THE INFLUENCE OF SAMPLES INCUBATION ON DETECTION OF PLRV AND THE EFFECT OF SOME EXTRACTION BUFFER'S ADDITIVES ON THE DETECTION OF POTATO VIRUSES Y, A, X AND S BY ELISA TECHNIQUE

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Abstract. The goal of this research was to examine and evaluate the effects of several modifications of ELISA (enzyme linked immunosorbent assay) technique on the detection of potato viruses Y, A, X, S and potato leafroll virus by this technique. These modifications consisted on: modification of the incubation modality of conjugate (IgG-AP), the use of several additives in extraction buffer, replacement of grinding buffer with McIlvain buffer.

The results show a better identification of PLRV in leaves and sprouting tubers using the co-incubation sample and IgG-AP conjugate. Compared with the classical method, the test safety and sensitivity increased. Testing leaves, the average values of OD at 405 nm was 5-6,6 times higher than those obtained by standard DAS ELISA method and using sap from sprouting tubers (dilution 1/10) this average was 1,5 times higher.

The detection of potato viruses Y and A by enzyme–linked immunosorbent assay (ELISA) can be improved using extraction buffers with new composition. Using McIlvain's phosphate-citric acid buffer (0,18M; pH 7), the absorbance values (A_{405nm}) increased significantly for PVY and PVA detection comparing with the classic extraction buffer. Sodium diethyldithiocarbamate (0,01M) in phosphate-buffered saline plus Tween 20 (PBS-T) used instead of the polyvinylpyrrolidone increased the sensitivity of potato virus Y but this additives decrease the absorbance values in case of PLRV identification. The same decrease was observed when we used sodium thioglicolat (0,01M) and sodium diethyldithiocarbamate (0,01M) in PBS-T. The co-incubation sample and conjugate and the use of McIlvain's buffer could save time and costs of potato viruses diagnostic tests.

Keywords: potato virus, co-incubation, extraction buffer, ELISA.

INTRODUCTION

The employment of high sensitive methods of detection and identification of nucleic acids, allowing virus detection directly in plant extract, is still difficult for routine indexation of potatoes seed because of the high cost, complicated samples preparation and need highly trained personal to perform this kind of work [5 -14]. So, ELISA is the most commonly assay for detection of virus particles in potato tissues. A lot of researches were targeted to make modifications of this assay their purpose being to increase its performance or to enhance its sensitivity [8, 15, 16].

The detection level of the viruses and the rate of immunological reaction depend on which part of the plant is used for assay and on several physico-chemical factors like: temperature, diffusion of components in reaction (mixing), buffers composition [3, 4, 15, 16].

The goal of this research was to examine and evaluate the effects of several modifications on the detection by ELISA for potato viruses Y, A, X, S and potato leafroll virus. These modifications are:

-the modification of IgG-AP conjugate incubation

-the use of several additives to extraction buffer

-the replacement of extraction buffer with McIlvain's phosphate-citric acid buffer (0.18M; pH 7).

MATERIALS AND METHODS

a. The effect of samples incubation on detection of PLRV (potato leafroll virus)

Potato material. All the biological material (healthy and infected) was obtained from the virus collection of our institute. We used 29 infected samples. The infection of this material was confirmed by using antiserum from Bioreba (Switzerland). A press with smooth roles was used for leaf samples preparation. For the tuber testing, the sap was extracted, diluted and dispensed directly into the plate using the extractor Microlab 500B/C **(**Hamilton**)**. We tested sprouting tubers after natural break of dormancy, when the sprouts were 2-3mm long.

Antibodies and conjugates dilution was as recommended by the manufactures 1:200 for Loewe (Germany) and 1:1000 for Bioreba AG (Switzerland).

Microplates - NUNC microplates were coated with antibodies for overnight incubation in the refrigerator.

DAS ELISA- (V1). The analysis was performed following essentially the protocol described by Clark and Adams (1977) [2]. We used 100 µl from each reactives solutions in each well of the plate [1, 2].

