Statistically reliable sampling and diagnosis of plant pathogens using a simplified "universal" protocol for sample preparation and analysis by (RT-)PCR

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Summary

Thanks to the higher sensitivity of molecular tests based on PCR techniques, researches have been conducted to show that processing of pooled samples is realistic and can bring a positive solution to sanitary control procedures by increasing the statistical reliability of the obtained results. Rate of pooling depends on sample type, batch size, required sensitivity, and expected and accepted level of infection. But the use of pooled samples always allows to test an increased number of samples without dramatically increasing the cost.

In parallel a new simplified "universal" protocol has been developed and patented for sampling and sample preparation. This protocol allows field sampling either on woody or herbaceous plant tissues, easy conservation and transport to laboratory where a few seconds are required for sample preparation before its inclusion in PCR mix. The same protocol can be applied on different plant species for detection of different pathogens (virus, viroïds, phytoplasms, bacteria, fungi,...) and is especially dedicated to laboratories that do not want to invest in an expensive routine sample preparation device.

Introduction

Until now, validation of official methods of analysis for phytosanitary certification has been exclusively based on the technique itself (sensitivity, specificity, reproducibility,...). The definition of sampling protocols was most generally not integrated in the validation step due to incompatibility between statistical requirements and workability. Treatment of large number of samples arises different problems such as increasing of the cost of analysis and complicating handling, storage and elimination of the processed samples.

This paper presents the approach followed by the Plant Pathology Unit of the Agricultural University of Gembloux during the last decade for the development of "user friendly" diagnostic techniques based on PCR. These works have been conducted on different plant materials and pathogens (mainly Latent and ILAR viruses on *Prunus* and *Malus* fruit trees, Potato virus Y, X and S and *Ralstonia solanacearum* on potato). Simplification of the PCR technique is essential and is a major aim of the laboratory while keeping the performances of the method such as sensitivity and specificity..

Rapid overview of statistical requirements for sampling

Whatever the diagnostic technique may be, statistical validation of the procedure of analysis is a test of hypothesis where two different risks of error are assessed: (i) a false positive risk (producer risk) if a non infected sample is declared infected after analysis and (ii) a false negative risk (consumer risk) if an infected sample is not detected. Numerous factors influence those risks among which sensitivity, specificity, reproducibility, accuracy, ...that can be considered as intrinsic properties of the technique of analysis. This paper will focus on the most important external factor influencing the consumer risk: the sampling.

Numbers of phytosanitary crisis have arisen because one or several false negative results have occurred at one step of the certification procedure.

The consumer risk could be considered as the most important in certification because, if it occurs, it can rapidly conduct to a crisis in which the producer himself supports the negative effects. Moreover, when positive results arise, confirmation analysis is generally performed for confirming or infirming false positive hypothesis. Whereas, no confirmation of negative results is performed as it is considered as the normal case.

A statistical approach is essential in phytosanitary certification scheme where low to very low infection rates are generally met in nuclear stocks and nurseries. Detection of one infected individual among thousands of non infected ones requires to pick up this individual during sampling otherwise a false negative result will be produced.

To secure consumer risk it is necessary to determine the minimum number of samples to be taken in function of the pathogen/host pair considered.

Works have previously been conducted (Chandelier *et al.*, 2001) that analyze consumer risk for distribution of PVY infection in potato tubers in the field. This risk has been related to binomial distribution. Table 1, representing the binomial distribution of consumer risk, gives the number of tubers that should be sampled and analyzed to detect (with various rates of confidence) an infected lot for various infection levels. Recent studies performed on apple trees infected by ASPV and ACLSV (Kummert *et al.*, 2003) show that, especially in case of heterogeneous infection by ACLSV, it can be necessary to test until 8 leaves for each tree to detect the virus with a confidence of 99.9 %. In this respect, values in Table 1 should be multiply by 8 for reliable monitoring of fruit tree multiplication material. Those results consider that each sample is analyzed individually.

Pooled sampling can therefore bring a solution by analyzing several individuals together as a unique sample. The consumer risk is similar when individual or pooled sampling are presented for analysis whatever the rate of pooling applied. But pooled sampling afford to test larger number of individuals keeping constant the number of analysis and therefore reducing the unitary cost of a diagnostic test.

