Journal of Plant Diseases and Protection (2021) 128:1101–1114
https://doi.org/10.1007/s41348-021-00448-9

ORIGINAL ARTICLE

# Protecting potato plants against PVX and PVY viral infections by the application of native and chemically modified legume proteins

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Received: 30 July 2020 / Accepted: 20 February 2021 / Published online: 19 March 2021


## Abstract

Methylated soy and chickpea proteins (MSP and MCP, respectively), and 11S globulin (a soy protein fraction), are characterized by net positive charges and a more hydrophobic nature, nominating them as antiviral proteins. Under greenhouse conditions, potato plants (cv. Spunta) mechanically infected with potato virus Y (PVY) and potato virus X (PVX) were treated with MSP, MCP, and 11S globulin at different concentrations, after ten days of infection. The three tested substances inhibited the viral propagation, where the concentration 500 µg $ml^{-1}$ produced the maximum antiviral action. This influence was further augmented by applying the material twice 10 and 20 days after the viral infection. Assessing the viral load by a double antibody sandwich ELISA (DAS-ELISA), dot-blot hybridization, and reverse transcription-polymerase chain reaction (RT-PCR) confirmed the viral inhibition, in the following order: MSP > MCP > 11S globulin. Bringing the virus in contact with MCP produced deformed TEM (transmission electron microscope) viral micrographs referring to a potential direct action of the substance on the virus. The tested materials' antiviral activity at 500 µg $ml^{-1}$ was positively reflected on the growth and yield of PVY or PVX-infected potato plants cultivated under open field conditions in January. Considerably high increases in the tuber yield of either PVY-infected (68, 42, and 36%) or PVX-infected plants (52, 41 and 30%), were produced by the treatment with MSP, MCP and 11S (500 µg $ml^{-1}$), respectively.

**Keywords** Methylated soybean protein · Methylated chickpea protein · 11S globulin · PVY · PVX · Potato plants

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## Introduction

The potato crop is an outstanding staple food in the Mediterranean region, producing about 30 million tons of tubers (Bradshaw and Ramsay 2009). Egypt is one of the Mediterranean countries that usually grow potatoes in the winter-spring cycle for early production, supporting competitive exportation to northern European countries (Ierna and Mauromicale 2012). So, great attention should necessarily be paid to the quality and safety of the food product. Additionally, potato tuber yield is considerably influenced by viral infections. Potato tubers (imported Alpha cultivar) grown in Sudan and infected by potato viruses (PVS, PVX and PVY) suffered about 20% and 36% decline in yield of the first and second generation, respectively. Highly significant negative correlation coefficients were established between the cumulative incidence of potato viruses and the associated yield losses manifested in leaf area index and tuber yield of a local potato cultivar Zalinge (Omer and El-Hassan 1992).

More than 40 plant viruses infecting potato crops are vegetatively propagated. Potato leafroll virus (PLRV),

potato virus Y (PVY) and PVS are profoundly affecting potato production worldwide (Lacomme and Jacquot 2017). Potato virus Y (PVY) belongs to the genus Potyvirus, family Potyviridae, order Patataviralesis, a critical pathogen that is substantially reducing the yields of potatoes (Gray and Power 2018). Potato virus X (PVX) is the type species of the genus Potexvirus, family Alphaflexiviridae, order Tymovirales (Komatsu et al. 2012). It is an important pathogen of potato and other Solanaceae being among the top ten economic plant viruses (Scholthof et al. 2011), whose control is difficult being easily transmitted mechanically between their hosts (Esfandiari and Sefidbakht 2018). Synergistic interaction was reported between PVY and PVX (Aguilar et al. 2017). In Norway, field collections of 170 potato cultivars were found heavily infected with potato virus, especially potato virus S (PVS) and potato virus X (PVX). Virus-free seed potatoes were set as a pre-requisite for further use of these cultivars due to their long preservation time in the open field (Zhang et al. 2019).

Eliminating or mitigating the crop losses incurred by viral infection may pass through either breeding of virus-resistant cultivars (Gray and Power 2018), insecticidal treatment of the viral vector (Yang et al. 2019). PVY is an aphid-borne virus (Rybicki 2015), which can be controlled by horticultural mineral oil (HMO), which reduces the binding and retention of virions (Yang et al. 2019). However, the need for frequent reapplication makes routine use of HMO impractical (Buteler and Stadler 2011). Chemical treatments using agents such as salicylic acid(SA) were used as exogenous treatments to control plant diseases by counteracting the virus-induced biochemical changes (Cueto-Ginzo et al. 2016), most probably by perturbing the viral replication through directly targeting a host component in the replication complex (Boom et al. 1990; Sitohy et al. 2001a, 2010a; Tian et al. 2015).

