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Full Length Article

Improved Indirect ELISA for Detection of some Plant Viruses

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Abstract

Addition of 0.1% Triton X-100 improved detection of *Bean yellow mosaic virus* (BYMV) by indirect ELISA, which was further increased the absorbance value by 35.04%, when Triton X-100 was added to carbonate buffer. Detailed studies with other three viruses; *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV), *Potato virus Y* (PVY) using three concentrations of cellulase, macerozyme or Triton X-100 and two extraction buffers (phosphate or carbonate buffer) were achieved. The absorbance values of CMV, ToMV or PVY infected sap were increased significantly by adding each of the additives tested to both extraction buffers. The most efficient additive was macerozyme when used at 0.1%, since the percent increase in absorbance values of CMV, ToMV and PVY was 40.91, 28.33 and 37.02, respectively when the samples extracted in phosphate buffer. While the percent increase was 40.06, 39.16 and 45.51, respectively when the samples extracted in carbonate buffer. Extraction of CMV, ToMV and PVY infected samples in carbonate buffer increased the absorbance values as compared with phosphate buffer. The maximum increases in absorbance values were 30.89, 29.19 followed by 27.32%, which obtained when PVY infected samples were extracted in carbonate buffer with Triton X-100 (0.25%), (0.1%) and macerozyme (0.1%), respectively. © 2013 Friends Science Publishers

Keywords: Improvement; Indirect ELISA; Extraction buffer; Triton X-100; Macerozyme; Cellulase

Introduction

Enzyme-linked Immunosorbent assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. ELISA since its use with plants (Clark and Adams, 1977) has become the most popular method for detection of viruses in plants, because of its simplicity and wide applicability (Copeland, 1998). Indirect ELISA has been used in plant pathology as a diagnostic tool for detection of many plant viruses in the infected samples.

Different extraction buffers such as phosphate buffer saline (PBS), PBS-Tween containing 2% polyvinylpyrrolidone (PBS-Tween-PVP) (Koenig, 1981) and carbonate buffer (Gillaspie and Hopkins, 1991; Fegla *et al*., 1997) were used to extract the sap of the samples.

Bean yellow mosaic virus (BYMV), *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV) and *Potato virus Y* (PVY) are worldwide in occurrence and among the most important viruses infecting economic crops in Arab region including Egypt (Fegla *et al*., 2008; Mansour *et al*., 2008 a, b; Fegla *et al*., 2009b). Using some additives like enzymes may enhance the detection of Pea seed borne mosaic virus (PSbMV) by ELISA (Ding *et al*., 1992).

Cellulase refers to a group of enzymes produced chiefly by fungi, bacteria, protozoa and some other organisms that can hydrolyze cellulose to glucose and other active ingredients. Also, it increased the permeability of plant cell walls, which led to improve the extraction rate (Shuangqi *et al*., 2011).

Macerozyme refers to an enzyme or a mixture of enzymes that has the capability to soften plant tissue, used experimentally to digest the plant cell walls. Cellulase and pectinase were considerably cheaper and effective for Potato leaf roll virus purification (Prins and Thompson, 1988). Macerozyme applied with hemicellulase or driselase during the protoplast isolation of the moss *Atrichum undulatum P. Beauv* in vitro (Li *et al*., 2005). Cellulase, macerozyme, driselase and pectolyase used to improve the protocols for isolation, culture and regeneration of protoplasts of *Lotus corniculatus* (Raikar *et al*., 2008).

Triton X-100 is a commonly used detergent in laboratories. Also, it used as a component of cell lysis buffers or other solutions intended to extract and solubilize proteins.

The present study was performed to test the effect of cellulase, Triton X-100 or macerozyme as additives and carbonate and phosphate as extraction buffers to improve the detection of some viruses by indirect ELISA.

Materials and Methods

Viruses

Four viruses, namely BYMV, CMV, ToMV and PVY used in this study were obtained from the collection of Virology

Lab., Plant Pathology Dept., Faculty of Agriculture, Alexandria University. ToMV was maintained on *Nicotiana tabacum* cv. Turkish, while CMV and PVY were maintained on *N. glutinosa* and BYMV on *Vicia faba* cv. Giza 461, which served as virus source plants for further studies. BYMV was used in preliminary study.

Source of Antisera

Antisera used in this study were locally produced in Virology Lab., Plant Pathology Dept., Faculty of Agriculture, Alexandria University, Egypt.

