Validation of Plant Virus Detection

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Abstract
Validation of test methods is required for laboratories seeking ISO 17025 accreditation. Recently developed manuals help choosing relevant performance characteristics to be studied for qualitative tests common in plant virus detection. For routine testing in certification schemes additional information on the plant material is important to determine the scope of the accredited application. For application of virus tests for diagnostic purposes the determination of certain performance characteristics is highly recommended. Furthermore, in case of detection of quarantine organisms a confirmation by an alternative test is indispensable.

INTRODUCTION

Testing for plant viruses includes making decisions. Stocks are classified, candidate plants are selected and ship loads of plants may be destroyed based on test results. Tests are performed to the best of our knowledge but this is not always enough. Using test kits and protocols from established suppliers does not release us from the responsibility to check the quality of the assays.

Validation is the key word for many laboratories seeking accreditation or extending the number of tests under the present accreditation. However, also for laboratories without accreditation validation becomes common practice. Validation means proving that the method is fit for the purpose we have in mind. Plant Research International (PRI), assisted by a panel of Dutch plant pathologists, published a manual for validation of plant pathogen tests (Van der Vlugt et al., 2006; see also ISO/IEC 17025). This manual describes performance characteristics that are important for qualitative assays and includes examples on how to obtain data. However, once aspects like detection limit, trueness, selectivity, specificity, repeatability, reproducibility, robustness etc. are determined, we realize that a report describing these facts is not complete without technical information. Plants are a difficult matrix for virus detection and occasionally evoke peculiar interactions with plant pathogens or components of the diagnostic assay. The successful detection of viruses in plants depends on season, distribution in plants, storage conditions and geographical distribution with possible false negatives. The plant material itself may cause false positives. Antibodies may fail to detect all serotypes and primers may not cover the whole range of strains. Information on these processes and characteristics is needed before we can define the test protocol, the quality of the assay, and its boundaries, in terms of season, type of plant material to be sampled etc.

VALIDATION OF TEST METHODS

Validation is defined as the process of checking if a test method is suited for its intended purpose. Before starting a process of validation it is necessary to determine:
- The purpose of the test, test method and matrix.
- Whether the test is quantitative or qualitative.
- Which performance characteristics need to be determined.

Performance Characteristics

As plant virus tests are generally qualitative tests, the following performance characteristics are relevant. Below is the general interpretation for ELISA:

- Trueness: performance of the test compared with an "ideal" test. In practise this will be a second test e.g. indicator plants or PCR.
- Detection limit: threshold value.
- Repeatability: correspondence between repetitive tests under the same circumstances.
- Reproducibility: correspondence between repetitive tests under different circumstances.
- Specificity: how does the test react with other viruses within the same crop and closely related viruses.
- Selectivity: how does the test distinguish between the target and the matrix.
- Measuring range: limits of detection on both ends of the scale.
- Robustness: insensitivity of the test to deviations of test circumstances.

When the validation of a method is already reported before in literature, only the non-determined characteristics should be validated. However, in plant virology usually all eight performance characteristics are included in validation reports because of its large variety of virus/crops combinations. The validation report should give details about the reliability of the method and its application in a specific laboratory. In practice, test laboratories often experience difficulties with many virus/plant combinations. This may result in narrowing the application to sampling certain parts of plants in a particular period or after a specific treatment. As a consequence, the respective reliability of the test method should be accepted as it is; as an alternative, it can be replaced by another method.

VALIDATION WITH RESPECT TO PLANT VIRUS DETECTION

The authors experienced during their research or in their daily practice with certification schemes phenomena that may be grouped in the following categories:

- Non-homogenous distribution of viruses in plants.
- False positives caused by interference of plant extracts in ELISA.
- False negatives caused by interference of plant extract in ELISA.
- False negatives related to the period of sampling.
- Inability to detect certain serotypes in ELISA or cross reactivity with other viruses.

The scope of the test method may be restricted by experimental data describing some of these aspects. It is useful to include these data in validation reports. To illustrate this we present two case studies.

