УДК 632.38:634.2

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DETECTION OF *PLUM POX VIRUS* ISOLATES IN THE ORCHARDS OF THE EASTERN STEPPE OF UKRAINE

Plum pox virus (PPV), the causal agent of Sharka disease, produces severe damage and significant economic losses to stone fruit production. The propagation and distribution of virus free materials into healthy growing areas is one of the main ways to reduce the spread of this virus. Given the aggressiveness of PPV, it is imperative that all infected by this virus plants must be excluded from planting materials. ELISA method now is one of the most popular and used techniques for PPV detection. Whereas the usage of polyclonal antibodies, which recognize multiple epitopes on any one antigen, is controversial due to problems with specificity and sensitivity because serum contains a mixture of antibodies of different affinity. To ensure the effectiveness of annual screening surveys of rootstocks and varieties nurseries to detect viral pathogens on guaranteeing healthy status of plant material, the minimalizaton of false-negative test results is important. The diagnostic characteristics of two commercially available serological diagnostic tests for the detection of Ukrainian isolates of plum pox virus in plant material of six stone fruit crops selected in the orchards of the Eastern Steppe were evaluated. The standardized methodology was used for the calculation of the parameters of the operational capacity of DAS-ELISA. The analyses of diagnostic data were performed with 2x2 contingency tables. For assessing the validity of two ELISA test systems for the detection of local PPV isolates, some diagnostic parameters were calculated: sensitivity and specificity, positive and negative predictive values, positive and negative likelihood ratios. It is shown that the Agdia test system is more specific and more reliably identifies the plant material PPV affected, allowing for a more comprehensive eradication of the virus-infected material. The Agdia test is therefore more useful for the screening of nursery orchards to guarantee the future propagation of PPV-free plant material. Incongruent diagnoses obtained by different diagnostic systems in all stone fruit plant material may evidence that PPV prevalence in the

local stone fruit crops orchards being significantly underestimated, may result in the unintended and harmful propagation of the disease.

Key words: likelihood ratio, Plum pox virus, polyclonal antibodies, PPV-strains, predictive value, specificity.

Plum pox virus (PPV), the agent responsible for Sharka disease, belongs to genus *Potyvirus*. The natural host range of this virus is restricted to Prunus spp. (stone fruits and ornamental trees). PPV is especially harmful for *P. armeniaca*, *P. domestica*, *P. persica* та *P. salicina*. The disease significantly reduces the quality and quantity of fruits in plants of these species. This virus is highly polymorphous. To date, seven strains/groups of PPV have been identified due to biological, serological, and molecular properties: D (Dideron), M (Marcus), C (Cherry), EA (El Amar), W (Winona), Rec (Recombinant) and T (Turkish) [4,14]. The comparison of complete genomic sequences revealed up to 27.7% of nucleotide divergence between representative isolates of seven strains of PPV. Most of the deposited virus isolates presented in the GenBank database of nucleotide sequences belongs to PPV D (Dideron) and PPV M (Marcus) [5].

Although many diagnostic tools are available for the sensitive and/or specific detection of PPV, its detection is significantly complicated by its uneven distribution in infected woody hosts, and its low titer outside of the active growth period. There are no effective control measures against plum pox virus. The use of certified planting material, the removal of wild hosts, and the control of aphid vectors will help to prevent outbreaks of the disease and reduce the risk of the disease spreading. Taking into consideration the aggressiveness of this virus, it is imperative that all PPV infected plants must be excluded from planting materials.

The annual screening surveys of rootstocks and varieties nurseries are conducted to detect viral pathogens, including the plum pox virus, using systematic sampling. To ensure the effectiveness of these screening surveys on guaranteeing healthy plant material, the minimalizaton of false-negative test results is important. The continued presence of affected plants can significantly reduce the effectiveness of the work and lead to unexpected losses in subsequent stages of virus-free planting material propagation. Consequently the objective of this study was to compare the accuracy of Loewe Phytodiagnostica (Germany) and AGDIA (USA) ELISA protocols in the detection of Ukrainian PPV isolates, with a particular emphasis on the false-negative indicators.