In the other variant (V2, cocktail ELISA), all the steps were the same as DAS ELISA except that the sample and the enzyme conjugate IgG were added together and incubated overnight at 4^0C. All experiments were repeated four times. Rinsed microplates were filled with substrate solution (p-nitrophenylphosphate) incubated 30, 60 and 120 minute and the absorbance values were estimated at 405 nm (A_{405}) on PR1100 reader. The samples having A_{405} values exceeding the cut-off (two times the average of healthy control samples) were considered virus infected.

b. The effect of extraction buffer's additives on the detection of potato viruses Y (PVY), A (PVA), X (PVX) and S (PVS) by ELISA technique

Virus samples were obtained from infected plant leaves (we used 18 plants infected from Record variety for PVY, 18 plants infected from Corona variety for PVA,18 plants infected from Amsel variety for PVS and 18 plants infected from Bintje variety for PVX) and negative control samples were obtained from leaves of healthy plants from the same variety. We used fresh tissues that produced relatively moderate reactions in ELISA (dilution of sap from infected

leaves in healthy potato leaves sap was 1/1) [1]. First we mixed the sap from infected leaves with sap from healthy potato leaves and next we mixed this sample with extraction buffer. End dilution of infected sample in extraction buffer was 1/10.

Excepting potato virus A detection, the antiserum and conjugated used for the other viruses detection were obtained in our laboratory.

The extraction buffers used were:

- classic buffer (2% polyvinylpyrrolidone in PBS-T; pH 7) [2],
- McIlvain's phosphate-citric acid buffer (0.18M; pH 7),
- sodium diethyldithiocarbamate (DIECA) (0,01M) in PBS-T,
- sodium diethyldithiocarbamate (DIECA) (0,01M) and sodium thioglicolat (0.01M)in PBS-T.

PBS-T = phosphate-buffered saline with Tween 20 (0.05%) [2].

The analysis was performed following essentially the protocol described by Clark and Adams (1977) [2]. We used 100 µl from each reactive solutions in each well of the plate. All experiments were repeated five times. The optical density (absorbance values) (OD) was measured after 60 minutes, on PR1100 reader.

Each set of comparable assay was conducted at the same time and with the same bulk sample. Analysis of variance (ANOVA) and Duncan's multiple range test were used to analyze the data. In the aim to illustrate the precision of the mean we use confidence interval (CI).

RESULTS

a. The effect of samples incubation on detection of potato leafroll virus (PLRV)

As shown in Table 1, the sensibility of detecting PLRV in leaves was correlated to the incubation modality of the samples and the incubation time with substrate solution. In comparison with the classical method, the test safety and sensitivity increased. Testing leaves, the mean values of OD at 405 nm was 5-6.6 times higher than those obtained by standard DAS ELISA method.

Significantly higher readings were obtained applying cocktail ELISA, this variant improving the detection of potato virus particles.

Fig. 1 shows that the co-incubation sample and IgG conjugate gave positive reaction in 100% of the testing

Table 1. Detection PLRV in leaves by DAS ELISA* and COCKTAIL ELISA.

	Incubation time with substrate solution					
	30 minutes		**60 minutes**		**120 minutes**	
	V1*	**V2****	**V1***	**V2****	**V1***	**V2****
Cut off	*0.065*	***0.084***	*0.072*	***0.113***	*0.097*	***0.171***
OD_{405nm}***	0.055 ± 0.014	**0.278 ± 0.119**	0.089 ± 0.030	**0.536 ± 0.230**	0.162 ± 0.063	**1.072 ± 0.450**
		x 5.0		x 6.0		x 6.6

Note: * V1 – DAS ELISA
** V2 – COCKTAIL ELISA (co-incubation samples and IgG-AP conjugate)
*** – mean values of OD at 405 nm for 4 repetitions ± standard deviation

