b	20.6 bc
3	3.8 c	25.0 ab
4	18.9 a	27.3a
5	3.7 c	18.5 c
6	... ^z	18.9 c

^w There was a significant effect ($P < 0.001$) of organ and test rank for all trials conducted.
^x Ratio between absorbance values of samples measured at 2 h and the mean absorbance value of at least five healthy controls.
^y Means within each column followed by the same letter are not significantly different based on a Newman-Keuls test ($P < 0.05$) conducted on the test rank effect.
^z All samples were ELISA negative.

(the frequency of positive samples is much higher) (Table 3) and quantitative (absorbance values of positive samples are greater) (Table 4), ensuring a more reliable detection and a better discrimination between infected and healthy plants. Testing of cuttings and in vitro shoots can be performed on whole plants or on commercial material (floral stems and rootstock stems for propagation) for sanitary control of the virus. It is compatible with large-scale testing and allows a high number of repeats per plant. It is also relatively independent of the season and can be applied year long for greenhouse-grown plants (Table 3; additional data not shown). In comparison, PNRSV-infected Anna plants grown in our greenhouse showed large fluctuations of virus titer. PNRSV concentration was particularly low in the summer with all 42 leaf samples from the mother plants (from seven different stems) being ELISA negative in the sixth test (Table 3). In the spring, the virus titer is much higher and 66% of the leaf samples from the mother plants were ELISA positive during the fourth test (Table 3). Winter data showed intermediate frequencies of positive samples (Table 3, tests 1 and 2).

The in vitro shoot test has several additional advantages compared with cuttings: (i) the test is more rapid; (ii) development of shoots is more homogeneous; and (iii) control of climatic and phytosanitary conditions is also easier for this test. There is a strong gradient of dormancy among axillary buds increasing from the top to the base of the floral stem (19). As a consequence, we observed a very heterogeneous shoot development in cuttings and we were obliged to perform two or three ELISA tests as these shoots developed (Table 1). No such effect was observed in vitro. Moreover, this method is efficient for cultivars known to be difficult to propagate by cuttings, e.g., *R. hybrida* cv. Ruidriko (=Vivaldi) (18) (data not shown).

A total of 89% (59 out of 66) of Anna stems was entirely PNRSV negative when axillary shoots from these stems developed after the

Fig. 3. Influence of the number and type of samples (leaves or cuttings and in vitro shoots from *Prunus necrotic ringspot virus* (PNRSV)-infected Anna rose plants) on the detection efficiency of enzyme-linked immunosorbent assay. For example, if a 99% probability of detecting PNRSV in an infected plant is desired, at least 5 to 18 leaves from the mother plants should be tested, depending on the season, or two cuttings and in vitro shoots from these plants (mean of the six tests).

decapitation of the floral stem, after the development of cuttings, or grown *in vitro* were tested by ELISA. ELISA tests of leaves from these stems were all negative. Since IC-RT-PCR conducted in parallel on all the explants from three (out of seven) of these stems also were negative, we suspect that these stems were virus free, even if they belonged to infected plants, or that the viral titer was very low in the stems.

We assessed the implications of our results on the design of a sampling strategy that should be used for diagnosis of rose plants, based on the results obtained with Anna. Depending on the relative proportion of positive samples in these PNRSV-infected plants, the minimal number of samples to ensure a given probability of detection can be estimated. Figure 3 shows the number of samples necessary for a 99% detection probability in three sampling situations: leaves of floral stems in a favorable (May, test 4) or unfavorable (July, test 5) season and a test of cuttings and *in vitro* shoots. Test of cuttings and *in vitro* shoots can significantly reduce the number of samples to be analyzed in comparison to the test of leaves of the mother plants for the same 99% detection efficiency. Whereas two to three samples are sufficient for cuttings and *in vitro* shoots (tests 1 to 6), 5 to 18 leaf samples from the mother plants are necessary in a favorable (test 4) or unfavorable (test 5) season, respectively. No detection at all could be ensured with leaf samples from the mother plants during test 6.

Modifications of environmental conditions have been reported to increase virus or viroid concentration and improve detection. In particular, growing plants at a higher temperature, higher light intensity, or both has often proven to be effective (11,14). However, our work is, to our knowledge, the first report of the use of cuttings or *in vitro* culture to enhance the efficiency of detection of a plant virus. *Iilarvirus* detection during *in vitro* culture has been reported in sour cherry for PDV (2), red raspberry for ApMV (3), and peach for PNRSV (15), but no estimation of its efficiency or comparison to detection on the mother plants has been done.

Cytokinins and virus concentrations are often negatively correlated (8,17,21,33). In addition, the level of virus infection diminishes markedly as the *in vitro* tissues proliferate (13). Our results may be linked to the property of PNRSV (and other ilarviruses) to accumulate in actively multiplying and growing plant tissues (meristem cultures or callus tissues) (5,24; B. Moury, L. Cardin, J.-P. Onesto, and A. Poupet, *unpublished data*); indeed, although there is no direct evidence for the presence of PNRSV in meristem tissues, elimination of PNRSV by meristem or shoot tip culture often failed when it was not associated with chemotherapy (5,24; B. Moury, L. Cardin, J.-P. Onesto, and A. Poupet, *unpublished data*). Use of *in vitro* tests to detect PNRSV and other viruses in various tree species should help to clarify the links between virus accumulation in these conditions and the taxonomic position of the virus.

Rose propagators who would like to control genotypes before multiplying them vegetatively could preferably use ELISA tests conducted on shoot tips grown *in vitro*. Some propagators already use *in vitro* culture, and ELISA tests are relatively easy to manage and more reproducible from one laboratory to another than is IC-RT-PCR. A laboratory that would be in charge of sanitary controls could rather use a combination of both methods: IC-RT-PCR detection in *in vitro* shoots to ensure a maximal sensitivity of detection.

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