Materials and methods

Research was done in Virology, Plant health and Propagation of Fruit and Berry Cultures Department of the Institute of Horticulture NAAS Ukraine, during 2011—2012. The samples were selected at the beginning of June,

during the period of intensive growth in both collection and old fruiting orchards, in Artemivsk Experimental Nursery-garden Station of IH NAAS. The samples plants included myrobalan, plum, peach, apricot, and sweet and sour cherry. Each sample consisted of 5 branches or 10 full-grown leaves selected from different locations in the internal section of the tree. There were selected 130 samples from trees suspected to contain PPV presence and divided into two groups. The first group included the samples of plum, peach, myrobalan and apricot (total 96 samples), the second group was comprised of sour and sweet cherries (total 34 samples).

Plum pox virus was detected in two tests by DAS-ELISA (DAS-Double Antibody Sandwich) [6] in two repetition using specific polyclonal antibodies produced by Loewe Phytodiagnostica (Germany) and AGDIA (USA). Compliant with recommendations for PPV detection, a sample was considered to be positive when the rate of its absorbance value was more than two times greater than the negative control value [12].

Analyses of diagnostic data were performed with 2x2 contingency tables, enabling indicators of the operational capacity of each test system to be calculated. This method allows to make the probabilistic assessment of positive and negative test results. For assessing the validity of two ELISA test systems for the detection of local PPV isolates, some diagnostic parameters were calculated (formulas 1–6): sensitivity and specificity were calculated according to Altman and Bland [2], positive and negative predictive values were estimated according to Altman and Bland [3], positive and negative likelihood ratios were estimated according to Deeks and Altman [7]. The conventional data layout for the 2x2 contingency table used to calculate parameters of laboratory test capacity, along with relevant formulas, are shown in Table 1.

Table 1

Contingency table created by comparing the results of the diagnostic test and the reference test

		Reference test	
		Positive	**Negative**
Test Outcome	Test outcome Positive	a True positive (TP)	b False positive (FP)
	Test outcome Negative	c False negative (FN)	d True negative (TN)

$$\text{Sensitivity} = a/(a+c) = \text{true positives} / \text{disease+} \quad (1)$$

$$\text{Sensitivity} = d/(b+d) = \text{true negatives} / \text{disease–} \quad (2)$$

$$\text{Positive predictive value (PPV)} = a/(a+b) = \text{TruePositives/Test+} \quad (3)$$

$$\text{Negative predictive value} = d/(c+d) = \text{TrueNegatives/Test–} \quad (4)$$

$$\text{Likelihood ratio positive (LR+)} = \text{sensitivity} / (1 - \text{specificity}) \quad (5)$$

$$\text{Likelihood ratio negative (LR-)} = (1 - \text{sensitivity}) / \text{specificity} \quad (6)$$

Calculations were performed using the program JawaStat 2-way Contingency Table Analysis, in which confidence intervals (95%) for selected indicators were calculated on the basis of binomial distribution.

Results and discussion

Phytovirological monitoring of Plum pox virus in orchards belonging to the Institute of Horticulture have been conducted since 2004 by ELISA, using polyclonal commercial test systems produced by Loewe Phytodiagnostica in Germany, which are among the most common test systems used by in relevant European laboratories. Between 2004 and 2012, more then 3.500 trees from the plum group and approximately 1.200 trees from the cherry group were tested for PPV presence. This testing initiated the selection of virus-free clones of the rootstocks of prospective varieties and the creation of a virus-free clones base, and allowed for the control of PPV spread in different types of nurseries and orchards. The PPV prevalence rate was determined in the main collection orchards of stone fruit crops (plum, peach, plum, apricot, sweet and sour cherry) as well as in nurseries of vegetative rootstocks of the plum and cherry groups. It was found that in collection plantings of myrobalan, plum, peach, and apricot, the spread of PPV ranges from 3.8% to 19.2%, and in sweet and sour cherry plantings it varies between 6.7% and 9.1%.