Using esterified or native protein has been generally investigated as a new trend for combating viral infection or propagation (Sitohy et al. 2010a, 2010b; Wang et al. 2013; Sitohy et al. 2020a). Some natural chemically basic proteins, e.g., lysozyme and lactoferrin, may have inherent antiviral activity. Other native proteins can be turned into an alkaline character by esterification, which neutralizes the carboxyl groups and thus intensifies the net positive charge on protein molecules (Abbas et al. 2020; Osman et al. 2014a, 2016a, 2016b, 2014b; Sitohy et al. 2011, 2013). Thus, esterified proteins can interact with many microorganisms, including viruses (Abdel-Shafi et al. 2016; Mahgoub et al. 2013, 2011; Sitohy and Osman 2019, 2011). By virtue of their intensified positive charge, the modified proteins can complex DNA fragments in vitro, as visualized by DNA electrophoresis (Sitohy et al. 2002, 2000). They can also complex DNA present in the media of PCR DNA amplification, impeding the amplification process (Sitohy et al. 2001a, 2001c). In vivo replication of M13 bacteriophage and lactococcal bacteriophages was inhibited by esterified proteins' presence (Sitohy et al. 2005, 2006).

Moreover, esterified milk proteins were noticed to inhibit the replication of human and plant viruses (Chobert et al. 2007; Sitohy et al. 2008, 2007). PVY is an aphid-borne virus (Rybicki 2015), which can be controlled by HMO that can effectively prevent its spread in potato seed production (Galimberti and Alyokhin 2018; MacKenzie et al. 2017) through the direct insecticidal activity and reducing binding and retention of virions (Yang et al. 2019). Thus, future studies may be required to investigate the efficiency of combinational treatments, including both MSP (methylated soy protein) or MCP (methylated chickpea protein) as direct controllers of virus and HMO as chemical control of the virus vector. Alternatively, possible direct effects of MSP and MCP or their derivatives on the aphids may also be investigated for a better holistic strategy to control both the virus and the virus vector. The present study was designed to assess the protective effect of native and esterified legume proteins (MSP and MCP) and 11S globulin isolated from soybean on the growth and yield of potato plants mechanically infected with PVY and PVX.

## Materials and methods

### Tested proteins

Soy protein isolate and chickpea protein isolate were separated from soy and chickpea seed as detailed in (Sitohy and Osman 2010). Soy protein fraction 11S was isolated from soy protein isolate, as stated in (Sitohy et al. 2012). Methylated soy protein (MSP) and methylated Chickpea protein (MCP) were prepared by esterification with methanol in the presence of HCl (50 MR) according to the procedure of (Sitohy et al. 2001b, 2000). The esterification extent was colorimetrically quantified by the reaction with hydroxylamine hydrochloride (Bertrand-Harb et al. 1991).

For SDS-PAGE, an amount of SP and CP was dispersed in 1 ml SDS 10%, combined with 100 µl β-mercaptoethanol and mixed for 10 min with vortexing. The dispersion was centrifuged at 10,000 *xg* for 15 min. A mixture of 50 µl extract, and 50 µl of SDS-loading sample buffer (SDS 4%, β-mercaptoethanol 3%, glycerol 20%, Tris HCl 50 mM pH 6.8 and bromophenol blue traces), was heated at 96 °C for 5 min. Then, 15 µl aliquot (per lane) of the mixture was electrophoresed according to (Laemmli 1970).

Native (SP and CP) and esterified (MSP and MCP) legume proteins were analyzed by urea-PAGE in 3 and 10% stacking and resolving gels, respectively, according to (Evans and Williams 1980). The isoelectric points for native (SP and CP) and esterified (MSP and MCP) were estimated

from the protein pH-solubility curves, as the pH corresponding to the least protein solubility (Osman et al. 2016a).

