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The ELISA analysis results in tomato (*Lycopersicon esculentum* Mill.) seed health testing for *Tobacco mosaic virus*

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Abstract

Seed health testing is an important component in disease management for quarantine and phytosanitary certification. The time of seed sampling is one of the most important factors to obtaining accurate seed health test results. Most seed health tests utilize qualitative data based on the presence or absence of the pathogen in the test sample, with the lot being rejected if the pathogen is detected in the sample and accepted if the sample is negative. Because of the low frequencies with which many important seed-borne pathogens occur in seed and the low to very low seed contamination/infection thresholds for economic loss or the initiation of an epidemic, sampling for seed health testing may appear to present special problems. Four sets of experiments were done to determine whether *Tobacco mosaic virus* (TMV) transmits through contaminated tomato seeds and to examine whether the plant growth stage affects the ability of an enzyme-linked immunosorbent assay (ELISA)-based seed health test to detect TMV in Laboratory of Antalya Agricultural Quarantine Service in 2008. The plant growth stages (days after sowing) at the time of the DAS-ELISA analysis significantly affected the absorbance values obtained in the ELISA test of the tomato lots. However, there were no significant differences in absorbance between the two seed lots.

Key words: virus, seed, seed-borne virus, phytosanitary, TMV.

Introduction

Seed is the most important generative production material. About 90% of all food crops in the world are propagated by seed and losses caused by seed-borne pathogens, including viruses, therefore, are of great significance (Maude, 1996). Approximately 20% of plant viruses are transmitted from generation to generation in the seed (Mink, 1993) and in many cases the rate of transmission is very low. Seed transmission of plant pathogens plays an important role for the early outbreak of crop diseases and for the survival of inoculum from one crop season to the next. Seeds are instrumental in an effective worldwide spread of a range of diseases through international exchange of seeds. Effective management of seed-transmitted viruses depends upon the use of healthy planting material, through seed quality control and by plant quarantine monitoring and testing. Tests of seed for viral infection are crucial for providing adequate supplies of virus-free seeds or seeds with very low infection rates to avoid intra-national and international dispersal, leading to potentially high losses (Erkan, 1998; Morrison, 1999). The increasingly recognized importance of seed transmission in plant virus ecology has led to the strengthening of seed health testing for viruses in certification and quarantine agencies internationally (Maude, 1996).

In contrast to the situation four decades ago, highly sensitive and reliable methods for virus detection, well suited for testing of seeds, are available today. Among antibody-based methods, the enzyme linked immunosorbent assay (ELISA) has become the principal one, being relatively simple to use, high in sensitivity and reliability and suited for large-scale testing and partial automation (Morrison, 1999). The nucleic acid-based methods, especially the polymerase chain reaction (PCR) still lacks simplicity of use and suitability for large-scale testing compared with the ELISA. However, intense effort in many places is under way to improve and simplify molecular detection techniques.

The earliest method used for seed health testing for viruses was examination of seedlings raised from seed, the growing-on test. Symptom development in infected seedlings is, however, also a function of the virus or virus strain and the host species. It was early realized that growing-on test must be supplemented by confirmative biological, serological or molecular tests. Still, a growing-on test has the indisputable advantage of revealing seeds carrying seed transmissible virus to plants.

When serological tests done directly on ungerminated seed samples are used in an attempt to determine the incidence of virus transmission through seed to progeny seedlings, such testing generally provides considerable overestimates due to viral contamination of seed coats in the absence of embryo infection (Albrechtsen, 2006). In such instances, direct testing of whole seed samples can be used routinely to establish virus seed transmission rates to seedlings (O'Keefe et al., 2007). For direct tests, individual seeds are assayed to determine if they are infected or healthy, and the results can be quantified. In indirect tests, seed sample units (subsamples) of a specific quantity are assayed to determine the presence or absence of the pathogen in the sample unit and, therefore, whether the lot is positive or negative. In commercial seed health testing programs, indirect testing methods are most often used because of increased time and cost constraints and decreased efficiency associated with the direct testing approach (Morrison, 1999). *Tobacco mosaic virus* (TMV) is probably the best known virus in the world and an economically important virus infecting tomato, causing moderate to heavy annual losses. Transmission through seed was first suspected for TMV in tomato by Westerdijk. The virus contaminates the seed coat of tomato. Seeds usually carry TMV externally but it has never been firmly concluded that TMV contamination on tomato seed leads to successful plant infection (Pradhanang et al., 2005).