Loewe Phytodiagnostica produced immunoglobulins were considered to be the «golden standard» for screening surveys of stone fruit crops plant material in the laboratory, and are comprised of an artificial mixture of antibodies which are effective against all the strains of PPV except PPV W. However, AGDIA produced polyclonal antibodies can detect all strains of PPV including the elusive PPV W. Detection of the virus in selected material using various test systems revealed some differences in the status of the tested materials (table 2).

The highest consistency in the testing results was observed for samples in the first group. The Pearson correlation between parameters of absorbance value (optical density) obtained using two diagnostic systems was significant ($cor = 0.856$ $p < 0.001$). The proportion of the samples with positive diagnosis was found by AGDIA and Loewe Phytodiagnostica test systems to be 0.478 and 0.270, respectively. All positive diagnoses obtained using Loewe Phytodiagnostica were validated by AGDIA. Mismatch of diagnosis was observed in 20.8% of samples which tested positive by AGDIA results only. The optical density of these samples was usually ranged from 2- to 3-fold value of the negative control. These findings indicate that the spread of PPV can be systemically underestimated using the test system of Loewe Phytodiagnostica in our regional screening surveys.

Table 2

ELISA results obtained with the AGDIA and Loewe Phytodiagnostica tests

Groups	Proportion of plants with test positive		Proportion of plants with inconsistent diagnosis
	Loewe Phytodiagnostica	AGDIA	
Group I (myrobalan, plum, peach, apricot)	0.270	0.479	0.208
group II (sour and sweet cherries)	0	0.375	0.375

There is much to be learned about PPV strains that circulate in the Ukrainian stone fruit orchards. In recent years there have been partial genome sequencing performed on select Ukrainian plum isolates which were identified as D-strains [1], including two isolates in our department (in preparation). Also known that the origin of the PPV W atypical isolates [8, 10] may be tied back to the region of the Eastern steppe of Ukraine. So it is within reason that in the Artemivsk district this strain could also be in circulation.

In sweet and sour cherry orchards samples were collected from the trees showing a decline of main shoots, expressed basal shoots, and reduction of leaf and fruit size. In only two cases clear mosaic symptoms on leaves (sweet cherry cultivars Krupnoplidna and Mahalebca) were observed. Analysis of this samples group using two testing systems showed 16.7% discrepancy in positive diagnosis. For one of the sweet cherry samples the optical density was equal to the optical density of the negative control obtained using the Loewe Phytodiagnostica testing system, and was equal to the positive control obtained using AGDIA one. There was no significant Pearson correlation between optical density rates in two different analyses (corr = -0.056, p=0.771). The results obtained using the two serological tests for cherry PPV detection were inconsistent with one another.

In reality, we face a mixture of virus isolates in our screening, as different PPV strains can be present not only in the same orchard but in the same plant as well [9]. The use of polyclonal antibodies which recognize multiple epitopes on the same antigen always raises the question of sensitivity and specificity, because the serum contains a mixture of antibodies of different affinities [13]. The screening test for PPV detection in nurseries may be evaluated using different indices. The more important among them are specificity, negative predictive value, and negative likelihood ratio, because these are indices which estimate the false-negative test results. Specificity in particular is defined as the probability of a negative test in plants free of the disease, while sensitivity is defined as the probability of a positive test in plants harboring the disease. The Loewe Phytodiagnostika antibodies were more specific for PPV detection in both groups of samples (table 3, 4).