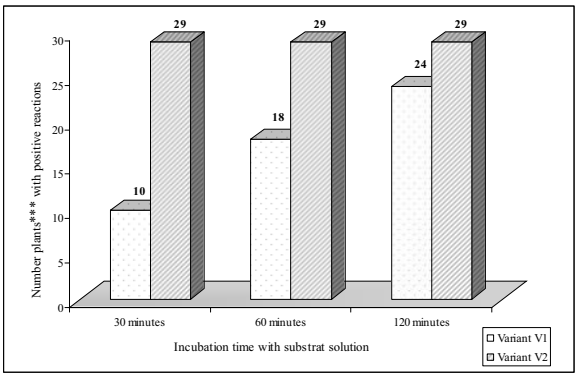

Figure 1. Detection of PLRV in leaves function on the samples incubation modality. Biological material used (***) for leaves samples =29 plants infected with PLRV. V1 = DAS ELISA ; V2= COCKTAIL ELISA (co-incubation samples and IgG-AP conjugate) Experiments were repeated on 4 occasions, with the same results.

plants even after the shortest incubation time with substrate solution.

When we used DAS ELISA, the percentage of infected plants grew with the time of substrate incubation from 37,5 to 82,8%, but the maximum percentage wasn't achieved not even after 2 hours despite the assays were made with plants sure infected. So, using the standard method, 17% of the samples didn't lead to positive reaction.

Testing sprouting tubers, the results showed a better identification of PLRV using the co-incubation sample and IgG-AP conjugate at all the sap's dilutions (Table 2). Using the co-incubation sample with conjugate, for sap's dilution 1/10 and 1/20, the infected tubers were easily distinguished from negative controls. PLRV has been practically undetectable by DAS ELISA in tuber extracts diluted more than 20 times, while in the cocktail version, readings differentiate extracts from infected tissues, but not very clearly.

The Fig. 2 shows that the highest values of OD at 405 nm were those obtained by cocktail ELISA. when the tuber extract was diluted 10 times, after 2 hours substrate incubation. The mean values was in this case 1.5 times higher than those obtained by standard DAS ELISA method.

Table 2. Virus detection in sprouting tubers fonction on the samples incubation modality.

Dilution of the sap	Incubation time with substrate solution(minutes)	V1*		V2**	
		Healthy	OD_{405nm} ± SD***	Healthy	OD_{405nm} ± SD***
1/10	60	0.025	**0.082 ± 0.036**	0.031	**0.103 ± 0.017**
	120	0.030	**0.155 ± 0.060**	0.037	**0.227 ± 0.042**
1/20	60	0.027	**0.050 ± 0.014**	0.030	**0.065 ± 0.007**
	120	0.036	**0.095 ± 0.043**	0.038	**0.132 ± 0.019**
1/40	60	0.026	**0.035 ± 0.009**	0.031	**0.048 ± 0.006**
	120	0.030	**0.058 ± 0.017**	0.037	**0.089 ± 0.014**

Note: * V1 – DAS ELISA
** V2 – COCKTAIL ELISA (co-incubation samples and IgG-AP conjugate)
*** – mean values of OD at 405 nm for 4 repetitions ± standard deviation
The sap was extracted from the rose end of 29 sprouting tubers (after natural break of dormancy).

b. The effect of extraction buffer's additives on the detection of potato viruses Y (PVY), A (PVA), X (PVX) and S (PVS) by ELISA technique. Using McIlvain's buffer, the absorbance values increased by 63.5% for PVA detection comparing with the classic extraction buffer, and by 32.46% in case of PVY detection (Table 3 & Fig. 3A).

Table 3. Effect of extraction buffer on the diagnostic sensitivity of DAS ELISA test* (mean values of OD at 405nm for 5 repetitions ±SD values).