## Virus and plants

### PVX

In most potato varieties, PVX causes mild or no symptoms, but when potatoes are infected with PVY, the synergy between these two viruses could cause serious symptoms. The observed symptoms included a mild mosaic with slight leaf crinkling. Leaf symptoms comprised mottling, vein-banding and clearing, and distortion. The plants may also be stunted with necrosis of the stems and apical buds. Samples from infected potato plants were collected from Al-Behera Governorate and directly transferred to the laboratory for detection. About 2 g of naturally infected potato leaf tissues were ground in 10 ml of 0.01 M phosphate buffer, containing 0.2% Diethyldithiocarbamate, at pH 7.2. They were then mechanically transmitted to each of the following host plant seedlings, *Gomphrena globosa L., Chenopodium amaranticolor L.* and *Chenopodium quinoa.* The plants were grown in clay pots containing sterilized soil and kept under greenhouse conditions. Four weeks later, seedlings were visually examined for viral infection and confirmed by DAS-ELISA (Bio-Rad Laboratories, Ind, California, USA). The virus isolate was biologically purified from a single local lesion on *G. globose,* according to (Taha et al. 2019). After successive single local lesion transfers in the host, the resulting virus isolate was propagated in *N. tabacum* cv. White Burley plants. The sap from systemically PVX-infected *N. tabacum* cv. White Burley were inoculated onto healthy potato leaves lightly dusted with carborundum 600 mesh and served as a source for in vitro and in vivo experiments.

### PVY

White burley tobacco plants infected by PVY were collected from the greenhouse farm from Al-Behera Governorate based on the visible viral infection symptoms, including mottling or yellowing of leaflets, leaf crinkling. Veins on the underside of leaves often show necrotic areas as black streaks. Mosaic mottling of leaves is another symptom. Infected plants may be stunted. The infected leaves were crushed in distilled water and squeezed through a double layer of muslin cloth. The mixture was centrifuged at 5000 *xg* for 10 min and the supernatant was used as the virus inoculum. The identity of the virus was verified and confirmed by Electron microscopy and biomolecular studies, i.e., double antibody sandwich ELISA (DAS-ELISA), dot-blot hybridization, and reverse transcription-polymerase chain reaction (RT-PCR).

## Experimental design

The study involved both greenhouse and field experiments following a randomized complete block design in a factorial arrangement using three replicates (5 plants each), totaling 15 plants per treatment. Scheme 1 presents the designed different treatments.

## Greenhouse experiments

Potato plants (*Solanum tuberosum* cv. Spunta) were cultivated under greenhouse conditions in 30 cm diameter pots. The potting soil consisted of peat moss and vermiculite (3:1). The plants grew under the following environmental conditions; the temperature ranged from 22 to 28 °C, the soil pH was in the range 5.2–5.5. The cultivating period started with a relatively long day and ended with a relatively shorter day. The relative humidity was in the range 60–80% with an average 70 %. No fertilizers were added. These plants were then subjected to artificial virus infection with PVY (100 µg $ml^{-1}$) propagated on plant tissue after 32 days of the plantation. Three plant leaves were inoculated with the virus inoculum, in the apex and the second two leaves. The same plants were treated with chemically modified proteins (MSP and MCP) solubilized in tap water at different concentrations (100, 500 and 1000 µg $ml^{-1}$) by foliar spray after ten days of viral infection. Samples were collected from leaves after 10 days of treatment and viral RNA was estimated by DAS-ELISA. In a second greenhouse experiment, potato plants (*Solanum tuberosum* cv. Spunta) were similarly prepared and virally infected with PVY. Plants were then divided into two groups; the first one received only one treatment with 500 µg $ml^{-1}$ after 10 days of the viral infection, while the second group received the same treatment twice, i.e., after 10 and 20 days of the viral infection. Potato leaves samples in the two groups were collected for viral determination after 10 days of the second substance treatment. A similar design to the second experiment was conducted in a third experiment except using virus PVX and the double treatment while the plant leaf samples were collected for viral analysis after 7 and 21 days of the second application of the substances.

