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DEVELOPMENT OF AN OPTIMAL VARIANT OF THE ELISA METHOD FOR THE DIAGNOSIS OF THE POTATO VIRUS X

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SUMMARY

Potato (*Solanum tuberosum* L.) is among the most economically important crops worldwide, yet its productivity acquires severe constraints from viral infections, especially the Potato virus X (PVX). This study aimed to develop an optimized double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for the rapid and reliable diagnosis of PVX in seed and field materials. Virus propagation in *Datura stramonium* and *D. tatula* enabled the preparation of purified antigen and polyclonal antiserum in rabbits. Optimization trials established the most effective dilutions for primary (1:2000) and secondary (1:5000) antibodies, achieving approximately 96% sensitivity and 94%–95% specificity. The improved ELISA demonstrated a strong diagnostic performance, ensuring early detection of PVX in potato cultivars grown under Uzbekistan conditions. The application of the test revealed high infection rates (80%–100%) in certain cultivars, whereas others, including Folva, Piskom, and Aureta, showed no infection, indicating their suitability for virus-free breeding. The developed DAS-ELISA provides a cost-effective, rapid, and reproducible diagnostic platform suitable for seed certification, monitoring, and phytosanitary programs. Its integration with RT-PCR and eco-safe vector management can further enhance potato yield stability and strengthen regional plant health protection systems.

Keywords: Potato (*S. tuberosum* L.), Potato virus X, ELISA, antigen, isotonic solution, polyclonal antiserum, conjugate

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Key findings: Potato (*S. tuberosum* L.) yields primarily rely on virus-free seeds and recommended crop husbandry. The optimized DAS-ELISA (1:2000/1:5000) achieved ~96% sensitivity and ~94%–95% specificity, enabling PVX detection for seed certification, monitoring, and virus-free programs. Pairing ELISA with RT-PCR and vector control strengthens the phytosanitary protection and stability.

INTRODUCTION

The potato (*Solanum tuberosum* L.) is one of the most widely cultivated food crops in the world. In global agriculture, it ranks fifth in cultivated area after wheat, rice, maize, and white sorghum, and fourth in overall production (Ekaterinskaya, 2019; Fayziev, 2019). At present, China, Russia, and India together contribute more than 40% of total global potato output. High yields achieved in major potato-producing countries are largely dependent on the use of healthy planting materials, systematic application of virus-free seeds, adherence to sound agronomic practices, and well-coordinated measures for protecting crops from phytopathogenic agents (Beisembina, 2021).

The most widely cultivated potato species, *S. tuberosum* L., originates from South America, where several other potato species also attained domestication. The natural distribution of both cultivated and wild potatoes covers broad regions of South and Central America (Correll *et al*., 1962).

The potato crop is susceptible to numerous diseases caused by diverse pathogens, with tuber-borne infections posing the most pronounced risk. Overall, viral diseases are the most crop-damaging. As potato stems and tubers are rich in carbon and water, these provide a favorable environment for virus persistence and their widespreadness (Oripov and Khalilov*,* 2007). Based on enhanced severity, the economically important potato viruses often listed include PVX, PVS, PVM, PVA, PVY, and PLRV. Mixed infections, such as PVX+PVS+PVY, PVX+PVA+PVY, and PVX+PVM+PVY, proved to be particularly harmful, frequently reducing tuber yield by 25%–30% and even more (Eskendirova *et al.,* 2014; Beisembina*,* 2022; Fayziev *et al.,* 2020, 2023; Jovlieva *et al.,* 2024).

Regional edaphic and climatic differences foster the emergence of aggressive viral isolates and intensify their effects on potato plants (Anisimov *et al.,* 2009; Eskendirova *et al*., 2014). The research work has shown that adverse environments exacerbate virus-induced declines in promising physiological traits in Uzbekistan (Fayziev *et al.,* 2020). Integrating strategies include applying environmentally benign bio-products derived from fungi and bacteria (Reghmit *et al.,* 2022) and critical application of modern diagnostics to remove infected seed lots and rapidly identify the phytopathogenic viruses (Berzina *et al.,* 2013; Jovlieva *et al.,* 2024). In Uzbekistan, the specific antisera have succeeded the generation for several viruses, with antibody titers characterized and the reagents implemented in diagnostics (Sattorov *et al.,* 2020; Sobirova *et al.,* 2020; Khusanov *et al.,* 2020; Fayziev *et al.,* 2020; Kholmatova *et al*., 2024).