Case Study 1: Virus Detection in Lily by ELISA

Lily is a bulb crop that is propagated by vegetative reproduction using the scales. In the Netherlands large numbers of samples from lily stocks are tested under a classification scheme. Three main groups are distinguished: Oriental Hybrids, Asiatic Hybrids and Longiflorums. A large amount of experimental data on immunological assays (ELISA) for *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV), *Cucumber mosaic virus* (CMV) and *Arabis mosaic virus* (ArMV) is available:

- Leaf tests on LSV and LMoV perform well on all varieties from the period of flowering till shortly before harvest and leaves are preferably picked at two-third of the heights of the plants. The rate of detection of primary infections beside secondary infections increases with time. ArMV leaf test performs poorly and CMV may only be detected before flowering, not after.
- In the case of testing Oriental Hybrids, bulb tests on LSV perform very well on freshly lifted bulbs while LMoV is detected very poorly. When Asiatic hybrids are tested, both LSV and LMoV tests perform very well under one condition: the bulbs must have been stored for a minimum of three weeks after lifting (Fig. 1). Bulb tests on Longiflorum types perform very poorly compared to the excellent leaf tests. However, for infected scales light treatment for two weeks helps to increase absorbance values in ELISA.

The same treatment does not have any positive effect on the outcome of bulb tests for other lily types.

- Storing of Oriental Hybrid bulbs for three weeks after lifting has a very positive effect on the detection of ArMV.
- High background reactions in ELISA as well as false positives may be caused by microbial growth on scales of lilies. Using micro pore plastic bags for samples and ventilated storage will effectively prevent this.

Above information and the results of the determination of performance characteristics was input for adjusting the scope for a range of virus tests on lily under ISO 17025 accreditation as follows:

- Leaf tests on LSV and LMoV after flowering, all varieties.
- Bulb tests on LSV:
  o all varieties except the group of Longiflorum types,
  o with specification on the storage period after harvest of Asiatic Hybrids.
- Bulb tests on LMoV, Asiatic hybrids only, with specification on the storage period.
- Bulb tests on ArMV, with specification on the storage period.

Other tests on lilies mentioned above are not performed under ISO 17025 due to poor performance characteristics. However, these tests may still be useful in diagnosis.

Case Study 2: Detection of *Prunus necrotic ringspot virus* (PNRSV) in Rose

PNRSV is the main virus responsible for the "rose mosaic" symptom. Screening for PNRSV in rose root stocks is desirable to prevent virus spread by grafting. Considering costs, screening is preferably done using ELISA. However, virus levels may differ through the seasons leading to false negatives, whereas specific plant components may cause false positives. Different test methods were compared to reveal possible drawbacks of ELISA. In this case, healthy root stocks were graft-inoculated with two strains of PNRSV varying in severity. Tests were carried out throughout the season, young full-developed leaves as well as older leaves from the same stem were tested with DAS-ELISA. Results showed that high extinction values were obtained for non-infected plants during summer, although reactions were higher for root stock 'Natal Briar' than for 'Burr' (Fig. 2). Furthermore, leaf age and the season of testing influenced the extinction values of healthy plants in DAS-ELISA. In addition, the various accessions of rose infected with PNRSV were tested with DAS-ELISA, Ilarvirus RT-PCR (Candresse et al., 1998), PNRSV RT-PCR (Marbot et al., 2003) and by chip-budding of small chips from the infected plants on *Prunus serulata* 'Shirofugen' and *Rosa multiflora* 'Burr' as indicators. RT-PCR and chip-budding showed the highest detection levels (Table 1). Since too many factors influenced the outcome of ELISA, RT-PCR is recommended and used for screening now. Testing of candidate plants is still carried out using above indicators.

Other Aspects Influencing the Scope of Virus Tests

Table 2 shows user experiences of plant-virus tests. Some of these will limit the scope of validated tests or even prevent proper validation of a test in such a way that the results for reproducibility, selectivity, trueness etc. are not acceptable. As a consequence, an alternative test may be required.