## Field experiment

This experiment aimed to determine the tested substances' efficiency to protect potato plants grown under open field conditions during plant growth and harvest against PVY and PVX. Potato plants were cultivated in the Faculty of Agriculture farm, Cairo University, Giza, Egypt for two successive seasons for each virus in October month in each season. The potato tubers were directly planted in the field of loamy soil, taking into consideration the environmental required conditions, e.g., irrigation and fertilization. The temperature

**Scheme 1** The experimental design

| Experiment location | | Viral infection ,32 d after planting | Antiviral application | | Plant Leaves sampling | Virus and plant analysis |
|---|---|---|---|---|---|---|
| | | | Dosage | Timing | | |
| Green house | 1st exp | PVY | CP, SP, MCP & MSP (100, 500 and 1000 µg ml$^{-1}$) | 10 d after viral infection | 10 d after treatment | Relative viral content by DAS-ELISA |
| | 2nd exp | PVY | SP, MSP, MCP & 11S (500 µg ml$^{-1}$) | Single treatment 10 d after viral infection Double treatment, 10 & 20 d after viral infection | 10 d after the final treatment | |
| | 3rd exp | PVX | | Double treatment, 10 & 20 d after viral infection | 7 & 21 d after the final treatment | |
| Open field | PVX exp | PVX & PVY | CP, SP, MCP, MSP & 11S (500 µg ml$^{-1}$) | Double treatment 10 & 20 d after viral infection | 21 d after the final treatment | • Viruses: DAS-ELISA, SEM, RT-PCR and dot-blot hybridization. • Plants: yield traits. |
| | PVY exp | | | | | |

varied between 15 and 25 °C. Generally, the plants enjoyed relatively long days during plantation in October (ca. 11 h) and relatively short days in the stage of tuber formation (ca. 10 h). The soil attained a moderate acidity range (pH 5.2–5.5). The plants in all treatments received the same rates of fertilization and surface irrigation. The fertilization rate was 286 kg Nitrogen, 178 kg phosphorus and 194 kg potassium per hectare and the irrigation rate was 476 mm. The field experiment followed a randomized complete block design in a factorial arrangement using three replicates (5 plants in one row and one-meter distance for each treatment), totaling 15 plants per treatment. These plants were then subjected to artificial virus infection with PVY or PVX (100 µg ml$^{-1}$) propagated on plant tissue after 32 days of the plantation. Three plant leaves of potato plants (*Solanum tuberosum* cv. Spunta), the apex and the second two leaves, were inoculated with the virus inoculum (100 µl / leaf) after dusting with carborundum. Then, the inoculated leaves were washed with distilled water. Negative control (NC) plants (healthy plants) were inoculated with buffer only. Viral positive control (PC) plants were inoculated by PVY or PVX only. The plants were treated twice by chemically modified proteins (500 µg ml$^{-1}$) after 10 and 20 days of infection. Every treated plant received 48 mL of the spray solution and the global rate of treatment was 1500 L/ha. Samples were collected from leaves after 21 days from the second treatment for detection of potato viruses in leaf by DAS-ELISA technique, TEM, RT-PCR and dot-blot hybridization. Plants were harvested and fresh weight (g), dry weight (g), the number of shoots, plant height (cm), the number of tubers/plant and weight of tubers/plant (g) were determined. The yield increase (%) was calculated relative to NC, as in the following equation:

$$\%\text{Increase} = \left[(\text{weight of treatment} - \text{weight of NC})/\text{weight of NC}\right] \times 100$$

And the yield increases relative to PC was calculated as in the following equation:

$$\%\text{Increase} = \left[(\text{weight of treatment} - \text{weight of PC})/\text{weight of PC}\right] \times 100$$

Scheme 1 summarizes the different viral infection experiments.

## Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for PVY

DAS-ELISA assay was used following (Clark and Adams 1977) to determine the relative viral load in potato leaves after different treatments. After interacting the leaves extracts with the polyclonal antisera from rabbit (LOEWE®Biochemica GmbH, Sauerlach, Germany), the developed color was measured at 405 nm. The DAS-ELISA

readings of the treatments (T), positive control ($C_p$) and Negative control ($C_n$) were used to calculate the viral inhibition % in the treated plants according to the following equation:

$$\text{Viral inhibition}\% = \left[(\text{Cp} - \text{Cn}) - (T - \text{Cn})/(\text{Cp} - \text{Cn})\right] \times 100$$