Breeding and physiological research programs increasingly focus on developing high-yielding and stress-resilient cultivars—particularly those tolerant to drought, salinity, and major diseases—and have extended these evaluations to other strategic crops, such as sunflower and soybean (Omonov *et al*., 2023). Among available diagnostic platforms, immunological assays are widely applicable due to their rapid performance, minimal labor requirements, and suitability for high-throughput screening (Isakov and Isakov, 2014; Jovlieva *et al*., 2024). These methods rely on specific interactions between antigens and antibodies (Gnutova, 1987; Isakov and Isakov, 2015). In particular, the enzyme-linked immunosorbent assay (ELISA) combines immune recognition with enzymatic signal amplification, providing both specificity and quantitative measurement (Egorov *et al*., 1991; Kharitonova and Israfilova, 2018). Globally, continuous efforts progress to enhance ELISA sensitivity for PVX detection by refining reagents, assay formats, and analytical conditions (Uhde-Holzema *et al*., 2010;

Lakshmipriya *et al*., 2016; Panferova *et al*., 2019; Soliman *et al*., 2019).

ELISA is a key component of phytosanitary systems that support meristem-based production of virus-free plants, particularly in potatoes. As regeneration and acclimatization require extended time, routine ELISA monitoring of mother plants and infection sources is essential to prevent reinfection (Jovlieva *et al*., 2024). However, globalization of potato trade is widening the geographical spread of viruses and driving the emergence of more aggressive isolates. These trends highlight the need for strengthened virus protection and continuous improvement of biotechnological diagnostic tools.

MATERIALS AND METHODS

Experimental site

For the experiment, the propagation of biologically purified potato virus X (PVX) first proceeded in *Datura stramonium* L. and *Datura tatula* L. plants. After completing the specific antibody preparation procedures, performing the ELISA assay transpired, as described by Jovlieva *et al.* (2024).

Keeping and caring for animals

For rabbits, selecting a separate insulated iron cage adapted to the animals ensued. The animals remained in a room with a temperature of 23 °C–24 °C, air humidity (55%–60%), and 12–14 h of light. Animals maintained were under daily clinical observations. Constant checking of their look comprised nutrition, regularity of breathing, appropriateness of their movements, and skin and ears.

Antigen preparation

After propagation of the virus in *D. stramonium* L. and *D. tatula* L. plants, the purified preparation proceeded under laboratory conditions using the physicochemical and gel chromatography methods, with the degree of purity and concentration determined and stored at +4 °C.

Immunization

The virus administration continued by immunization of the antigen between the two subscapularis and hind leg muscles of rabbits. Injections entailed five repeats, with an interval of three days.

Antiserum separation and storage

After keeping the collected blood in a thermostat at +37 °C for two hours and then at +4 °C for 20 hours, the careful separation of the antiserum fraction took place into another sterile container using a glass rod. In removing hemolyzed elements, the antiserum received centrifugation at 1500 rpm for 15 min (Egorov *et al.,* 1991). Then dispensing the clarified serum into 2 ml sterile tubes followed by storage at −4 °C and −20 °C for further use. All manipulations performed were under aseptic conditions to prevent contamination (Jovlieva *et al.,* 2024).

By optimizing the immunoenzyme analysis methodology, the diagnostic assay evaluation relied on its sensitivity and specificity. These concepts' introduction first came from the American biostatistician, Jacob Yerushalmi, in 1947. Test outcomes for infected organisms entailed classification as either positive or negative, which fall into four categories: true positive, false positive, true negative, and false negative. The distribution of these outcomes mathematically defines the accuracy of a diagnostic test, allowing the calculation of sensitivity and specificity (Kovalchuk *et al*., 2010; Biernacka *et al*., 2018).

In the optimization of the ELISA kit for the detection of PVX, the necessary antibody, conjugate (goat anti-rabbit-Ab+peroxidase), and healthy and PVX-infected samples (Ag) underwent dilution and titration. Ag dilution was in concentrations ranging from 0.1 to 5.0 µg/ml before being sorbed onto polystyrene plates. The titer of antibody and conjugate reached determination in the same order, with calculations following these parameters.

$$Specificity = \frac{\text{TNR}}{\text{TNR} + YoIN} \times 100\%$$

Where TNR (true negative results) is the number of negative results, and FPR (false positive results) is the number of false positive results;

$$Sensitivity = \frac{\text{TPR}}{TPR + FNR} \times 100\%$$

Where TPR (true positive results) is the number of positive results, and FNR (false negative results) is the number of false negative results.