Where molecular tests can bring answer to statistical requirements…

Sample pooling

Testing of pooled samples requires to use techniques with high sensitivity to detect one infected plant among 10, 20 or even 100 non infected ones when analyzed together. RT-PCR tests developed at Plant Pathology Unit of the Agricultural University of Gembloux on crude extract of different plant material (potato tubers and leaves, fruit tree twigs and leaves) have shown the possibility to reach high pooling rates. Figure 1 shows the results obtained by pooling 20, 40 and 80 twigs of apple tree together for the detection of ASPV where the specific band is clearly observable on the agarose gel. Worst case is presented where only 1 infected twig is mixed with respectively 19, 39 and 79 non infected ones. Pooling of 150 twigs (1 infected + 149 non infected) have also been successfully conducted to detect PNRSV in *Prunus* (data not shown).

Multiplex analysis

Pooling of samples brings undeniable advantages to reduce the number of analysis for detecting one pathogen in plant material. When two or more pathogens have to be tested in the same sample, multiplex analysis can also offer a good solution to reduce the number of tests to be performed. Therefore single RT-PCR techniques have been improved to allow Duplex analysis for fruit tree viruses (ASPv-ASGv; ACLSv-ApMv, PDv-PNRSv). The Duplex PDV-PNRSV (Figure 2) was equally sensitive as the single test reaching dilution 10^6 for both single (data not shown) and duplex runs. It is therefore reasonable to think that a combination of pooling and duplex can be useful to be integrated in a certification procedure by increasing number of samples to be tested without increasing the cost.

However, in this approach the only cost reduction considered is directly related to the analysis itself once samples are ready to be included in the analytical run. Costs related to sample preparation are not considered at this step but are still an important bottleneck for large number sample preparations.

Simplification of sample preparation

Beside the cost of PCR reagents, sample preparation is one of the major bottleneck for applicability of PCR technique in routine analysis at a competitive price especially when nucleic acid extraction and purification is necessary. In those cases, sample preparation can be time and workforce consuming. For a couple of years, different protocols have been proposed to work with crude plant extracts to simplify sample preparation. However, inhibitory effects on PCR appeared, caused by plant components or by oxidization of plant extracts.

These problems are particularly met when processing of woody plant tissues and occur with different type of grinding device.

Therefore a specific protocol was initially set up for sampling and preparation of woody tissues for the detection of ILAR and Latent fruit tree viruses. Figure 3 shows results for comparison of detection sensitivity of ASPV following two different protocols for crude extract preparation from apple twigs. The classical protocol follows *Kummert et al.*, 2001 with Homex grinder and the second one is the new patented protocol presented in Figure 4. Similar maximum dilution was reached for both protocols.

This new protocol has rapidly shown its "universal" application whatever the type of plant material (fruit trees, banana trees, Potatoes, Tomatoes, Sweet Potato, Tobacco, Catharantus, ...) or tissue (leaves, stems, woody tissues, tubers) or pathogen (viruses, phytoplasm, viroïd, fungi, bacteria).

This original simplified protocol (Figure 4) is based on a sampling device plus the "universal" KAJI extraction buffer especially developed for that protocol.

If necessary, the sampling device can easily be used in field. Only few milligrams of infected plant tissues are sampled and can be stored during at least 3 weeks at room temperature. Thanks to this device, sampled tissues can easily be sent by standard courier to the laboratory for analysis. Before inclusion in (RT-)PCR Mix, sampled tissues are placed in a 10 ml tube with 1,5 ml of the extraction KAJI buffer, vigorously agitated during 30 seconds. Clarified crude extract can easily be directly pipetted out of the tube and diluted in distilled water according specifications for plant and tissue type. This diluted extract is ready to be included in the (RT-)PCR mix. A wide range of various pathogens can be tested from the same extract.

If necessary (e.a. for research purposes) a Total Nucleic Acid extraction and purification protocol has been adapted for application after crude extract has been prepared.

