Method for the Detection of Pospiviroids on Tomato Seed

Crop	Tomato (*Solanum lycopersicum*)
Pathogens	Pospiviroidae: Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), Mexican papita viroid (MPVd), Pepper chat fruit viroid (PCFVd), Potato spindle tuber viroid (PSTVd), Tomato apical stunt viroid (TASVd), Tomato chlorotic dwarf viroid (TCDVd), Tomato planta macho viroid (TPMVd)

ISHI-Veg recommends using the Naktuinbouw protocol

Sample and sub-sample sizes

The Naktuinbouw protocol uses 3,000 or 20,000 seeds in the sample. A sample size of 3,000 seeds with a maximum sub-sample size of 1,000 seeds is adequate for detecting pospiviroidae in tomato seed.

NOTE: An important factor that determines the sample size of the seed to be tested is the epidemiology of the disease, i.e.

1. the rate of disease transmission from infected seed to seedling and
2. the spread of the disease in a seed production unit

1. Rate of disease transmission

Several studies have experimentally demonstrated variable rates of transmission of pospiviroids from infected seed to seedlings (Kryczynski et al., 1988 for PSTVd, Singh and Dilworth, 2009 for TCDVd, Antignus et al., 2007 for TASVd). However, there are also studies in which no seed transmission was observed (Singh et al., 1999 and Matsushita et al., 2011 for TCDVd). In all these studies the infected seeds were obtained by artificially infecting mother plants.

In the few studies using naturally infected seed lots, transmission rates are very low; 0,08 % for TCDVd based on 2500 seeds (Candresse et al., 2010) and 0,27% for PSTVd based on 370 seeds (Brunschot et al., 2014). Koenraadt et al. (2009) found no transmission of PSTVd (based on 100 seeds) and TCDVd (based on 4000 seeds). Verhoeven (pers. comm.) also reported no transmission in a grow-out study using a seed lot that tested positive for PSTVd when using a PCR method. In addition, he observed no infection in healthy seedlings that were artificially infected using an extract of infected seed.

A strong argument supporting low transmission rates is the fact that only very few outbreaks of pospiviroids in tomato production in greenhouses have been traced back to infected seed (Candresse et al., 2010, Brunschot et al., 2014). The majority of outbreaks studied could not be traced back to seed (Verhoeven et al., 2007 for PSTVd) but to ornamental solanaceous species (Navarro et al., 2009 for PSTVd and Verhoeven et al., 2010 for pospiviroids).

Yet another argument in favour of low transmission rates is that a very small percentage of commercial seed lots tested have been found to be positive for pospiviroids, from 2 in 2000 seed lots tested by Naktuinbouw in the Netherlands to a few percent of those tested by CSP Labs in USA. For reasons that are not understood a much higher percentage of positive seed lots - up to 40% - have been reported when tested in Australia.

2. Spread of the disease in a seed production unit

Instead of only working with epidemiological thresholds when dealing with quarantine pathogens, it is useful to test seed in a production unit for them. There are statistical tools that determine sample size and calculate the probability of detecting a low number of infected seeds in a seed lot (Gu and Novak, 2004; GSPP, http://www.GSPP.eu).

In experiments performed at Naktuinbouw in 2013, fruits from plants showing symptoms of PSTVd-infection were studied. The study showed that every fruit from a truss of the infected plant was infected and also that every seed from an infected fruit was infected. Thus, it is likely that every seed of an infected plant is infected with PSTVd.

For a seed lot originating from *n* plants in the production unit, to find at least one seed of every plant in a sample with 95% confidence level, the amount of seeds to be tested is 3*n* (where *n* is the number of plants used to produce the lot). A tomato plant typically produces around 3,300 seeds. A tomato seed lot of 10 kg consists of ca. 3,300,000 seeds, originating from ca 1,000 plants. Assuming a single plant in 1,000 was infected and all seeds of this plant are infected, there is a 95% probability that a representative sample of 3,000 seeds will contain an infected seed.

In practice at least three factors strongly increase the chances for detecting the pathogen in a sample:

i. Infection will not be limited to a single plant in a crop cycle.

ii. Seeds act as a nutritional sink in a plant and pathogens tend to disperse to these sinks.

iii. A viroid is readily transmitted to seeds from non-infected plants during seed extraction processes being used in the seed industry (experiments performed by Naktuinbouw in 2013).

Considering these factors a sample size of 3 times the number of plants used to produce the seeds will generally provide a confidence level higher than 95% for determining whether this seed crop was infected with a pospiviroid.

Principle

In the Naktuinbouw protocol RNA from seed extract of tomato is isolated and purified with a KingFisher™ kit. The putative presence of viroid RNA is demonstrated by means of Reverse Transcriptase TaqMan PCR using selective sets of primers and labeled probes. Each subsample is spiked with Dahlia Latent Viroid as an internal amplification control (IAC) to monitor the performance of RNA extraction and RT Taqman PCR.

Note: Other RNA-extraction methods can also be used. The user must, however, be able to show that the performance criterion of detecting one infected seed in 1000 seeds can be met.

Restrictions on use

- This test method is suitable for untreated seed.
- This test method is suitable for seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for such inhibition by analysis, sample spiking, or experimental comparisons.
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a user chooses to test treated seed using this method, it is his/her responsibility to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

Notes

- This protocol has been validated for the detection of PSTVd and TCDVd. In 2014, the protocol has been validated for all viroids in the EU TESTA-project (see https://secure.fera.defra.gov.uk/testa).
- More information about the validation reports can be obtained at Naktuinbouw (m.ebskamp@naktuinbouw.nl).
- Due to the low number of positive findings and the fact that pospiviroids are quarantine pests in many countries ISHI-Veg has not been able to validate the test.
- The pospiviroids MPVd and TPMVd have not been found on tomato seed.
- It should be noted that this is an "indirect" test. The seed industry has adopted a position paper about indirect tests (http://www.worldseed.org/isf/seed_health_testing.html). With an indirect test, i.e. Immuno-Fluorescence, DAS-ELISA or PCR the presence of viable pathogens is not demonstrated. Viability and pathogenicity should normally be confirmed in a second, direct test by inoculation of assay plants with the isolate. At this moment, no confirmatory method is available. Confirmation of the presence and identity of viroids can be obtained by sequencing the amplicons obtained after the Taqman PCR. This does not give information about viability or pathogenicity.

Link to Naktuinbouw's protocol: http://www.naktuinbouw.com/protocols-pospiviroids

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