Virus	Sample	Extraction buffer			
		Classic buffer	McIlvain buffer	DIECA(0.01M) in PBS-T	DIECA+sodium thioglicolat in PBS-T
PVY	Infected	1.251 ± 0.317 b**	**1.668 ± 0.259 a**	1.784 ± 0.189 a	1.675 ± 0.260 a
	Healthy	0.037 ± 0.004 -	0.034 ± 0.002 -	0.033 ± 0.001 -	0.033 ± 0.002 -
	Buffer	0.027 ± 0.002 -	0.028 ± 0.001 -	0.029 ± 0.001 -	0.027 ± 0.001 -
PVA	Infected	0.648 ± 0.293 c	**1.146 ± 0.109 a**	0.899 ± 0.115 b	0.699 ± 0.122 c
	Healthy	0.038 ± 0.009-	0.036 ± 0.012-	0.038 ± 0.008-	0.035 ± 0.010-
	Buffer	0.027 ± 0.007-	0.026 ± 0.009-	0.030 ± 0.010-	0.029 ± 0.008-
PVS	Infected	>2.000	**>2.000**	>2.000	>2.000
	Healthy	0.032 ± 0.009	0.035 ± 0.012	0.033 ± 0.008	0.032 ± 0.010
	Buffer	0.028 ± 0.007	0.026 ± 0.009	0.024 ± 0.010	0.024 ± 0.008
PVX	Infected	>2.000	**>2.000**	>2.000	>2.000
	Healthy	0.045 ± 0.011	0.048 ± 0.012	0.044 ± 0.014	0.047 ± 0.012
	Buffer	0.030 ± 0.009	0.029 ± 0.011	0.031 ± 0.009	0.029 ± 0.008

Note: * For the samples we used fresh tissues that produced relatively medium ELISA reactions (dilution of the sap from infected plants in sap from health plants was 1/1)
** Values not followed by the same letter are significantly different (P=0.05) according to Duncan's test.

Sodium diethyldithiocarbamate (0.01M) in PBS-T (phosphate-buffered saline + Tween 20) used instead of the polyvinylpyrrolidone (2%) in PBS-T increased the sensitivity of potato virus Y (by 38.4%) but this additives decrease the absorbance values in case of PLRV identification. The same decrease was observed when we used sodium thioglicolat (0.01M) and sodium diethyldithiocarbamate (0.01M) in PBS-T. The detection of potato virus X and S was not significant influenced by the composition of extraction buffer.

DISCUSSIONS

The results presented in this paper show a better identification of PLRV in leaves and sprouting tubers (after natural break of dormancy) using the co-incubation sample and IgG-AP conjugate, together in the wells of the ELISA microplates. This method has been used by others researchers, in different conditions [15, 16]. Particles of IgG immobilized on the well surface are used to entrap virus particles, which bind conjugate particles at the same time, resulting in formation of multilayer structure of antibody-antigen-enzyme complex. This system allows binding higher amounts of virus and conjugate particles than occurs in regular DAS-ELISA [15, 16].

Significantly higher readings were obtained applying cocktail ELISA, improving the detection of potato virus particles. Compared with the classical method, the test safety and sensitivity increased. Testing leaves, the average values of OD at 405 nm was 5-6.6 times higher than those obtained by standard DAS ELISA method and using sap from sprouting tubers (dilution 1/10) this average was 1.5 times higher. Testing tubers, comparative with the other results reported [4, 15] our extinctions values (Table 2) were not so high because we don't made the pre-incubation of samples. Like in the other research experiments [15], the results obtained in the detection of PLRV in leaves were better than in tubers (because of the low concentration of the virus in tubers).

As shown in this paper, the detection of potato viruses Y and A by enzyme–linked immunosorbent assay (ELISA), can be increased using extraction buffers with new composition tested in our research work. The effect of additives like sodium diethyldithiocarbamate and sodium thioglicolat were tested by other researchers too [3].