Table 3

Discriminatory power of applying test systems for PPV detection in samples group I

	AGDIA		Loewe Phytodiagnostica	
Indicators	**Parameters**	**95% confidence intervals**	**Parameters**	**95% confidence intervals**
Sensitivity	1.000	0.859÷1.000	0.565	0.485 ÷0.565
Specificity	0.714	0.662 ÷0.714	1.000	0.926 ÷1.000
PPV	0.565	0.485 ÷0.565	1.000	0.859 ÷1.000
NPV	1.000	0.926 ÷1.000	0.714	0.662 ÷0.714
+LR	3.500	2.538 ÷3.500	inf	7.893 ÷inf
-LR	0.000	0.000 ÷0.214	0.435	0.435 ÷0.556

Table 4

Discriminatory power of applying test systems for PPV detection in samples group II

	AGDIA		Loewe Phytodiagnostica	
Indicators	**Parameters**	**95% confidence intervals**	**Parameters**	**95% confidence intervals**
Sensitivity	0.333	0.063 ÷0.781	0.111	0.021 ÷0.260
Specificity	0.814	0.795 ÷0.845	0.946	0.924 ÷0.982
PPV	0.111	0.021 ÷0.026	0.333	0.063 ÷0.781
NPV	0.946	0.924 ÷0.982	0.814	0.795 ÷0.845
+LR	1.792	0.305 ÷5.047	2.056	0.275 ÷14.678
-LR	0.819	0.259 ÷1.179	0.940	0.753 ÷1.060

It is known that the laboratory test has a high capacity level if the sum of its sensitivity and specificity exceeds the value of 1.4. [15]. In our study, both test systems have sufficient capacity levels for PPV detection in plant material of the first group. The value of this sum is 1.714 for AGDIA test system and 1.565 for Loewe Phytodiagnostica. For the second group these values are much lower, resulting in 1.147 and 1.057 respectively. Low sensitivity of both diagnostic tests for the detection of local cherry PPV isolates should be noted. At the same time the level of its specificity to these virus strains is sufficient. Other authors also observed discordant results in determining PPV status in cherry plant material by ELISA [11].

But specificity and sensitivity serve only to provide a rough distribution of results into "positive" and "negative" categories, because these indices are significantly influenced by the prevalence of infected plants in the population investigated. Positive and negative predictive values are estimates of the probability that the infected plants have a defined diagnosis (posterior probability). Its value is less dependent on the disease prevalence in the population investigated. Positive predictive value is defined as the proportion of the plants showing positive results given by the method, which have been correctly diagnosed. Negative predictive value was the proportion of the plants showing negative results by the method, which were correctly diagnosed. The higher the NPV, the lower the rate of false-negative test results. For test results in both groups this indicator is higher for the Agdia test system. The NPV values indicate that a negative diagnosis in first group of samples was more reliable (NPV =1.000) in comparison with the second one (NPV= 0.946).

Potential utility of the test can be objectively evaluated using the likelihood ratio (LR). This indicator offers important advantages over sensitivity and specificity in characterizing diagnostic tests, since it does not depend on the virus prevalence in the population. Likelihood ratio can be positive (+LR) or negative (- LR). LR+ is defined as the probability of a plant *with* disease having a positive test result, divided by the probability of a plant *without* disease having a positive test result. LR is defined as the probability of a plant *with* disease having a negative test result, divided by the probability of a plant *without* disease having a negative test result. The positive likelihood ratio (LR +) must be greater than 1, and the negative likelihood ratio (LR-) — ranged from 0 to 1. The larger the value of LR+, the stronger the relationship between a positive result and the probability that the plants carry the disease. The smaller value of LR-, the stronger the relationship between a negative result and the probability that the plants are disease-free.

The antibodies provided by AGDIA is therefore more discriminative than the Loewe Phytodiagnostica test, as it demonstrated the lowest probability of false-negative diagnoses a wider range of PPV isolates in group I (LR-=0.000). In the plant material of the second group of the samples the lowest share of false-negative diagnoses were also obtained with the same test system.

Conclusion. The test system of AGDIA is more specific for the screening of nursery orchards of six stone fruit crops (plum, myrobalan, peach, apricot, sour and sweet cherry) for the presence of PPV. Incongruent diagnoses obtained by different diagnostic systems in all stone fruit plant material may evidence that PPV prevalence in the local stone fruit crops orchards has been significantly underestimated, resulting in the unintended and harmful propagation of the disease. Both test systems have low discriminatory abilities in the detection of local cherry PPV isolates. The local PPV strains require further study by more specific methodes.

