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THE EFFECT OF DROUGHT STRESS AND SUPER RESEARCH REGARDING THE IMPROVEMENT OF METHODS USED FOR POTATO VIRUSES Y, A, X , S AND PLRV IDENTIFICATION

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ABSTRACT - Obtaining health and safety food impose the improvement of identification's techniques of pathogen agents. 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 (PLRV). These modifications consisted on: the use of sap extracted directly from the tubers, modification of the incubation modality of conjugate (IgG-AP), the use of several additives in extraction and conjugate buffers, replacement of grinding buffer with McIlvain buffer. The results show a better identification of PLRV in sprouting tubers using the co-incubation sample and IgG-AP conjugate. Compared with the classical method, the test safety and sensitivity increased. Using sap from sprouting tubers (dilution 1/10) the average values of OD at 405 nm was 2.5 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.Presence of proteins in conjugate buffer improve safety of viruses identification. Food gelatin was more efficiently like the bovine serum albumine (BSA) for PLRV identification. The use of new equipement, the use of McIlvain's buffer and gelatin food could save time and costs in routine diagnostic of viruses wich affect potato plants.

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Key words: Potato virus; Co-incubation; Extraction; Conjugated; Buffer; Gelatin food, ELISA.

REZUMAT – Cercetări privind îmbunătățirea metodelor utilizate pentru identificarea infecțiilor cu virusurile cartofului Y, A, X, S şi PLRV. Obținerea de alimente sigure şi curate, sănătoase, libere de boli, impune, printre altele, perfecționarea metodelor de identificare a agenților patogeni. Optimizarea tehnicii ELISA (enzyme linked immunosorbent assay), de identificare a principalelor virusuri ale cartofului (prin modificarea modalității de prelevare şi incubare a probelor, a compoziției soluțiilor tampon de extracție şi conjugat), a constituit unul dintre obiectivele acestei lucrări. Probele au fost prelevate direct din tuberculi, cu un echipament special, şi s-a modificat modul de incubare a conjugatului (IgG-AP). Rezultatele au evidențiat o identificare mai sigură a virusului răsucirii frunzelor (PLRV) în probele extrase din cartofi încolțiți (diluția 1/10), media DO la 405nm fiind de 2,5 ori mai mare decât la varianta clasică. Identificarea virusurilor Y şi A ale cartofului prin tehnica ELISA poate fi îmbunătățită prin utilizarea unor soluții tampon cu compoziție modificată. În cazul substituirii tamponului de extracție clasic cu soluție tampon McIlvain (acid citric 0.18M; fosfat monopotasic 0.18M; pH 7), la diagnosticarea virusurilor Y şi A s-a remarcat o creştere a valorilor densităților optice (DO_{405nm}) cu 63,5% la diagnosticarea virusului A şi cu 32,46% la diagnosticarea virusului Y. Substituirea polivinilpirolidonei (tampon clasic) cu dietilditiocarbamat de sodiu (0,01M) a condus la o creştere a DO_{405nm} cu 38,4% la diagnosticarea virusului Y, dar această variantă nu a fost benefică pentru identificarea probelor infectate cu PLRV. Un efect asemănător l-a avut şi utilizarea unui amestec de tioglicolat de sodiu şi dietilditiocarbamat de sodiu. Diagnosticarea virusurilor X şi S nu a fost semnificativ influențată de noua compoziție a tampoanelor de extracție. Prezența proteinelor în tamponul conjugat a condus la o identificare mai sigură a virusurilor restate.Gelatina alimentară s-a dovedit a fi mai eficientă decât albumina bovină (BSA), în cazul identificării virusului răsucirii frunzelor. Substituirea soluției tampon de extracție cu soluția McIlvain, a albuminei serice bovine (BSA) cu gelatina alimentară (în soluția tampon conjugat), utilizarea noilor modalități de prelevare şi incubare a probelor ar putea contribui la reducerea costurilor aferente testelor efectuate pentru identificarea virusurilor cartofului.