The sensitivity of ELISA for PVY and PVA were especially enhanced by using McIlvain's buffer, its composition maybe stabilize the nucleocapside of these viruses and thereby enhance its serological reactivity

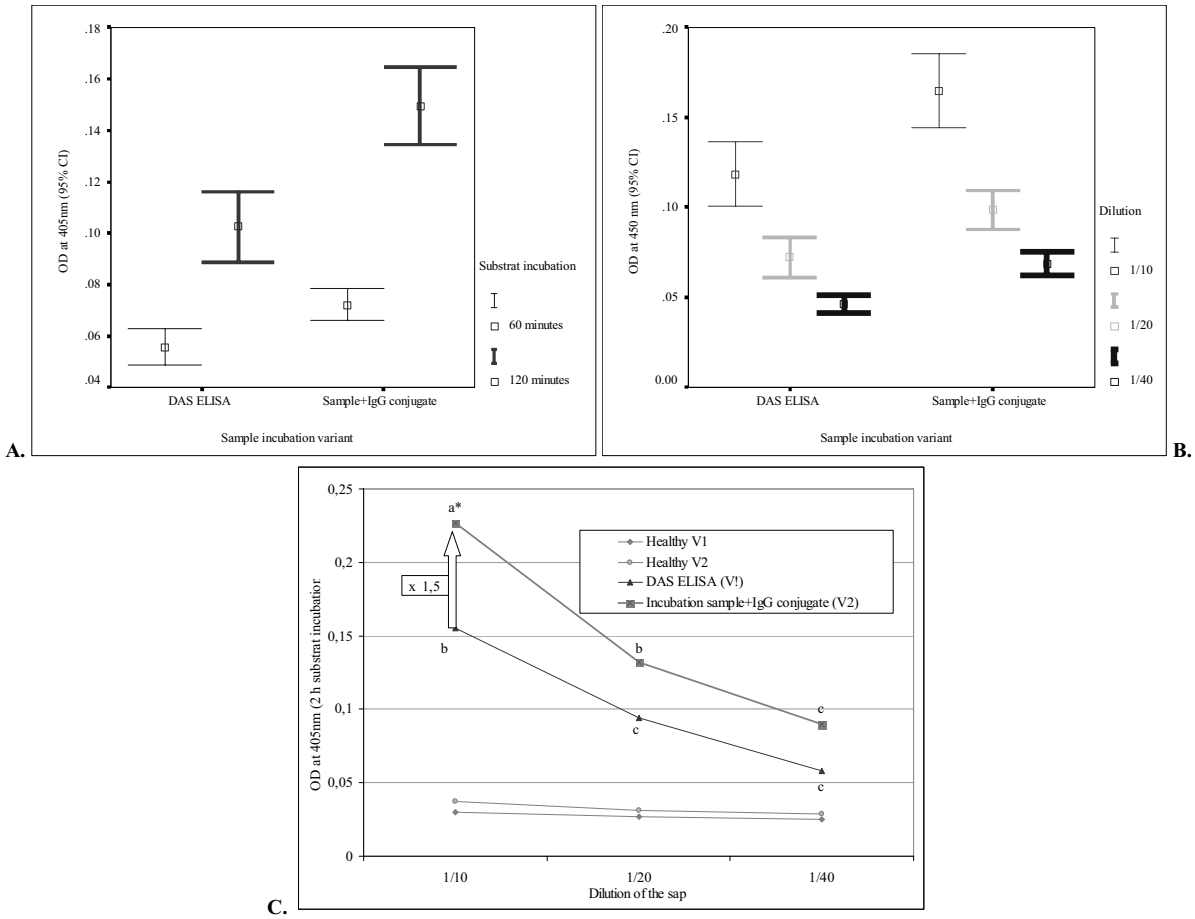

Figure 2. PLRV detection in sprouting tubers: **A.** Influence of substrat incubation time and sample incubation variant on potato leafroll virus detection (infected samples, dilution of the tuber's sap in extracting buffer=1/10); **B.** Influence of sap dilution and sample incubation variant on potato leafroll virus detection (infected samples, substrat incubation time =2 hours); The sap was extracted from the rose end of sprouting tubers; Dilution = dilution of the tuber's sap in extracting buffer (v/v); 95% CI=95% confidence interval of the difference; **C.** The effect of sap dilution and sample incubation variant on optic density (OD_{405nm}) values obtained in PLRV detection by ELISA (healthy and infected samples; substrat incubation time =2 hours) Values not followed by the same letter (*) are significantly different (P=0.05) according to Duncan's test; experiments were repeated on 4 occasions.