The optimal concentrations for the interaction of the components of the test kit, developed for the determination of the ELISA diagnostic titer, were successful in determining and presenting the results section (Dmitriev*, 2020*).

RESULTS

The enzyme immunoassay process is the most sensitive method, especially used for the diagnosis of viruses (Al-Mrabeh *et al.,* 2009; Lakshmipriya *et al.,* 2016). Therefore, for this purpose, it is important to first determine the specific polyclonal antibody concentration for potato (*S. tuberosum* L.) virus X (PVX). The reason is that determining the sensitivity of the necessary reagents in the experiment provides the quality of the diagnosis. However, several versions of ELISA exist, with the polyclonal antibody titer to the virus determined using the wrong version of the ELISA test.

In the experiment, leaving a polystyrene tablet designed for ELISA (Thermo Fisher Scientific, USA) overnight was at +4 °C for sorption of Ag (purified PVX). On the next day, a 1% bovine serum albumin (BSA) solution served to close the open pores. Then, the polyclonal Ab (antibody) reached dilutions of 1:100, 1:1000, 1:10,000, 1:100,000, 1:150,000, 1:200,000, and 1:300,000 before absorbance at 37 °C for one hour. After each sorption, using carbonate buffer (PBS pH 9.5) served for washing. Washing off the excess antibody, the addition of the conjugate (goat antirabbit Ab+peroxidase) proceeded before sorbing for one hour at 37 °C. The excess conjugate also received washing off five times, followed by adding TMB (tetramethylbenzidine chromogen) and keeping it in a thermostat for one hour at 37 °C. After the specified time, the addition of reaction stop reagent (0.2M sulfuric acid) continued, and after 20 minutes at 37 °C in a thermostat, the result examination by ELISA analyzer (ELX808) for OD value at 450 nm and visualization ensued (Table 1).

The results entailed determination by diluting the polyclonal Ab, as given in Table 1. According to the said process, Ab titer diluted 100, 200, and 500 times appeared to be 'OUT'; when diluted 100,000 times, it was 1251; and when diluted 150,000 times and above, the Ab titer proved to be low. The results detection was by the '+' sign by counting the virus in the samples based on the formation of light and dark colors upon visual examination. It was also distinct from the said table that the sign

Table 1. Determination of specific polyclonal Ab titer obtained for PVX using ELISA.

Antibody dilution indicator		OD	Visual signs
K		OUT	++++
100	($1{,}0\times10^{-2}$)	OUT	++++
200	($2{,}0\times10^{-2}$)	OUT	++++
500	($5{,}0\times10^{-2}$)	2.901	++++
1000	($1{,}0\times10^{-3}$)	2.589	++++
10.000	($1{,}0\times10^{-4}$)	1.722	+++
100.000	($1{,}0\times10^{-5}$)	1.251	+++
150.000	($1{,}5\times10^{-5}$)	0.389	-
200.000	($2{,}0\times10^{-5}$)	0.134	-
300.000	($2{,}0\times10^{-5}$)	0.087	-

Note: here 0.500–0.799 nm "+"; 0.800–1.199 nm "++"; 1.200–1.499 nm "+++"; 1.500 nm and above means "++++" and "-" means low titer. The recording of "OUT" occurs when the optical density exceeds 3,000 when checking ELISA.

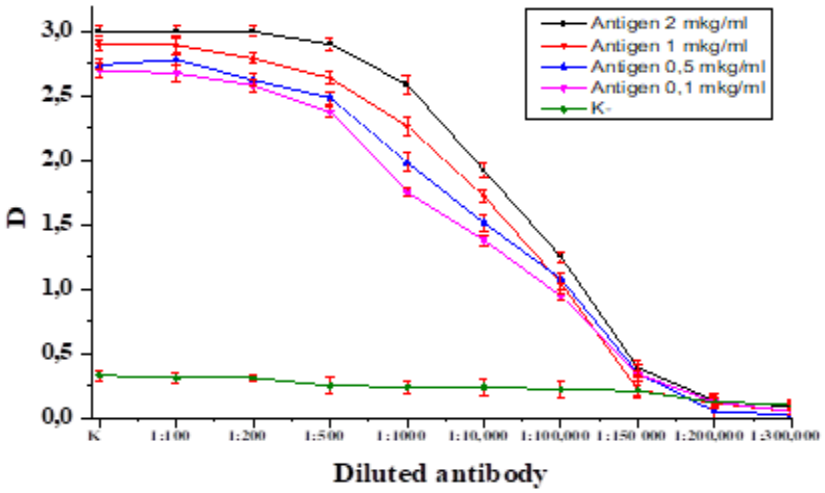

Figure 1. Graphic representation of the Ab titer obtained for PVX using the ELISA method. Control – BSA (bovine serum albumin, 100 µg/ml). K – represents the undiluted state of the antibody, D (450 nm).