Indirect ELISA

The indirect ELISA first reported by Koenig (1981) and modified by Fegla *et al*. (1997) was used. Infected or healthy leaves were ground in extraction buffer tested as mentioned later using a mortar and pestle, The ELISA values, measured by Universal automated microplate reader ELx 800, were expressed as absorbance at 405 nm. In each ELISA plate, six wells containing extracts from healthy samples were used to determine the negative threshold value. Sample was considered to be positive when its absorbance value (at 405 nm) was higher than the control plus three or five standard deviations (Cebolla-Cornejo *et al*., 2003; Fegla *et al*., 2009a; Fu *et al*., 2012).

Also, six wells containing extracts from infected samples extracted in buffer without any additions per each virus (positive control) were used to determine the effect of additives on the absorbance values. Increase in absorbance value at 405 nm of sap extracted from virus infected sample with additive was considered significant when it was higher than the positive control plus three or five standard deviations (Cebolla-Cornejo *et al*., 2003; Fegla *et al*., 2009a; Fu *et al*., 2012).

Effect of some Additives and Extraction Buffers

Preliminary study was carried out on BYMV using extraction buffers; carbonate and phosphate buffer saline (PBS) with or without 0.1% Triton X-100. In further studies cellulase (derived from *Trichoderma viride*) (C234), Macerozyme R-10 (a mixture of pectinase, cellulase and hemicellulase derived from *Rhizopus* sp.) (M481) obtained from Phyto Technology Laboratories, USA, and Triton X-100 was used. They were added individually to each extraction buffer at dilutions of 0.25%, 0.10% and 0.05% immediately prior to grinding of plants leaves separately infected with the tested viruses.

Comparison between some Extraction Buffers for Detection of CMV, ToMV and PVY

Two extraction solutions, 0.5 M carbonate, pH 9.6 and 0.02 M phosphate buffer saline, pH 7.4 were used in extraction ratio of 1:10 (w/v). Leaves were ground in each buffer with or without the above mentioned additives to compare their ability to improve detection of the tested viruses.

The experiments were repeated twice and ELISA absorbance values at 405 nm are average of two replicates each. Also, six wells with extract of virus infected tissue in each buffer without any additives were used to determine the significance of increase in ELISA absorbance values.

Results

Preliminary Study

First trial on BYMV revealed that, addition of Triton X-100 (0.1%) improved detection of BYMV by indirect ELISA as the absorbance value increase was 27.97 and 35.04% when the samples extracted in phosphate and carbonate, respectively (Table 1).

Effect of some Additives and Extraction Buffers

A more detailed experiment was carried out using three concentrations of cellulase, macerozyme or Triton X-100 with two extraction buffers (phosphate and carbonate buffer) with CMV, ToMV and PVY. Results showed that, the absorbance values of infected sap were increased, significantly, by adding cellulase, macerozyme and Triton X-100 to both phosphate and carbonate extraction buffers. The percent increase differed according to the extraction buffer, the additive, its concentration and the virus used.

CMV: Data presented in Table (2) indicated that using phosphate buffer, increase the absorbance values from 14.75 to 22.52%, 34.97 to 40.91% and 12.62 to 29.08% when cellulase, macerozyme and Triton X-100 were added, respectively.

The most efficient additive was macerozyme, since the increase was 34.97, 40.91 and 40.79% when macerozyme was used at 0.05, 0.1 and 0.25%, respectively. However, regardless the additive, 0.1% concentration gave relatively higher absorption values and the most efficient in this respect was 0.1% macerozyme (40.91%).

Adding cellulase at a concentration of 0.25% and 0.1% to phosphate buffer led to increase in the absorbance values reached to 21.71% and 22.52%, respectively. While, adding Triton X-100 with dilutions 0.25% and 0.1% increase the difference percentage to 26.90% and 29.08%, respectively (Table 2).

The same trend was observed with carbonate buffer. Results showed that, macerozyme was much more effective than cellulase and Triton X-100 in increasing the absorbance values of infected extracts (Table 2). Macerozyme had maximum effect at a concentration of 0.1% (40.06%). As with phosphate buffer, the most appropriate dilution of additives with carbonate buffer was 0.1%.