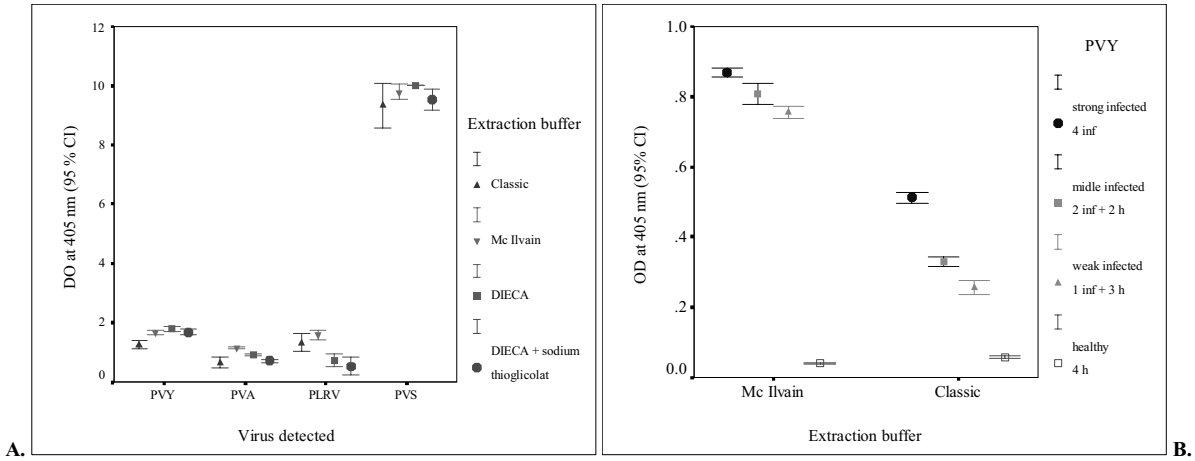

Figure 3. Influence of McIlvain's buffer and of some extraction buffer's additives on the detection of potato viruses Y, A, X and S by ELISA technique **A** - Influence of extraction buffer on potato leafroll virus, potato viruses Y, A, S detection (fresh samples); **B -** Influence of McIlvain's buffer on PVY detection (frozen leaves) (tissue samples that produced different levels of infection); virus source plant were selected to produce relatively strong. moderate or weak virus specific reactions in ELISA; 95%CI - 95% confidence interval of the difference.

[3]. We observed that using McIlvain's buffer, the absorbance values increased by 63.5% for PVA detection comparing with the classic extraction buffer and by 32.46% in case of PVY detection. Comparative with the results reported by other researchers [3, 15], in our experiments, sodium diethyldithiocarbamate (0.01M) in PBS-T used instead of the classic buffer increased the sensitivity of potato virus Y (by 38.4%) but this additive decrease the extinctions values in case of PLRV identification. Comparative with other

experiments [3, 15], for PVY detection we used sap from frozen leaves too.

The results achieved testing extraction's buffer additives show a better identification (significant differences) for PVY and PVA in potato leaves (sap's dilution 1/10) using McIlvain's phosphate-citric acid buffer (0.18M; pH 7). This buffer don't use polyvinylpyrrolidone. an expensive additive and difficult to dissolve in PBS-T. The McIlvain's buffer improves the detection for PVY even in frozen potato leaves. The results presented in Fig. 3B show a better identification (significant differences) using McIlvain's phosphate-citric acid buffer, comparatively to the classic variant. for all infection levels. Using this buffer, the test was more sensitive that the standard test. It detects low concentration of virus with greater reliability even for the weak infection level of sample.
The co-incubation sample and conjugate and the McIlvain's phosphate-citric acid buffer used as extracting buffer could be two possibilities for saving time and costs of potato viruses diagnostic tests.