'++++' emerged when Ab was not diluted and diluted 100, 1000, and 100,000 times during visual inspection. However, no reaction was evident when diluted 150,000 and above, marked with the sign'-'. An 'OUT' was notable when an optical density reading was above 3,000, which revealed that the antibody concentration was high. The antigen amount was also important in determining the titer of specific polyclonal antibody obtained for PVX. For this reason, different amounts of antigen, i.e., purified PXV, sustained addition to the tablets intended for ELISA. The bull serum albumin used served as a control, with the results shown graphically in Figure 1.

As shown in Figure 1, the antibody titer was highest in the undiluted state and gradually decreased with increasing dilution, approaching zero at high dilutions. However, a low but detectable titer was still evident at a dilution of 1.5×10^{-5} (1:150,000). A healthy plant sample and buffer served as negative controls, with no optical density signal recorded in these samples. The results indicated the optimal working antibody titer was 1.0×10^{-5}, allowing a larger number of samples for efficient testing. This optimized titer was subsequently applicable in developing the PVX immunodiagnostic assay based on the produced polyclonal antibodies.

Rapid Sandwich-ELISA kit development for PVX detection

It is typical that in the sandwich variant, the ELISA test kits are distinctive with their sensitivity and specificity, and that is why the said method has had more usage than others. Antigen is mainly identifiable by the sandwich method. For this purpose, antibodies against the antigen first underwent sorbing, and then test samples (antigens), specific antibodies against the antigen, and finally, the conjugate sustained dripping. The primary and secondary antibodies' amount was also optimal in this research work.

First, the primary antibodies attained adsorption to obtain an immunosorbent. In determining the optimal concentration of antibodies, the antiserum obtained by immunizing animals entailed diluting to 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000 dilutions in carbonate buffer, and after sorption, the open pores gained closing with a 1% bovine serum albumin (BSA) solution. Afterward, ELISA analysis proceeded with 25 healthy and 25 PVX-infected potato plant samples. The test results continued by evaluating the sensitivity and specificity of the analysis, which increased (Figure 2).

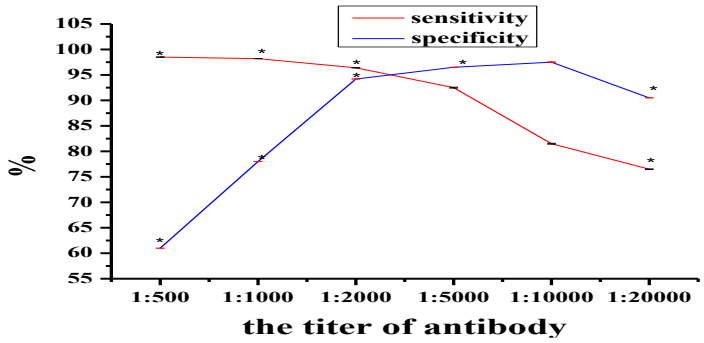

Figure 2. Optimization of primary antibody titer for sandwich variant ELISA assay.

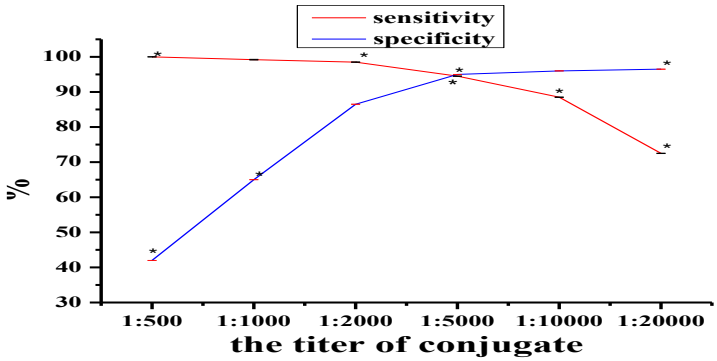

Figure 3. Optimization of secondary antibody titer for sandwich variant ELISA.