Table 1: Effect of Triton X-100 (0.1%) and extraction buffers on absorbance values of ELISA for BYMV

Extraction buffer		Additives	ELISA values at 405nm	Percent Increase *	Significance of increase	
					More than positive control value + 3 S.D.**	More than positive control value + 5 S.D.***
Phosphate buffer	Infected	Triton X-100	0.579	27.97	+	+
		Without Triton X-100	0.417			
	Healthy control****		0.084			
Carbonate buffer	Infected	Triton X-100	0.759	35.04	+	+
		Without Triton X-100	0.493			
	Healthy control****		0.089			

*Percent increase in ELISA absorbance values when Triton X-100 was used with each extraction buffer

**Mean positive control value + 3 Standard deviation= 0.426 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 3 Standard deviation= 0.502 (If carbonate buffer used as an extraction buffer)

***Mean positive control value + 5 Standard deviation= 0.432 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 5 Standard deviation= 0.508 (If carbonate buffer used as an extraction buffer)

****Negative threshold values were not included here because absorbance values of virus infected extracted sap with or without additives were greatly higher than that of healthy sample

Table 2: Effect of some additives and extraction buffers on absorbance values of ELISA for CMV

Extraction buffer		Additives	Conc.	ELISA values at 405nm	Percent Increase *	Significance of increase	
						More than positive control value + 3 S.D.**	More than positive control value + 5 S.D.***
Phosphate buffer	Infected	Cellulase	0.25%	0.760	21.71	+	+
			0.1%	0.768	22.52	+	+
			0.05%	0.698	14.75	+	+
		Macerozyme	0.25%	1.005	40.79	+	+
			0.1%	1.007	40.91	+	+
			0.05%	0.915	34.97	+	+
		Triton X-100	0.25%	0.814	26.90	+	+
			0.1%	0.839	29.08	+	+
			0.05%	0.681	12.62	+	+
	Positive control (without additives)			0.595			
	Healthy control****			0.173			
Carbonate buffer	Infected	Cellulase	0.25%	0.918	25.38	+	+
			0.1%	0.967	29.16	+	+
			0.05%	0.852	19.60	+	+
		Macerozyme	0.25%	1.122	38.94	+	+
			0.1%	1.143	40.06	+	+
			0.05%	0.957	28.42	+	+
		Triton X-100	0.25%	0.949	27.81	+	+
			0.1%	0.987	30.59	+	+
			0.05%	0.819	16.36	+	+
	Positive control (without additives)			0.685			
	Healthy control****			0.237			

*Percent increase in ELISA values when the additives were used with each extraction buffer

**Mean positive control value + 3 Standard deviation= 0.607 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 3 Standard deviation= 0.697 (If carbonate buffer used as an extraction buffer)

***Mean positive control value + 5 Standard deviation= 0.615 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 5 Standard deviation= 0.705 (If carbonate buffer used as an extraction buffer)

****Negative threshold values were not included here because absorbance values of virus infected extracted sap with or without additives were greatly higher than that of healthy sample

ToMV: Detection of ToMV in leaf extract improved by the addition of additives. Maximum improvement in absorbance values was obtained when macerozyme was used as compared with cellulase or Triton X-100 added either to phosphate buffer or carbonate buffer (Table 3). The maximum rate was obtained for all additives when used at a concentration of 0.1%.

Increase in absorbance values was 21.02, 28.33 and 16.35% with extraction phosphate buffer and 22.68, 39.16 and 16.95% with carbonate buffer when cellulase, macerozyme and Triton X-100 were used, respectively at a concentration of 0.1%.

PVY: Data concerning PVY presented in Table (4) revealed that, the three concentrations of macerozyme used (0.05, 0.1 and 0.25%) gave higher absorbance values as compared with the corresponding concentrations of the other two additives. The maximum increase was observed with 0.1% macerozyme when phosphate or carbonate extraction buffer was used.

The percent increase of absorbance values reached 28.06, 37.02 and 20.83 with phosphate buffer and 30.80, 45.51 and 33.26 with carbonate buffer when cellulase, macerozyme and Triton X-100 were used, respectively at 0.1%.

Table 3: Effect of some additives and extraction buffers on absorbance values of ELISA for ToMV