The advantages of this new protocol are:

- its **universality** (different tissues, different plants, different pathogens) which greatly simplifies lab management especially within quality control procedure.
- its **easiness** of use (particularly for difficult samples as woody tissues), which reduces time consuming steps, limits manipulation mistakes, facilitates transport and storage of the samples and does not requires any specific grinding devices.
- Its **high trough put** capacity. Hundreds of samples could be processed in the same time without expensive automation. As tissue sampling can directly be performed on the field, lab is not overcrowded with plant material. Moreover, this protocol is fully compatible with Pooled sampling and Duplex analysis.
- Its **reliability**

This universal protocol has been recently patented and is on the way for official evaluation and commercialization.

Conclusions

Thanks to the development of a "universal" simplified protocol for sample preparation, processing of numerous plant samples of any type is possible. This protocol allied to the power of (RT-)PCR techniques for treatment of pooled samples and multiplex analysis offers new perspectives for phytosanitary certification: statistically reliable sampling becomes realistic and economically workable.

	Pourcentage of infection (p)						
Sample size (n)	1,00%	0,50%	0,20%	0,10%	0,05%	0,02%	0,01%
200	0,86466	0,63212	0,32968	0,18127	0,09516	0,03921	0,0198
300	0,95021	0,77687	0,45119	0,25918	0,13929	0,05824	0,02955
400	0,98168	0,86466	0,55067	0,32968	0,18127	0,07668	0,03921
500	0,99326	0,91792	0,63212	0,39347	0,2212	0,09516	0,04877
1000	0,99995	0,99326	0,86466	0,63212	0,39347	0,18127	0,09516
2000	1	0,99995	0,98168	0,86466	0,63212	0,32968	0,18127
3000	1	1	0,99752	0,95021	0,77687	0,45119	0,25918
5000	1	1	0,99995	0,99326	0,91792	0,63212	0,39347

Table 1: Probability of detection of an infection in an infinite population for various levels of infection and sample sizes.

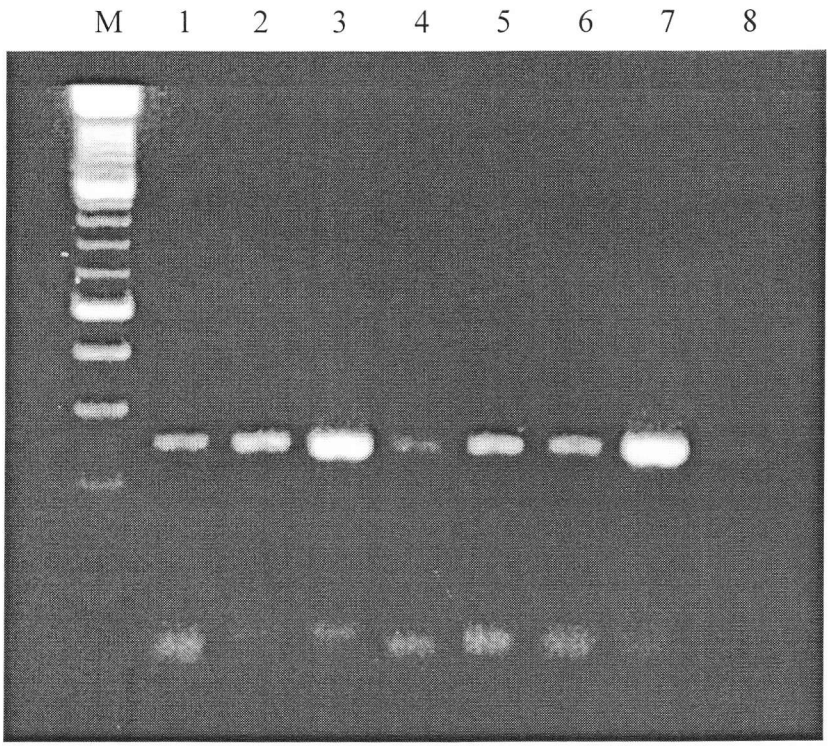

Figure 1: RT-PCR Detection of ASPV in crude extract prepared from apple twigs following the "universal" patented protocol. Lane 1, Lane 2 and Lane3: dilutions of 1 infected twig respectively in 79, 39 and 19 healthy ones before extraction. Lane 4, Lane 5 and Lane 6.: Dilutions of 1 part of infected crude extract respectively in 79, 39 and 19 parts of healthy one. Lane 7: Infected crude extract non diluted. Lane 8: Negative control.
Crude extracts were