According to the obtained results, selecting 1:2000 diluted antiserum-sorbed samples was the most optimal option. The levels of sensitivity and specificity were 96.4% and 94.2%, respectively. However, in other variants, it was apparent that one of the results of sensitivity and specificity showed a higher value, but the variants with high sensitivity showed low specificity and vice versa. Moreover, this determined the optimal amount of antibody, which was also taken as the optimal concentration for the antibody instilled after the antigen.

After optimizing the primary antibody concentration, the secondary antibody dilution also received refinements. Given the technical limitations, however, the antigen concentration could not be optimized. An IgG–peroxidase enzyme conjugate raised against rabbit IgG (Thermo Scientific, USA) served as the secondary antibody. The conjugate underwent testing at the following dilutions to determine the optimal working concentration: 1:500, 1:1000, 1:5000, 1:10000, 1:15000, and 1:20000. The results gained assessment by calculating the sensitivity and specificity of the assay (Figure 3). Sensitivity and specificity evaluations used ELISA readings from 25 healthy and 25 PVX-infected potato samples. To further enhance assay specificity and reduce nonspecific binding, a 1% BSA succeeded its incorporation into the procedure (Figure 3).

According to Figure 3, the sensitivity and specificity of the samples performed with secondary antibodies diluted 1:5000 became selected as the optimal option. The levels of sensitivity and specificity were 94.5% and 95.0%, respectively. In samples with high levels of secondary antibody, the sensitivity level reached 100%; however, conversely, the specificity level also decreased. The results further revealed the level of sensitivity was relatively low in the samples with a level of specificity higher than 95%. Therefore, the

above-mentioned option resulted in the final selection for the best option.

Moreover, in Figure 3, the use of secondary antibodies diluted at 1:5000 provided the optimal diagnostic balance, yielding sensitivity and specificity values of 94.5% and 95.0%, respectively. Although higher antibody concentrations increased sensitivity to 100%, this occurred at the cost of reduced specificity. Conversely, samples with specificity above 95% showed a noticeable decline in sensitivity. Therefore, the 1:5000 dilution became the choice as the most suitable and reliable option.

Selected tubers came from each of the existing potato cultivars, viz., Folva, Romantika, Piskom, Aureta, Evolution, Bog'izag'on, Umid, Spunta, Royal, Smega, Tuvong, Gala, C-02, C-5, C-12, C-2, C-09, and C-1. Based on the developed ELISA test kit for the diagnosis of PVX, a study proceeded to determine the incidence rate of PVX in the tubers by selecting five tubers of each potato cultivar. Based on the sandwich version of the developed ELISA, the research results are available in Table 2.

For this purpose, the growing point of each selected nodule sustained cutting, homogenization, and centrifugation, with a test sample prepared. By examining the samples with the sandwich variant of the optimized ELISA, first, the virus-specific primary antibody received sorbing on polystyrene plates with the liquid extracted from potatoes grown under the conditions of Uzbekistan, namely Ag, before immobilizing the conjugate. The results, determined by using an ELISA analyzer (Elx808), had a specified time after adding the substrate.

In this study, the promising results obtained came from the research conducted for the development of ELISA test kits for the diagnosis of PVX. The concentration of PXV was not determined in potato cultivars such as Folva, Romantika, Piskom, Aureta, C-02, C-5, C-12, and C-2. Studies on sandwich variants have shown high sensitivity and specificity (>95%) and appeared crucial for the accurate detection of PVX in potato genotypes.

Table 2. Determination of PVX in potato variety samples using the ELISA method.

No.	Potato cultivars	Optical density 450 nm (number of tubers taken for verification)					Tubers'average incidence rate (%)
		1	2	3	4	5	
1	Folva	0.304	0.301	0.289	0.296	0.300	10.0
2	C-02	0.265	0.261	0.252	0.280	0.256	8.7
3	C-5	0.242	0.245	0.235	0.180	0.210	7.4
4	Romantika	0.217	0.185	0.205	0.210	0.192	6.7
5	Aureta	0.173	0.160	0.170	0.176	0.164	5.6
6	Piskom	0.217	0.218	0.225	0.204	0.210	7.2
7	C-12	0.167	0.165	0.163	0.168	0.167	5.5
8	C-2	0.261	0.263	0.261	0.280	0.251	8.8
9	Bog'izag'on	2.850	2.905	OUT	2.865	2.874	96.0
10	C-09	2.874	2.874	2.850	2.864	OUT	83.1
11	C-1	2.575	2.600	2.575	2.578	2.575	86.0
12	Umid	OUT	OUT	2.950	OUT	OUT	99.7
13	Spunta	OUT	OUT	OUT	OUT	2.985	100.0
14	Royal	OUT	OUT	OUT	2.997	OUT	100.0
15	Evolution	2.374	2.386	2.565	2.400	2.381	80.7
16	Smega	OUT	OUT	OUT	OUT	OUT	100.0
17	Tuvong	OUT	OUT	OUT	OUT	OUT	100.0
18	Gala	OUT	OUT	OUT	OUT	OUT	100.0