Cuvinte cheie : virus al cartofului; coincubare; extracție; conjugat; tampon; gelatină alimentară; ELISA.

INTRODUCTION

For routine indexation of potatoes seed is still difficult the employment of high sensitive methods of detection and identification of nucleic acids, that allow for virus detection directly in plant extract, because of the high cost, complicated preparation of samples and highly trained personal needed to perform this kind of work (Leone, G. *et al*.,1997; Loebenstein *et al.,* 1997; Nie, 2000; Nie *et al*., 2001 and 2005; Nolasco *et al*., 1993; Schoen *et al*., 1996; Spiegel *et al*., 1993; Teverovsky *et al*., 1997; He *et al*., 2006). 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 (Nolasco *et al*., 1993;

Singh *et al.,* 1992 and 2003; Van den Heuvel , 1989).

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), composition of buffers (Martin, 1990; Teverovsky *et al*., 1997; Treder *et al*., 2004).

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 (Martin, 1990; Treder *et al*., 2005; Teverovsky *et al*., 1997).

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 (Treder *et al*., 2004 and 2005; Van den Heuvel *et al*., 1989; Bădărău *et al*., 2008 and 2009).

As shown in this paper, the virus absorbance (A_{405nm}) of enzyme–linked immunosorbent assay (ELISA) for potato leafroll virus, potato viruses Y and A could 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 (Goodwin *et al.*, 1984).

Suppression of unspecific reactions and stabilization of reaction components like: bovine serum albumine, gelatin, ascorbic acid, sodium sulfite play a key role in this matter, increasing the level of viruses detection. The effect of several of these reactives were examinate in our researches, in the aime to increase the performance of the ELISA tests and to decrease their costs.

MATERIALS AND METHODS

a. The effect of extraction and conjugate buffer's additives on the detection of potato viruses Y, A , X and S by ELISA technique. Virus samples were obtained from infected plant leaves (we used 18 plants infected from Ackersegen 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 (Bădărău *et al.,* 2008 and 2009). 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) (Clark, Adams, 1977);
- McIlvain's phosphate-citric acid buffer (0.18M; pH 7) (Bădărău *et al.,* 2009),
- Sodium diethyldithiocarbamate (DIECA) (0,01M) in PBS-T (Goodwin, 1984; Treder, 2005),
- Sodium diethyldithiocarbamate (DIECA) (0,01M) and diethyldithiocarbamate (DIECA) (0,01M) in PBS-T (Bădărău *et al.,* 2009).

PBS-T=phosphate-buffered saline with Tween 20 (0.005%)

The proteins added to the conjugate buffers for suppression unspecific binding were: bovine serum albumin (BSA from Flucka, 0.2%), gelatin obtained from local market (cheap product used for food preparation , 0.2%).

The analysis was performed following essentially the protocol described by Clark and Adams,1977. We used 100 µl from each reactive in each well of the plate. All experiments were repeated five times.The optical density (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).

b. The effect of samples incubation on detection of PLRV. 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 antisera from Bioreba (Switzerland). A press with smooth rolles 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**)** (Clark, Adams, 1977; Hill *et al*., 1984). We tested sprouting tubers after natural break of dormancy, when the sprouts were 2-3mm long (*Fig. 1*).

Figure 1- Sample's prelevation for testing sprouting tubers

Antibodies and conjugates dilution 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. We used 100 µl from each reactives solutions in each well of the plate (Bădărău *et al*., 2008 and 2009).

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 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.

RESULTS AND DISCUSSIONS

a. The effect of extraction and conjugated buffer's additives on the detection of potato viruses Y, A, X and S by ELISA technique. Using McIlvain's buffer, the absorbances increased by 63.5% for PVA detection comparing with the classic extraction buffer, and by 32.46% in case of PVY detection (*Table 1 and Fig. 2A*).