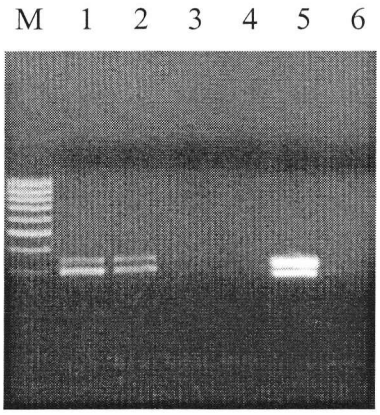

Figure 2: Detection of PDV-PNRSV in crude extract from Prunus twigs and revelation on 2% agarose gel after duplex RT-PCR.Lane 1, Lane 2, Lane 3, Lane 4: dilutions of respectively 10^5, 10^6, 10^7 and 10^8 times of crude extract in sterile water. Lane 5: dilution 10^2 as positive control. Lane 6: negative control.
Crude extracts were prepared foolowing the "universal" patented protocol.

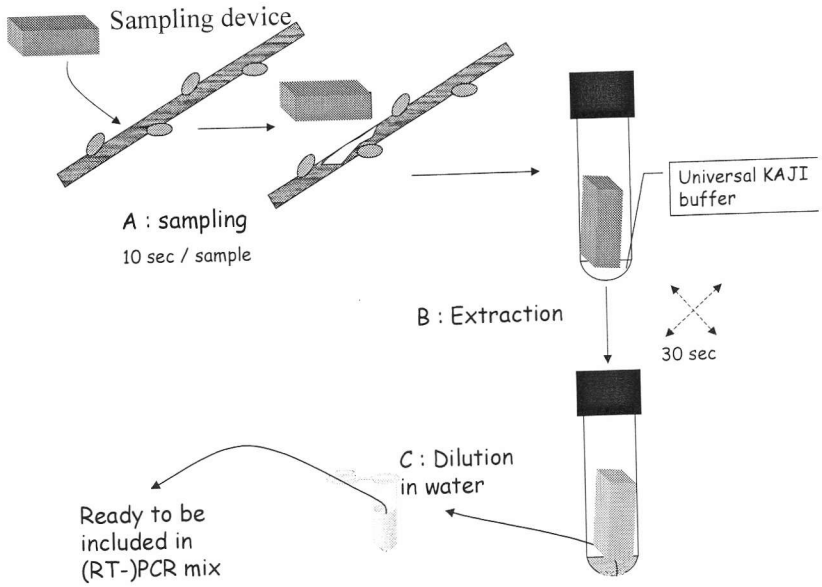

Figure 4: Description of the sampling and sample preparation procedure with the patented "universal" protocol.
Sampling device is represented as a blind box for confidential reasons.

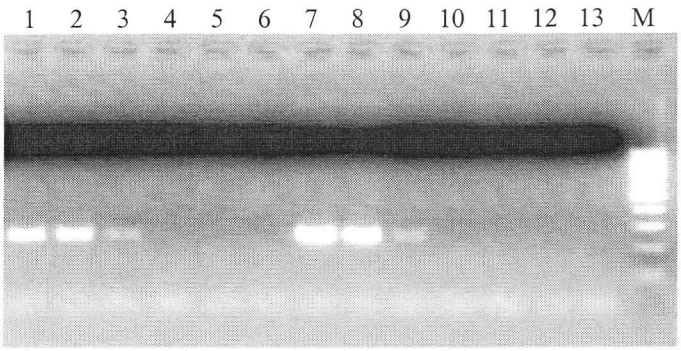

Figure 3: Detection of ASPV in 2 crude extracts (A and B) from apple twigs and revelation on 2% agarose gel after RT-PCR. Lane 1 to Lane 6: Crude extract A prepared with classical procedure : 200 mg of tissues in 2 ml buffer crushed in nylon mesh bag with Homex grinder and diluted in sterile water 10^2, 10^3, 10^4, 10^5, $10^{6.}$ and 10^7 times respectively. Lane 7 to Lane 12: Crude extract B prepared with new universal protocol: 20 mg of tissue in 1.5 ml KAJI buffer, agitated 30 sec. and diluted in sterile water 10^2, 10^3, 10^4, 10^5 $10^{6.}$ and 10^7 times respectively. Lane 13: negative control.