### RT-PCR detection for PVY

Potato leaves representing eight different treatments were tested for the PVY infection using RT-PCR (Bio-Rad Laboratories, Ind, California, USA), six potato plants from each treatment were tested. Total RNA was isolated from potato leaves using gene jet™ RNA purification kit (Fermentas, Waltham, MA, USA) according to the instruction manual. The one-step RT-PCR was performed using Verso™ one-step RT- PCR kit (Thermo Scientific, Waltham, MA, USA). RT-PCR mix (25 µl) included: 3 µl RNA (4 ng/µl), 12.5 µl of one-step PCR master mix (2x), 3 µl 10 µM of each primer, 0.5 µl Verso enzyme mix, 1.25 µl RT-Enhancer and 4.75 µl of nuclease-free water. The PCR file included 35 cycles of (2 min at 94 °C, 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C) and 10 min at 72 °C. PCR primers specific for PVY were used for RT-PCR detection as follows:

PVYCPvBamH1: TCAAGGATCCGCAAATGACAC AATTGATGCAGG.
PVYCPcEcoR1: AGAGAGAATTCATCACATGTTCTT GACTCC.

The RT-PCR products were stained with gel star (Lonza, USA) and analyzed on 1% agarose gels in 0.5X TBE buffer, then visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA). The expected size for the RT-PCR product of the positive samples was ~ 801.

### Transmission electron microscopy (TEM) for PVX and PVY

Transmission electron microscopy (JEOL-Ltd, Tokyo, Japan) was used to evaluate MCP's antiviral activity against PVY and PVX as described in (Derrick and Brlansky 1976).

### Dot-blot hybridization test for PVY

The non-radioactive hybridization technique was used to detect the presence of PVY virus in the tested plants and evaluate the antiviral activity of the used tested proteins. Five µl of extracted PVY-RNA (PCR product) samples were dot onto the positively charged nylon membrane. The specific probe was prepared as described in (Forster et al. 1985). The hybridization experiments were carried out using Gene Images AlkPhos and Chemiluminescent Detection System signal generation and detection with CDP-Star (Amersham, Biosciences, UK Limited, Uppsala, Sweden). (Forster et al. 1985; Abdelbacki et al. 2010).

#### Statistical analysis

The data were analyzed by using SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, USA) and expressed as the mean ± standard deviation (± SE). The variation was assessed by one-way (ANOVA) and the differences between experimental groups were calculated by Duncan's multiple range test (Duncan 1955).

## Results

### Antiviral proteins characterization

The data in Fig. 1 present the SDS-PAGE patterns of soybean (SP) and chickpea (CP) proteins. The electropherogram shows two main fractions (7S and 11S globulins) with their constituting subunits. The molecular weight of 11S subunits is 21 and 34 kD, corresponding to the basic and acidic subunits, respectively. The molecular weight of 7S globulin subunits ranges from 50 to 65 kD. The esterification extents of the esterified products, MSP and MCP were 80% and 83%, respectively. Grafting methyl groups on the free carboxyl groups of the protein molecules has two effects. First, it abolishes some of the protein molecules' negative charges, resulting in a more positive net charge on the protein molecule surface. Secondly, it augments the hydrophobic nature of the protein molecule, apparently through abolishing the electrostatic charge. These two factors may probably participate in the potential antiviral activity of these two proteins. The migration in Urea-PAGE into cathode direction indicated that MSP and MCP were much faster than their respective native proteins (lanes 1 and 2), referring to more significant positive net charges (Fig. 1) and validating the hypothesized effect. Furthermore, the isoelectric points (PIs) of the native (SP and CP) and methylated (MSP and MCP) proteins, deduced from the protein pH solubility shown in Fig. 1, refer to the change of the net global protein charges. The PI shifted from 4.5 for the native forms (SP and CP) to 8 for the methylated forms (MSP and MCP), evidencing further the expected effect of esterification.

### Protecting potato plants against PVX and PVY viral infections

#### Greenhouse experiments

The results in Fig. 2 are extracted from potato plants grown under greenhouse conditions, mechanically