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Стаття надійшла до редакції 27.02.2013 р.

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ВИЯВЛЕННЯ ІЗОЛЯТІВ ВІРУСУ ШАРКИ СЛИВИ В НАСАДЖЕННЯХ СХІДНОГО СТЕПУ УКРАЇНИ

Реферат

Метою роботи був порівняльний аналіз результатів дослідження маточників клональних підвоїв і маточно-черенкових насаджень на наявність вірусних патогенів та підтвердження безвірусного статусу рослинного матеріалу з використанням ІФА-діагностикумів. В роботі оцінено діагностичні характеристики двох комерційних тестових систем для виявлення українських ізолятів вірусу шарки сливи в рослинному матеріалі шести кісточкових культур, відібраному в насадженнях Східного Степу. Для розрахунку показників операційної потужності методу DAS-ELISA було використано стандартну методологію. Аналіз діагностичних даних було проведено з використанням 2x2 таблиці спряженості ознак. Для оцінки валідності двох тестових систем для ідентифікації локальних ізолятів вірусу шарки сливи було розраховано наступні діагностичні показники: чутливість, специфічність, негативне та позитивне предиктивні значення, негативне та позитивне відношення правдоподібності. Було показано, що тестова система виробництва AGDIA є більш специфічною та більш надійно визначає статус інфікованого вірусом шарки сливи рослинного матеріалу, що дозволяє більш ефективно його використовувати. Ця тестова система є більш придатною для скринінгових обстежень в локальних маточних насадженнях і більш надійно забезпечує вирощування вільного від вірусу шарки сливи садивного матеріалу. Неузгодженість діагнозів, отриманих різними системами для всіх шести кісточкових культур свідчить про те, що поширення вірусу шарки сливи в локальних насадженнях може бути значно недооціненим, що сприяє небезпечному поширенню цього захворювання.

Ключові слова: відношення правдоподібності, вірус шарки сливи, поліклональні антитіла, предиктивне значення, специфічність, штами вірусу шарки сливи.

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ВЫЯВЛЕНИЕ ИЗОЛЯТОВ ВИРУСА ШАРКИ СЛИВЫ В НАСАЖДЕНИЯХ ВОСТОЧНОЙ СТЕПИ УКРАИНЫ

Реферат

Целью данной работы явился сравнительный анализ результатов исследования маточников клоновых подвоев и маточно-черенковых насаждений на наличие вирусных патогенов и подтверждение безвирусного статуса растительного материала с использованием ИФА-диагностикумов. В статье оценены диагностические характеристики двух коммерческих тестовых систем для выявления украинских изолятов вируса шарки сливы в растительном материале шести косточковых культур, отобранном в насаждениях Восточной Степи. Для расчета показателей операционной мощности метода DAS-ELISA была использована стандартная методология. Анализ диагностических данных был проведен с использованием 2x2 таблицы сопряженности признаков. Для оценки валидности двух тестовых систем для идентификации локальных изолятов вируса шарки сливы были рассчитаны следующие диагностические показатели: чувствительность, специфичность, отрицательное и положительное предиктивные значения, отрицательное и положительное отношения правдоподобия. Было показано, что тестовая система производства AGDIA является более специфической и более надежно определяет статус инфицированного вирусом шарки сливы растительного материала, что позволяет более эффективно его искоренять. Эта тестовая система является более подходящей для скрининговых обследований в локальных маточных насаждениях и более надежно обеспечивает выращивание свободного от вируса шарки сливы посадочного материала. Несогласованность диагнозов, полученных разными системами для всех шести косточковых культур свидетельствует о том, что распространение вируса шарки сливы в локальных насаждениях может быть значительно недооцененным, что способствует опасному распространению этого заболевания.

Ключевые слова: отношения правдоподобия, вирус шарки сливы, поликлональные антитела, предиктивные значения, специфичность, штаммы вируса шарки сливы.