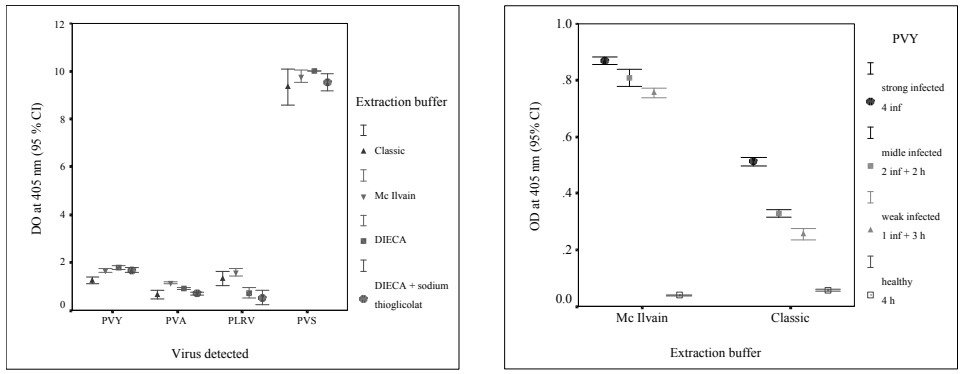
Table 1 - Effect of extraction buffer on the diagnostic sensitivity of DAS ELISA test* (mean values of OD at 405nm for five repetitions ±SD values)

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 sap from infected plants in sap from healthy plsnts was 1/1).

** Values not followed by the same letter are significantly different (P=0.05) according to Duncan's test.



A **B**

Figure 2 - Influence of McIlvaine 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.

Sodium diethyldithiocarbamate (0.01M) in PBS-T used instead of the polyvinylpyrrolidone (2%) in PBS-T increased the sensitivity of potato virus Y (by 38.4%) but this additives decrease the absorbances 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.

The sensitivity of ELISA for PVY and PVA were especially enhanced by using McIlvain buffer, their composition maybe help stabilize the nucleocapside of these viruses and thereby enhance its serological reactivity (Bădărău *et al*., 2009). Other researchers (Kaniewski *et al*., 1988; Treder *et al.,* 2005) reported other increase in sensitivity for PVY. Comparatively with their results, we observed in our experiments that using McIlvain's buffer, the absorbances increased by 63,5% for PVA detection comparing with the classic extraction buffer and by 32,46% in case of PVY detection. 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 (by 38,4%) but this additives decrease the extinctions values in case of PLRV identification. Comparatively with other experiments (Kaniewski *et al.,* 1988; Treder *et al.,* 2005) we used sap from frozen leaves too *(Fig. 2B)*.

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 additive expensive 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 *Figure 2B* 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.

Presence of proteins in buffer solution improve the detection of PLRV in extracted leaves, while detection of PVY was slightly affected by the proteins in buffer solution (*Fig. 3*). The results achieved testing conjugate buffer's additives show a better identification (significant differences) for PLRV in potato leaves (sap's dilution 1/10) using food gelatin (1%; pH 7.4) and a mixture gelatin (1%) + Congo Red (40 mg/l).This buffer don't use bovine albumine serum an additive expensive. Gelatin food was more efficient as more expensive bovine albumine serum (BSA) (*Fig. 3*). Therefore all further assays have been done (for PLRV detection) using conjugate buffer supplemented with 1% food gelatin.