The aims of this study were (i) to determine whether TMV transmits through contaminated tomato seeds, (ii) to estimate the rate of seed-to-seedling transmission of TMV by ELISA, (iii) to determine optimum detection time (plant growth stage) in tomato seeds or seedlings.

Materials and methods

Seed materials. Two raw commercial F1 hybrid seed lots (2 varieties) that tested positive for *Tobacco mosaic virus* (TMV) with a specific antibody were used in the study. TMV-infected seeds from two commercial tomato (*Lycopersicon exulentum* Mill.) hybrids were sown in disposable plastic pots (7.5 × 5.5 × 6.0 cm) containing autoclaved potting mixture.

Seed-to-seedling transmission of TMV. The experiments were conducted in the Laboratory of Antalya Agricultural Quarantine Service in 2008. To determine transmission rates from tomato seeds to seedlings, 1000 seeds from seed lots were germinated in small sterile plastic pots containing autoclaved potting mixture and placed in a climatic chamber with a 16-h light, 8-h dark cycles at 23°C and irrigation with distilled water. A total of 200 seedlings each at the cotyledon stage from lots 1 and 2, were randomly selected and transferred to the plastic pots. From every sample 0.1 g were taken to be analysed by DAS-ELISA, to detect TMV infection in tomato plants for the period of 15 growth days.

Biological testing. Inocula were prepared by grinding infected tomato seedling tissue in phosphate buffer (0.01 M, pH 7.0) and were applied on carborundum-dusted leaves of *Nicotiana tabacum* L. cv. Xanthi nc at the 2–4 leaf stage (Jezewska, Trzmiel, 2005). Inoculated plants were kept in a growth chamber at 23°C. Symptoms on inoculated plants were recorded during the following 4 weeks. The presence of viruses in the test plants was determined by testing both inoculated and non-inoculated top leaves by ELISA as described below.

Serological testing. DAS-ELISA (double antibody sandwich-enzyme linked immunosorbent assay) method was used to detect TMV in tomato seed or seedling samples. DAS-ELISA method was applied according to Clark and Adams (1977) and instructions of the antisera's manufacturer ("Agdia", USA) for the polyclonal antisera of TMV. In DAS-ELISA method, seed and leaf samples were ground (0.1 g sample / 0.5 ml buffer) in extraction buffer (PBST: 0.13 M NaCl, 0.014 M KH_2PO_4, 0.08 M Na_2HPO_4 x 12 H_2O, 0.002 M KCl, pH 7.4) containing 0.05% Tween-20 added to wells of microplate ("Nunc Microwell", Denmark) after coating with TMV-specific polyclonal antisera diluted in carbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed three times with PBST-Tweeen-20 buffer and coated with alkaline phosphatase conjugated antibody diluted in extraction buffer and incubated for 2 h at 37°C. After washing, p-nitrophenyl phosphate in diethanolamine substrate buffer (pH 9.8) was added to wells and incubated at room temperature for 30–180 min. Absorbance values were read at 405 nm using a microplate reader ("Tecan", Switzerland). Virus-free tomato plants grown in an insect-proof growth chamber were used as negative controls. Samples were considered to be positive when the absorbance at 405 nm (A_{405}) values exceeded the mean of the negative controls at least a factor of two (Svoboda et al., 2006; Kutluk-Yilmaz, 2010).

Statistical analysis. In all experiments, ELISA absorbance data and the results of ELISA were recorded and analysed using the statistical package SPSS 12.0 for *Windows* (SPSS Inc., USA).

Results

DAS-ELISA analysis. The virus was identified by DAS-ELISA in the samples from tomato seed and seedling. A total of 93 samples from the four experiments gave strong ELISA reactions with antisera to TMV. 93 samples out of 400 seedlings grown from

infected seeds were TMV-positive, corresponding to a seed-to-seedling transmission rate of 23.5%. Absorbance values for duplicate samples of 16 negative controls ranged from 0.085 to 0.155, and for duplicate samples of 16 positive controls from 0.295 to 2.368 (Table 1).