REFERENCES

[1] Bădărău, C.L., Cojocaru, N., Ianosi, M., Rusu, S.N., (2008): The effect of samples incubation modality on detection of PLRV by ELISA technique in leaves and sprouting tubers. pp. 558-561, In: Chiru S.N., Olteanu G., Badarau C.L. (eds.) Potato for a changing world. 17th Triennial Conference of the EAPR. Transilvania University of Brasov Publishing House, Brasov.

[2] Clark, M.F., Adam, A.N., (1977): Characteristics of microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. J. Gen Virol., 34: 475-483.

[3] Goodwin, P.H., Bantarri, E.E., (1984): Increased sensitivity of ELISA for potato viruses S, X and Y by polystyrene pretreatments. additives and modified assay procedure. Plant Diseases. vol 68(11): 944-948.

[4] Kaniewski, W.K., Thomas, P.E., (1988): A two-step ELISA for rapid, reliable detection of potato viruses. Am. Potato Journal. 65: 561-571.

[5] He C., Molen T. A., Xiong X., Boiteau G., Nie X. (2006): Cytocrome c oxidase mRNA as an internal control for detection of Potato virus Y and Potato leafroll virus from single aphides by coamplification RT-PCR assay. J. Virol. Methods 138: 152-159

[6] Leone, G., van Schijndel, H.B**.,** van Genien, B., Schoen, C.D., (1997): Direct detection of potato leafroll virus in potato tubers by immunocapture and the isothermal nucleic acid amplification method NASBA. J. Virological. Meth., 66 (1): 19-27.

[7] Loebenstein G., Akad F., Filatov V., Sadvakasova G., Manadilova AS., Bekelman H., Teverovsky, E., Lachmann, O., David, A., (1997): Improved detection of potato leafroll luteovirus in leaves and tubers with a digoxigenin-labeled cRNA probe. Plant Dis., 81 (5): 489-491.

[8] Martin, R.H., (1990): ELISA methods for plants viruses. pp. 179-196. In Hampton, R., Ball, E., De Boer, S. (ed), Serological methods for detection and identification of viral and bacterial plant pathogens: Laboratory Manual. Amer Phytopathological Society.

[9] Nolasco, G., De Blas, C., Torres, V., Ponz, F., (1993): A method combining immunocapture and PCR amplification in microtiter plate for the detection of plant viruses and subviral pathogens. J. Virol. Meth., 45: 201-218.

[10] Nie X. and Singh R. P. (2000) Detection of multiple potato viruses using an oligo(dT) as the common cDNA primer in multiplex RT-PCR. J. Virol. Methods 86: 179-185

[11] Nie X. and Singh R. P. (2001) A novel usage of random primers for multiplex RT-PCR detection of virus and viroid in aphids, leaves and tubers. J. Virol. Methods 91: 37-49

[12] Nie X. (2005): Reverse Transcription Loop-Mediated Isothermal Amplification of DNA for Detection of *Potato virus Y*. Plant Dis., 89 (6):605-610.

[13]Singh R.P. and Nie X. (2003): Multiple virus and viroid detection and strain separation via multiplex reverse transcription-polymerase chain reaction. Canadian Journal Plant Pathology 25:127-134

[14]Singh R.P., McLaren D. L., Nie X. and Singh M. (2003). Possible escape of recombinant Potato virus Y by the serological indexing of certified seeds and methods of its detection. Plant Dis., 87 (6): 679-685

[15] Treder, K., Plilecki, T., Lewosz, J., (2005): Detection of PVY, PVM and PLRV in the sap of dormant tubers by COCKTAIL ELISA. Bulletin of Papers and Posters. The 16th Triennial Conference of the EAPR. July 17-22, Bilbao. Spain : 1010-1014.

[16] van den Heuvel, J.F.J.M., Peters, D., (1989): Improved detection of potato leafroll virus in plant material and in aphids. Phytopathology 79: 963-967.