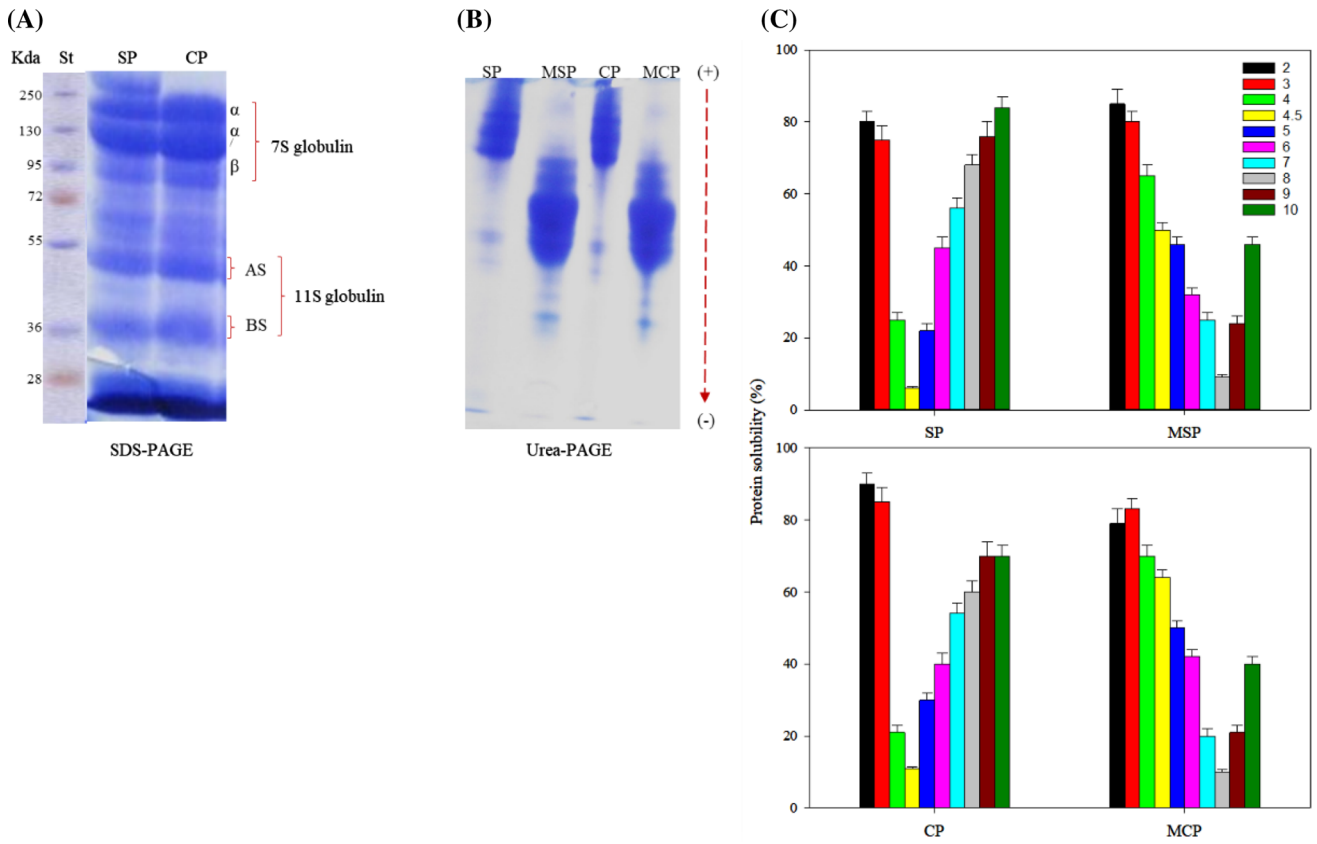


**Fig. 1** SDS-PAGE (**A**) of soybean (SP) and chickpea (CP) proteins compared to protein marker (St), where AS: acidic subunit and BS: Basic subunit. Urea-PAGE (**B**) of SP and CP before and after esterification [methylated soybean (MSP) & methylated chickpea (MCP)]. pH-protein solubility (%) (**C**) at different pHs (2–10) before and after esterification. The iso-electric points were deduced from the protein pH-solubility curves as the pH at which the protein is least soluble. The variation bars refer to ± SD

infected with PVY, and then treated by native (SP and CP) or chemically modified proteins (MSP and MCP) at different concentrations (100, 500 and 1000 µg ml$^{-1}$) after ten days of infection. The data herein show that MSP and MCP treatments healed plants infected with PVY in a concentration-dependent manner. The native forms seemed nearly void of antiviral activity. The antiviral potencies seem similar for MSP and MCP, exhibiting the maximum