The mean absorbances of the seed and seedling stages were 0.006–0.105 and 0.008–0.214, respectively, in seed lot 1, and 0.004–0.139 and 0.015–0.294, respectively, in seed lot 2. All maximum and mean ELISA A_{405} values exceeded the mean of the negative controls by at least a factor of three (0.254). Samples with $A_{405} > 0.250$ were considered as positive, while samples with $A_{405} < 0.254$ were rated as negative.

Table 1. Distribution of absorbance values when infected seed of lot 1 and 2 were assayed for TMV using an enzyme-linked immunosorbent assay (ELISA)-based seed health test

Range of absorbance values*	Lot 1 (variety 1)		Lot 2 (variety 1)	
	negative	positive	negative	positive
0–0.033	200	0	200	0
0.033–0.1	0	0	0	0
0.1–0.2	0	0	0	0
0.2–0.5	0	4	0	3
0.5–1.0	0	6	0	7
1.0–1.5	0	18	0	14
1.5–2.0	0	13	0	9
2.0–2.5	0	8	0	5
2.5–3.0	0	4	0	1
3.0 <	0	1	0	0
Total	200	54	200	39

Note. * – absorbance at 450 nm. Absorbance values below 0.1 were considered negative outcomes (samples free of TMV), those between 0.1–0.2 were classified as elevated samples, and those above 0.2 were positive outcomes (samples containing TMV-infected).

Detection of TMV in test plants by DAS-ELISA. TMV is transmitted principally by mechanical inoculation (Zaitlin, 2000). The infected tobacco plants started to develop the TMV symptoms after 7 days. Samples of newly developed leaves of the inoculated tobacco plants were tested for TMV infection using ELISA kit. The ELISA results indicated that tobacco samples were infected with TMV.

Concentration of TMV in seeds, leaves and seedlings. All the leaf and seed extracts of tomato inoculated with TMV produced local lesions on *Nicotiana tabacum*. In tomato seedlings, the concentration of TMV was greater than in seeds (Table 2).

The plant growth stage at the time of the DAS-ELISA analysis significantly affected the absorbance values obtained in the ELISA test of the tomato lots ($P < 0.05$). However, there were no significant differences in absorbance between the two seed lots ($P > 0.05$) (Table 2).

Table 2. The mean ELISA absorbances of the tomato samples at days after sowing

Days after sowing		Range of absorbance values		
		Means of positive samples	Negative controls	Positive samples
Seed	Lot 1 (variety 1)	0.960A* (0.403–1.873)	0.033–0.047	0.335–0.472 C
Day 5			0.044–0.053	0.255–1.847 B
Day 10			0.026–0.035	0.571–3.173 A
Day 15			0.025–0.050	0.256–0.771 C
Seed	Lot 2 (variety 1)	0.764A (0.351–1.528)	0.047–0.089	0.313–0.514 C
Day 5			0.032–0.077	0.415–1.113 B
Day 10			0.050–0.063	0.354–2.702 A
Day 15			0.035–0.071	0.270–0.432 C

Note. * – means followed by the same letter in the columns are not significantly different ($P > 0.05$) according to Duncan test.

Further, the concentration of virus established in seedlings was greater 10 days after sowing (Figure).

Concentration of the virus during the incubation period. P-nitrophenyl phosphate in diethanolamine substrate buffer (pH 9.8) was added to wells and microplate incubated at room temperature for 30–180 min. The absorbance values, A_{405}, were read using a microplate reader ("Tecan") at 30-minute intervals (30, 60, 90, 120, 150, 180 min). ELISA absorbance data were recorded and analysed. There was difference between mean absorbance values. The differences between results were statistically significant ($P < 0.05$) (Table 3).