Note: "OUT" was notable when the peak reading was above 3.000 readings when checked with the ELISA analyzer. A reading below 0.400 means no virus detected. The average indicator of the results of each tested node appears in %. In this case, the highest (100%) was calculated against 3.000.

DISCUSSION

In this research, the optimized DAS-ELISA protocol for detecting potato virus X (PVX) in *S. tuberosum* L. demonstrated strong diagnostic performance, achieving approximately 96% sensitivity and 94%–95% specificity. These results align with expectations for well-optimized ELISA systems that adhere to established immunochemical principles (Egorov *et al*., 1991; Kharitonova and Israfilova, 2018). Efficient antigen adsorption onto polystyrene plates, blocking with 1% BSA, and the use of carefully optimized dilutions for both primary and secondary antibodies significantly enhanced the signal-to-noise ratio while minimizing nonspecific reactions. Overall, the findings are consistent with global efforts to improve ELISA sensitivity for PVX detection through advanced reagents and refined assay formats (Uhde-Holzema *et al*., 2010; Lakshmipriya *et al*., 2016; Panferova *et al*., 2019).

Field samples revealed the highest infection frequencies (≈80%–100%) in certain potato cultivars and clones, underscoring the phytosanitary importance of PVX under Uzbekistan conditions. Study results also attained considerable support from previous studies that regional edaphic and climatic factors can favor the emergence of aggressive isolates (Anisimov *et al*., 2009; Fayziev *et al*., 2023). Conversely, several potato cultivars and clones examined emerged negative for PVX, indicating that 'clean' genetic material is available for breeding and seed production, consistent with the practice that virus-free planting stock underpins the highest yields (Ekaterinskaya, 2019; Beisembina, 2021).

From a diagnostic perspective, the optimized DAS-ELISA proved to be well-suited for seed certification, monitoring of mother plants and reservoirs, and supporting meristem-based 'virus-free' schemes (Jovlieva *et al*., 2024). Owing to its affordability, procedural simplicity, and high throughput, ELISA enables rapid screening of large sets of plant samples (Isakov and Isakov, 2014). Nevertheless, because mixed infections (PVX+PVS+PVY) were common in potatoes, researchers should consider potential cross-reactivity and antigenic variability (Eskendirova *et al*., 2014; Fayziev *et al*., 2020). In such scenarios, confirmatory testing, such as parallel assays using locally produced high-titer polyclonal antisera, can also strengthen the results (Sattorov *et al*., 2020).

Operationally, early ELISA-based detection, combined with vector control, field sanitation, and cultivar rotation, can substantially limit crop yield losses (Oripov and Khalilov, 2007). Looking forward, ecologically safe bioproducts derived from fungal and bacterial systems represent a promising pillar of integrated PVX management (Channarayappa and Nayaka, 2020). The packaging diagnostics and monitoring, in concert with virus-free seed systems, will be equally important to interrupt transmission cycles. In conclusion, the presented DAS-ELISA provides a rapid, reliable, and cost-effective tool for PVX detection under local conditions, informing critical decisions across the breeding and seed supply chains and reinforcing regional phytosanitary security (Fayziev, 2019; Panferova *et al.,* 2019; Jovlieva *et al.,* 2024).

CONCLUSIONS

In Uzbekistan, mixed infections of PVX and other viruses can cause yield losses of 25%–30% and even more in potato (*S. tuberosum* L.) crops. The presented study aimed to maintain potato yields based on the virus-free planting material and strict agronomic practices. The recommended DAS-ELISA settings (primary 1:2000, secondary 1:5000) achieved the highest sensitivity (~96%) and specificity (~94%–95%), enabling early field detection, rapid segregation of high-risk cultivars and clones, and assembly of 'clean' initial breeding materials. This approach strengthens the seed certification, monitoring of mother plants and reservoirs, and the effectiveness of meristem-based 'virus-free' programs. By integrating a two-tier ELISA+RT-PCR scheme with vector control, field sanitation, and eco-safe bioactive products, scientists will further enhance the potato yield stability and regional phytosanitary security.

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