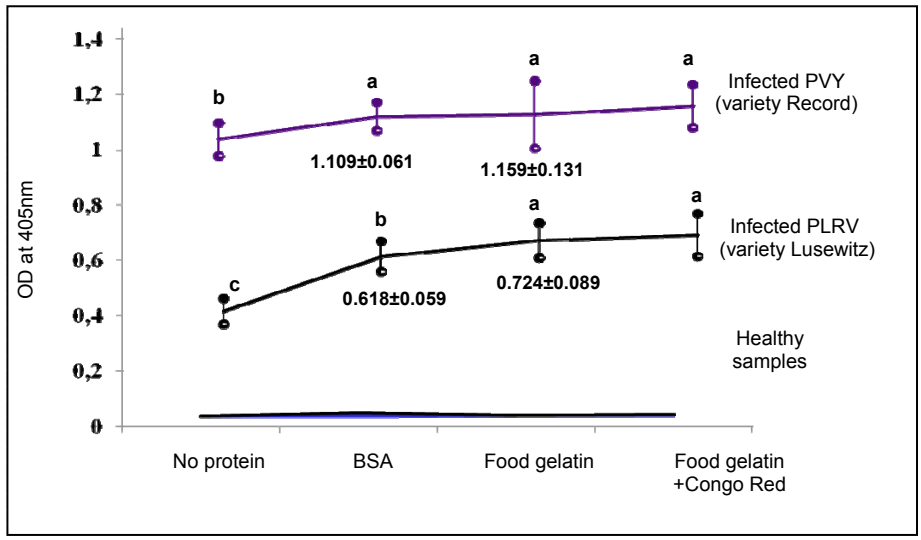


Figure 3 - Influence of several additives of conjugate buffer on the detection of potato viruses Y and potato leafroll virus by ELISA technique

OD= mean values of optic density for four repetitions; ± SD (standard deviation values); BSA= bovine albumin serum 2%; food gelatin from local market 1%; Congo Red was added on the solution for colour them, for easyer work with them. For the samples we used fresh tissues that produced relatively medium ELISA reactions (dilution of sap from infected plants in sap from healthy plsnts was 1/1). Values not followed by the same letter are significantly different (P=0.05) according to Duncan's test. Experiments were repeated on four occasions.

b. The effect of samples incubation on detection of PLRV. As shown in *Table 2*, 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 detectability of potato virus particles.

Table 2 - 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 four repetitions **±** standard deviation

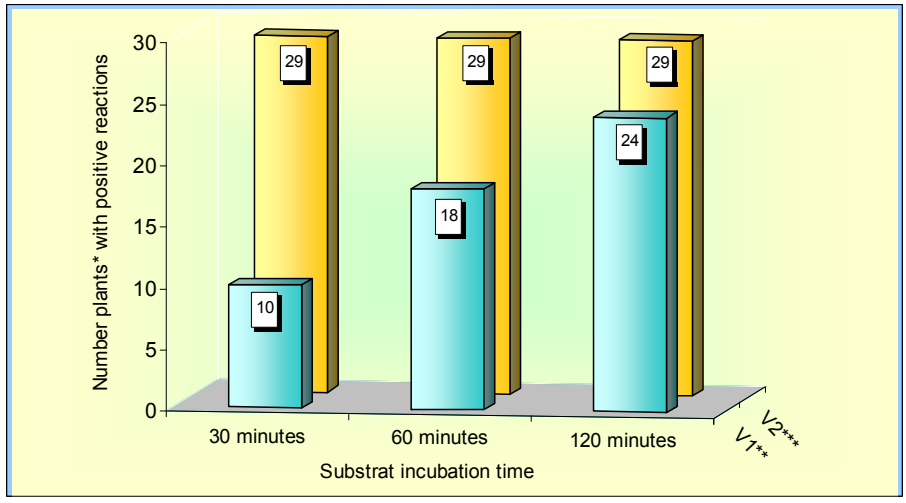


Figure 4 - Detectability of PLRV in leaves function on the samples incubation modality; biological material used* for leaves samples =29 plantes infected with PLRV; V1 – DAS ELISA; V2*** – cocktail ELISA (co-incubation samples and IgG-AP conjugate).** Experiments were repeated on four occasions, with the same results.

Fig. 4 and Table 2 show that the co-incubation sample and IgG conjugate gave positive reaction in 100% of the testing 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 two 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.