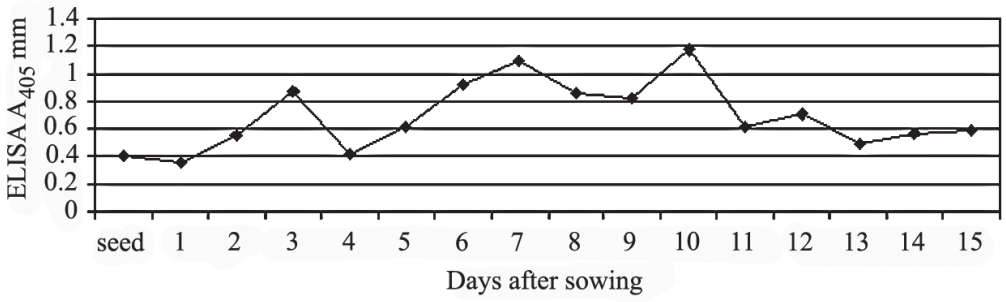

Figure. Range of absorbance values at days after sowing

Table 3. Absorbance values of ELISA obtained at 30-minute intervals after adding the substrate to the wells

Lot / Minutes	Mean absorbance value					
	30	60	90	120	150	180
Lot 1	0.088 a*	0.211 b	0.710 c	1.153 d	1.469 e	2.302 f
Lot 2	0.099 a	0.205 b	0.803 c	1.247 d	1.760 e	2.550 f

Note. * – means followed by different letters in the rows are statistically different (at $P < 0.05$) by the Duncan test.

Discussion

Many plant viruses are known to be borne by seeds (Chitra et al., 1999). Most seed transmitted viruses remain viable in seeds for years and often as long as the seeds remain viable (Erkan, 1998). Thus seeds are an obvious means of dispersing viruses over long distances by trade and exchanges of seeds (Degirmenci, Açikgöz, 2005). Several seed transmitted viruses have undoubtedly been spread worldwide in this way (Jones, 2000). Increased trade in seed and other agricultural products spawned some legitimate concerns about risks of pathogen movement between nations; however, some phytosanitary regulations were not based on pest risk but instead were enacted as substitutes for previously existing trade barriers. Faced with these new challenges, several organizations with interest in international seed trade began to address the scientific basis of the burgeoning lists of quarantine pests, using pest risk analysis processes (Munkvold, 2009). Because only a portion of the seed lot is actually tested, a negative test result does not guarantee that the seed lot is completely free of the specified pathogen(s), only that the sample of seed tested was found to be negative. This study also supported that observation.

In the present study, naturally infected tomato seeds were obtained from commercial seed lots derived from susceptible tomato plants. The experiments were done to examine whether the time of seed sampling (plant stage of development) affected the ability of an ELISA-based seed health test to detect TMV and to estimate the rate of seed-to-seedling transmission of TMV by ELISA. Detection of virus depends on the concentration of virus in seeds, and the concentration in samples in turn depends on the growth stage at which the samples are tested. The present study showed that infection ratio can vary in the samples of tomato according to growth stage at the time of testing. Similarly, the stage of plant growth at the time of ELISA analysis significantly ($P = 0.0000$) affected the *Pepino mosaic virus* (PepMV) infection rate (measured as absorbance obtained in ELISA) of the untreated tomato lots (Cordoba-Selles et al., 2007). Seed transmission studies revealed that seedlings grown from infected seeds were TMV-positive in seed lots 1 and 2, respectively, corresponding to seed-to-seedling transmission rates of 27.00 and 19.50%, respectively. When compared to seed, ELISA absorbance values were higher in seedlings, whereas Chitra et al. (1999) reported that the concentration of virus in seeds will be higher, if the plants are infected at an early growth stage. Since TMV occurs in the seed coat, even late infection of the host leads to establishment of virus particles in the seed.

The presence of a virus in a seed, even in the embryo (Varma et al., 1992), does not always lead to seedling infection. This property distinguishes a seed-borne virus that is carried by the seed but does not

infect the seedling, from a seed transmitted virus that does infect the seedling produced from the seed. TMV is not a seed-transmitted virus, as it cannot be carried within the embryo. However, it is so readily infecting its host that it can successfully invade young seedlings from testa during germination (Mink, 1993). Seed-transmitted viruses generally invade the seeds during embryonic development, resulting in high rates of natural infection among the germinated seedlings (Wang, Maule, 1994). The seed transmission of members of the genus *Tobamovirus* is an exception. These pathogens, which are mainly carried on tomato and pepper seed coats, do not infect the developing embryos; however, they can infect germinating seedlings as a result of mechanical contact with contaminated seeds (Chitra et al., 1999). Transmission on or in the seed coat without infection of the embryo is comparatively rare among viruses (Lewandowski, 1999) and might be related to the high stability of the virus particles. TMV is highly stable and can survive in soil and in seed. Accordingly, seedling infection by tobamoviruses occurs primarily through mechanical contact with contaminated sources. Sakamoto and Matsuo (1972) reported that TMV was detected in the testa or seed coat of infected pepper seeds. In addition, they showed that a TMV-contaminated seed coat spontaneously released from pepper seedlings in bud did not lead to infection during germination.