Extraction buffer		Additives	Conc.	ELISA values at 405nm	Percent increase*	Significance of increase	
						More than positive control value + 3 S.D.**	More than positive control value + 5 S.D.***
Phosphate buffer	Infected	Cellulase	0.25%	0.868	16.93	+	+
			0.1%	0.913	21.02	+	+
			0.05%	0.810	10.98	+	+
		Macerozyme	0.25%	1.002	28.04	+	+
			0.1%	1.006	28.33	+	+
			0.05%	0.963	25.12	+	+
		Triton X-100	0.25%	0.830	13.13	+	+
			0.1%	0.862	16.35	+	+
			0.05%	0.796	9.42	+	+
	Positive control (without additives)			0.721			
	Healthy control****			0.145			
Carbonate buffer	Infected	Cellulase	0.25%	1.063	22.10	+	+
			0.1%	1.071	22.68	+	+
			0.05%	1.035	20.00	+	+
		Macerozyme	0.25%	1.274	35.00	+	+
			0.1%	1.361	39.16	+	+
			0.05%	1.086	23.75	+	+
		Triton X-100	0.25%	0.964	14.10	+	+
			0.1%	0.997	16.95	+	+
			0.05%	0.935	11.44	+	+
	Positive control (without additives)			0.828			
	Healthy control****			0.211			

*Percent increase in ELISA values when the additives were used with each extraction buffer.
**Mean positive control value + 3 Standard deviation= 0.733 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 3 Standard deviation= 0.882 (If carbonate buffer used as an extraction buffer)
***Mean positive control value + 5 Standard deviation= 0.741 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 5 Standard deviation= 0.890 (If carbonate buffer used as an extraction buffer)
****Negative threshold values were not included here because absorbance values of virus infected extracted sap with or without additives were greatly higher than that of healthy sample.

Table 4: Effect of some additives and extraction buffers on absorbance values of ELISA for PVY

Extraction buffer		Additives	Conc.	ELISA values at 405nm	Percent Increase *	Significance of increase	
						More than positive control value + 3 S.D.**	More than positive control value + 5 S.D.***
Phosphate buffer	Infected	Cellulase	0.25%	0.688	19.91	+	+
			0.1%	0.766	28.06	+	+
			0.05%	0.620	11.12	+	+
		Macerozyme	0.25%	0.805	31.55	+	+
			0.1%	0.875	37.02	+	+
			0.05%	0.704	21.73	+	+
		TritonX-100	0.25%	0.671	17.88	+	+
			0.1%	0.696	20.83	+	+
			0.05%	0.624	11.69	+	+
	Positive control (without additives)			0.551			
	Healthy control****			0.011			
Carbonate buffer	Infected	Cellulase	0.25%	0.925	29.08	+	+
			0.1%	0.948	30.80	+	+
			0.05%	0.809	18.91	+	+
		Macerozyme	0.25%	1.084	39.48	+	+
			0.1%	1.204	45.51	+	+
			0.05%	0.904	27.43	+	+
		TritonX-100	0.25%	0.971	32.44	+	+
			0.1%	0.983	33.26	+	+
			0.05%	0.811	19.11	+	+
	Positive control (without additives)			0.656			
	Healthy control****			0.015			

*Percent increase in ELISA values when the additives were used with each extraction buffer.
**Mean positive control value + 3 Standard deviation= 0.563 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 3 Standard deviation= 0.668 (If carbonate buffer used as an extraction buffer)
***Mean positive control value + 5 Standard deviation= 0.571 (If phosphate buffer used as an extraction buffer) and Mean positive control value + 5 Standard deviation= 0.676 (If carbonate buffer used as an extraction buffer)
****Negative threshold values were not included here because absorbance values of virus infected extracted sap with or without additives were greatly higher than that of healthy sample.

Comparison between some Extraction Buffers for Detection of CMV, ToMV and PVY

Results presented in Tables (5, 6 and 7) revealed that, carbonate buffer without additives increased absorbance values of infected extracts with CMV, ToMV or PVY as compared with phosphate buffer. The differences in absorbance values observed after adding additives to extraction buffers varied according to the virus, additive and additive concentration. However, all additives with carbonate buffer led to much more increase in absorbance values of PVY infected extracts over those of phosphate buffer. The increase varied from 19.19 to 25.62% with cellulase, 22.12 to 27.32% with macerozyme and 23.05 to 30.89% with Triton X-100 as compared with 16% for positive control without additives.

Discussion

The main goal of this work was to improve the detection of some viruses by indirect ELISA. Improvement of detection was investigated by studying the effect of two extraction buffers (carbonate buffer and phosphate buffer) and three concentrations of three additives; TritonX-100, cellulase and macerozyme. Little work has been done on the effect of such additives in improving virus detection by ELISA (Ding *et al*., 1992).

Triton X-100 is nonionic detergent used in the extraction and purification of a wide range of viruses (Noordam, 1973; Hull, 2004), and is also sometimes added to diluents and washing buffers used in ELISA to help prevent nonspecific reaction (Walker, 1987). Cellulase and macerozyme or other pectinases are also often used in the extraction and purification of some plant viruses that are difficult to purify by normal procedures, particularly the phloem-limited viruses (Singh *et al*., 1984; Govier, 1985; Jones *et al*., 1991).