DISCUSSION

Validation requires great efforts and as a consequence, most validation reports are related to large scale virus testing. For these applications often additional information about the matrix is available like complicating factors such as false positives and/or negatives. In case of virus diagnosis on lesser known crops, knowledge of performance characteristics is often limited to information from the supplier of the antibodies. Although the information on specificity for commercial kits is useful in most cases, given cut-off values for ELISA only relate to certain crops and need to be determined experimentally for other crops. Trueness may be studied comparing ELISA with PCR or

indicator-plant results or sequencing PCR products. This even is a necessity in case of detection of quarantine organisms or other situations were stakes are high.

Accreditation bodies often require validation as is the case for ISO 17025. Validation reports often reveal limitations of the test method, not only in the scope but also in their performance characteristics. 100% reliability is not always realistic in plant-virus testing especially, when tests should also be practical and affordable.

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Tables

Table 1. Test results of collection rose with symptoms (1992-2004). Samples were tested with indicator plants *Rosa multiflora* 'Burr', *Prunus serulatus* 'Shirofugen', DAS-ELISA and RT-PCR. Not all roses accessions are infected with PNRSV (ApMV is excluded).

Method	Year	Number accessions tested	PNRSV detected	% detection
ELISA	2004	28	22	78.6
	2005	28	20	71.4
RT-PCR Ilarvirus	2004	28	26	92.9
	2005	27	21	77.8
RT-PCR PNRSV	2004	28	27	96.4
	2005	28	28	100.0
Indicator 'Burr'	2005	26	25	96.2
Indicator 'Shirofugen'	2005	27	27	100.0

Table 2. Examples of matrix-virus combinations, which may lead to false positives or false negatives during testing.

| Crop	Virus	Material	Test method	Remarks
Bouvardia	CMV	Leaf/flower	ELISA	Testing leaves may lead to false negatives, better test flowers
Dahlia	TSWV	Tuber	ELISA	Unequal distribution may lead to false negatives
General	TRV	Leaf	ELISA	False negatives due to unequal distribution, diversity in serotypes, isolates lacking protein coat, low virus levels. It's better to use (real-time) RT-PCR
Aromatic herbs e.g. *Lavandula*	Virus general	Leaf	ELISA	False positives, buffer adjustments needed
Nemesia	Poty	Leaf	ELISA	Unequal distribution may lead to false negatives
Osteospermum	CMV, TMV and potyvirus	Leaf	ELISA	False positives with material from (sub)-tropical origin but not with material from the same varieties from the Netherlands. Buffer adjustments needed
Tulipa	TBV	Leaf	ELISA	Poor detection before flowering resulting in false negatives
Verbena	Virus general	Leaf	ELISA	False positives, buffer adjustments needed
Zantedeschia	Virus general	Tuber	ELISA	False positives
Zantedeschia	Virus general	Leaf	ELISA	Unequal distribution may lead to false negatives