Testing of progeny rather than seeds provides obvious advantages in disease management for quarantine agencies. Only transmissible virus is detected and the antigen titre is generally higher in progeny than in seeds. For example, when testing soybean for *Soybean mosaic virus* (SMV) by ELISA, Lister (1978) found higher absorbances in leaves than in seed extracts. In addition, growing-on tests provide the opportunity to disclose possible symptom developments in the seedlings prior to ELISA. One of the factors implicated by direct testing of seeds is the great variation of virus concentrations existing among embryos of different virus-host combinations (Maury et al., 1998). Although virus concentrations are generally higher in the progeny, such a variation also occurs in seedlings. A disadvantage as compared to direct testing of seeds is the time it takes to raise seedlings (1–2 weeks for most vegetable crops). Phytosanitary certification requires the timely and quick processing of many samples, as achieved by a rapid and sensitive detection method devised in the laboratory (Gallitelli, 2000).

Conclusion

Seed health testing is an important component in disease management for quarantine and phytosanitary certification. Summarising the above presented data it may be concluded that: infection of tomato seeds with TMV may result in the virus transmission to the progeny. These results indicate that there was TMV transmitted to seedlings from the contaminated tomato seed. Tomato seeds may serve as a potential inoculum source for the long distance movement and local spread of TMV in the field, and could affect tomato production. From the description of the epidemiological characteristics of seed-borne viruses, which may occur in trace amounts and be present latently in their host, it is obvious that test methodology is critical.

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ELISA analizės rezultatai tiriant pomidoro (*Lycopersicon esculentum* Mill.) sėklų užsikrėtimą tabako mozaikos virusu

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Santrauka

Atliekant sėklų karantininį ir fitosanitarinį sertifikavimą, svarbus ligų valdymo komponentas yra sėklų sveikatingumo tyrimas. Siekiant gauti tikslius sėklų sveikatingumo tyrimų rezultatus, vienas svarbiausių veiksnių yra sėklų ėminio paėmimo laikas. Atliekant sėklų sveikatingumo tyrimus, daugeliu atvejų nustatomi tik kokybiniai rodikliai, t. y. tiriama, yra ar nėra jose tam tikrų patogenų. Sėklų partijos, kuriose nustatoma patogenų, yra atmetamos, o kuriose nenustatoma – priimamos. Kadangi daugelis svarbių su sėkla perduodamų patogenų sėklose pasitaiko nedažnai, dėl mažų arba labai mažų sėklų užterštumo/užsikrėtimo ribų, dėl kurių gali būti patiriama ekonominių nuostolių arba kilti ligos epidemijos, ėminių paėmimas sėklų sveikatingumo tyrimui gali būti problemiškas. Atlikti keturi tyrimai siekiant nustatyti, ar tabako mozaikos virusas (TMV) perduodamas per užterštą pomidorų sėklą, ir ištirti, ar augalų augimo tarpsnis turi įtakos sėklos tyrimų dėl TMV nustatymo rezultatams, tyrimus atliekant ELISA metodu. Tyrimai atlikti 2008 m. Antalijos žemės ūkio karantino tarnybos laboratorijoje. Augalų vystymosi tarpsniai (dienos po sėjos) DAS-ELISA analizių metu turėjo didelę įtaką absorbcijos reikšmėms, gautoms pomidorų sėklų partijas tiriant ELISA metodu. Tačiau tarp dviejų tirtų pomidorų sėklų partijų nebuvo nustatyta esminių skirtumų tarp absorbcijų.

Reikšminiai žodžiai: virusas, sėklos, su sėkla pernešamas virusas, fitosanitarija, TMV.