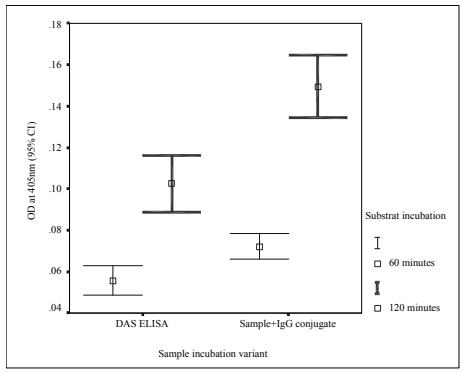
Testing sprouting tubers, the results showed a better identification of PLRV using the co-incubation sample and IgG-AP conjugate at all the sap's dilutions (*Table 3*). Using the co-incubation sample with conjugate, for sap's dilution 1/10 and 1/20, the infected tubers were easily distinguished from 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.

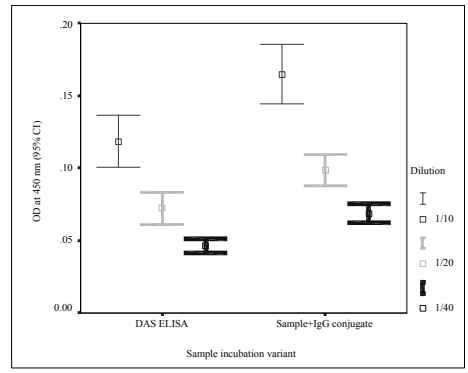
The *Fig. 5* shows that the highest values of OD at 405 nm were those obtained by cocktail ELISA. when the tuber extract was diluted 10 times. after two hours substrate incubation. The mean values was in this case 1.5 times higher than those obtained by standard DAS ELISA method. Therefore all further assays have been done (for PLRV detection) using cocktail ELISA technique.

Table 3 - 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 four repetitions **±** standard deviation.The sap was extracted from the rose end of 29 sprouting tubers (after natural break of dormancy).





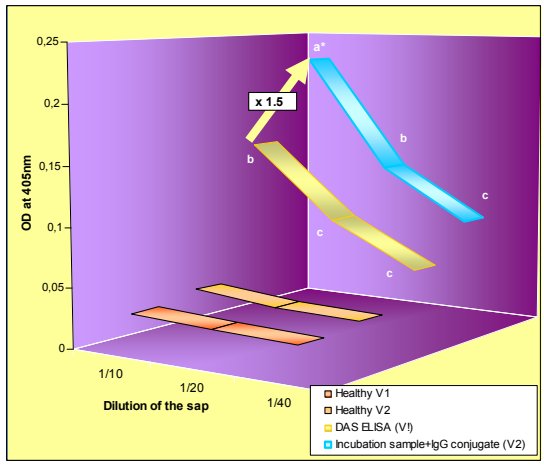


Figure 5 - PLRV detection in sprouting tubers. 95% CI= 95% confidence interval of the difference; Values not followed by the same letter (*) are significantly different (P=0.05) according to Duncan's test. Experiments were repeated on four occasions.

CONCLUSIONS

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

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 additive expensive and difficult to dissolve in PBS-T.

Presence of proteins in conjugate buffer improve safety of viruses identification. Food gelatin was more efficiently like the bovine serum albumine (BSA) for PLRV identification. So, the results achieved show a better identification (significant differences) for PLRV in potato leaves (sap's dilution 1/10) using gelatin (1%; pH 7.4) and a

mixture gelatin (1%) + Congo Red (40 mg/l). This buffer don't use bovine albumine serum (an additive expensive).

The co-incubation sample and conjugate, the use of gelatine food instead of other proteins in the composition of conjugate buffer and the McIlvain's phosphate-citric acid buffer used like extracting buffer could be possibilities for saving time and costs of potato viruses diagnostic tests.

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