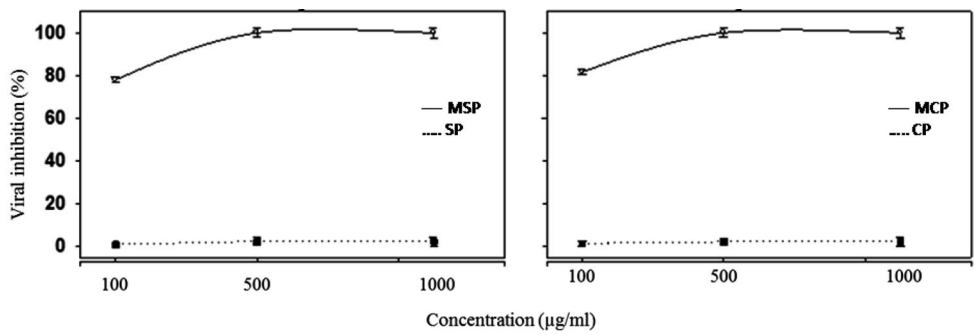


**Fig. 2** Viral inhibition (%) of PVY by the esterified soy and chickpea seed protein (MSP and MCP, respectively) as compared to their respective native forms (SP and CP) at different concentrations (100, 500 and 1000 µg ml$^{-1}$). The values were estimated 15 days after substance application. Relative viral content was estimated by DAS-ELISA (the double antibody sandwich linked immunosorbent assay). Viral inhibition was calculated according to the following equation: Viral inhibition % $= \left[(Cp - Cn) - (T - Cn)/(Cp - Cn)\right] \times 100$ where Cp is the positive control reading, Cn = the negative control reading and T is the treatment reading. The variation bars refer to ± SD

at 500 µg $ml^{-1}$. So, this concentration was used for the rest of the experiments.

In another experiment, the influence of the application frequency of the antiviral proteins was investigated by dividing the PVY-infected plants into two groups; the first group receiving a single foliar spray (after ten days of viral infection) and the second one receiving a double foliar spray (after 10 and 20 days of viral infection) treatment of 500 µg $ml^{-1}$ of MSP, MCP, 11S or SP. Potato leaf samples collected ten days after the second spray showed considerably higher antiviral action of MSP, MCP, and 11S in the double treatment than the single one (Fig. 3A). So, the double foliar spray application treatment was recommended for the open field experiment.

The stability of the antiviral activity in potato plants was tested in the leaves of the double treated plants infected with PVX after 7 and 21 days of the second application. It is noticed that the maximum viral inhibitory effect was observed after seven days of the last application, while only slight reductions were observed after 21 days (Fig. 3B). This preliminary experiment showed that viral activity was generally stable after 21 days of treatment.

## Field experiment

Based on the preliminary greenhouse results, an experiment was conducted under open field conditions during the plant whole life cycle up to the harvest stage. The objective was to determine the tested substances' efficiency to protect potato