Figures
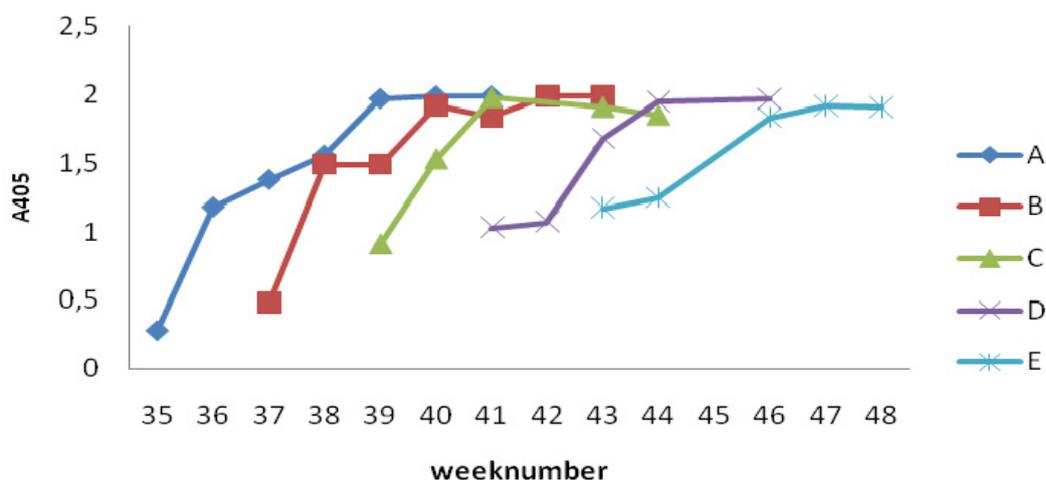
Fig. 1. ELISA-detection of LMoV in lily bulbs; average ELISA absorbances from 100 infected bulbs improve with later lifting dates and during storage. For optimal test results bulb samples are stored from 3 to 6 weeks after lifting. A-E different lifting dates.
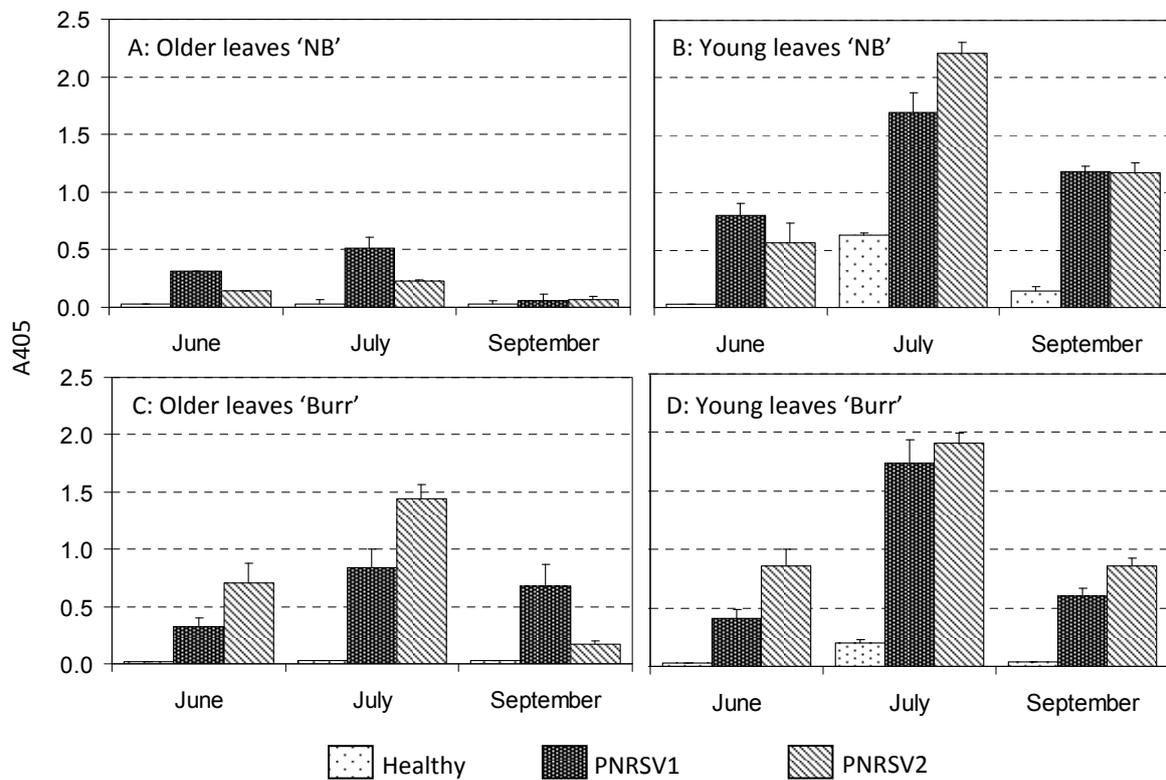
Fig. 2. Absorbance values of ELISA after substrate incubation of 1 hrs (A405), leaves were sampled in June, July and September. Plants were graft-infected with two different isolates of PNRSV (PNRSV1 and PNRSV2) or not (healthy plants); older and young leaves were taken from the same shoots, older leaves were basal leaves, young leaves were fully expanded leaves on the top of the shoot. A/B: leaves of 'Natal Briar'; C/D leaves of 'Burr'. Error bars: standard deviation (n=10).