Our results showed that additives used significantly increased ELISA absorbance values of the tested viruses. However, macerozyme was much more effective than cellulase and Triton X-100. Maximum effect of the three additives occurred at a concentration of 0.1%. However, effectiveness of the additives could vary according to the plant tissue and the virus. Ding *et al*. (1992) found that cellulase was much more effective than macerozyme in improving detection of PSbMV. Cellulase is used extensively in the isolation of plant protoplasts, protoplast transfection, frequently in combination with Macerozyme R10 (Martínez *et al*., 2011; Zhang *et al*., 2011).

The beneficial effect of macerozyme and cellulase as well as TritonX-100 was possibly to aid the release of virus particles from host materials. Cellulase was used at 1-1.5% when using standard luteovirus purification method (Brunt *et al*., 1996). Hull (2004) reported that, Triton X-100 and Tween 80 are often used in the initial extraction medium to assist release of virus particles from insoluble cell components and to dissociate cell membrane that may contaminate or occlude virus particles. TritonX-100 was as effective as cellulase in improving detection of PSbMV (Ding *et al*., 1992). However, increased measurable content was recorded by ELISA of nerve growth factor (NGF) antigen by using Triton X-100, pH 10.5 and Sodium chloride (Hoener and Varon, 1997).

Data concerning extraction buffers revealed that, carbonate buffer was much more effective than phosphate buffer with all viruses and additives used. Such results are in line with those by Fegla *et al*. (2000) who tested four extraction solutions to optimize dot blot immunobinding

Table 5: Comparison between absorbance values of ELISA when phosphate buffer and carbonate buffer were used as an extraction media for CMV

Additives	Concentration	Buffer used		Percent Increase
		Phosphate	Carbonate	
Cellulase	0.25%	0.760	0.918	17.21
	0.1%	0.768	0.967	20.57
	0.05%	0.698	0.852	18.07
Macerozyme	0.25%	1.005	1.122	10.42
	0.1%	1.007	1.143	11.89
	0.05%	0.915	0.957	4.38
Triton X-100	0.25%	0.814	0.949	14.22
	0.1%	0.839	0.987	14.99
	0.05%	0.681	0.819	16.84
Positive control (without additives)		0.595	0.685	13.13

Table 6: Comparison between absorbance values of ELISA when phosphate buffer and carbonate buffer were used as an extraction media for ToMV

Additives	Concentration	Buffer used		Percent Increase
		Phosphate	Carbonate	
Cellulase	0.25%	0.868	1.063	15.52
	0.1%	0.913	1.071	14.75
	0.05%	0.810	1.035	21.73
Macerozyme	0.25%	1.002	1.274	21.35
	0.1%	1.006	1.361	26.08
	0.05%	0.963	1.086	11.32
Triton X-100	0.25%	0.830	0.964	13.90
	0.1%	0.862	0.997	13.54
	0.05%	0.796	0.935	14.86
Positive control (without additives)		0.721	0.828	12.92

Table 7: Comparison between absorbance values of ELISA when phosphate buffer and carbonate buffer were used as an extraction media for PVY

Additives	Concentration	Buffer used		Percent Increase
		Phosphate	Carbonate	
Cellulase	0.25%	0.688	0.925	25.62
	0.1%	0.766	0.948	19.19
	0.05%	0.620	0.809	23.36
Macerozyme	0.25%	0.805	1.084	25.73
	0.1%	0.875	1.204	27.32
	0.05%	0.704	0.904	22.12
Triton X-100	0.25%	0.671	0.971	30.89
	0.1%	0.696	0.983	29.19
	0.05%	0.624	0.811	23.05
Positive control (without additives)		0.551	0.656	16.00

assay for detection of ToMV and found carbonate buffer the best among them. This could be attributed better efficiency of carbonate buffer than phosphate buffer in extraction of virus particles from infected tissues and/or by facilitating the adsorption of much more virus particles to the wells than do phosphate buffer.

It can be concluded that, detection of some plant viruses by indirect-ELISA could be enhanced by adding Triton X-100, macerozyme and cellulase to the extraction buffer. Maximum effect of the three additives occurred when added at a concentration of 0.1%, and the first in this respect, was macerozyme. Also, using carbonate buffer as a coating buffer was much more effective than phosphate buffer with viruses and additives used.

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