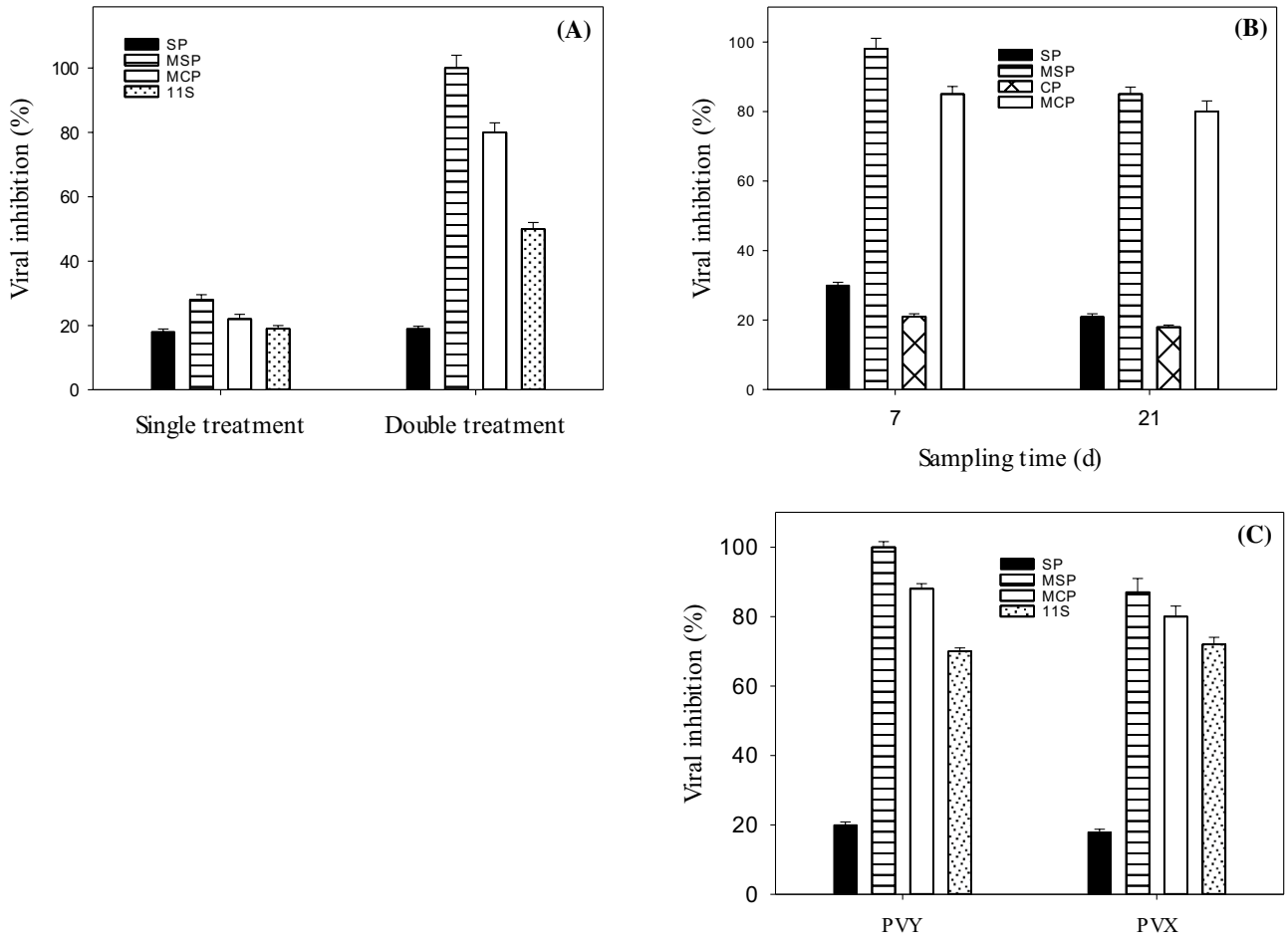


**Fig. 3** Potato virus inhibition (%) by methylated soy and chickpea proteins (MSP and MCP, respectively), 11S soy globulin, and native soy protein (SP) applied at 500 µg $ml^{-1}$. Relative viral content was estimated by DAS-ELISA (the double antibody sandwich linked immunosorbent assay). **A** Comparison between the single and double application of different proteins (10 and 20 days from viral infection) on the inhibition of potato virus Y (PVY) under greenhouse conditions. **B** Compare the sustainability of the antiviral action against PVX during 21 days after the second application of the antiviral substance, under greenhouse conditions. **C** Comparison between the antiviral action against potato virus Y (PVY) and potato virus X (PVX) under field conditions. Viral inhibition was calculated according to the following equation: Viral inhibition% $= \left[(Cp - Cn) - (T - Cn)/(Cp - Cn)\right] \times 100$ where Cp is the positive control reading, Cn = the negative control reading and T is the treatment reading. The variation bars refer to ± SD

plants from the potential damages caused by PVY and PVX under normal available conditions when using a double foliar spray of MSP and MCP (500 µg $ml^{-1}$) after 10 and 20 days of infection. The data in Fig. 3C show that the viral propagation in potato plants (determined by DAS-ELISA), grown under open field conditions and mechanically infected with either PVX or PVY was reduced by treating potato plants with 500 µg $ml^{-1}$ of MSP, MCP, 11S after 10 and 20 days of infection. Generally, the action of MSP, MCP against PVY was more evident than against PVX, while the native protein showed the least antiviral action in the leaf samples collected after 21 days from the second treatment. The three substances' antiviral action came in the following order; MSP > MCP > 11S, with slight differences between the three active substances.

Assessing PVY virus in 6 representative plants in each treatment by RT-PCR (Fig. 4A) showed that four out of six plants were free of the virus in MSP-treated plants against only one and two plants in case of 11S- and MCP-treated plants, respectively. On the other hand, the plants treated with the native soy protein (SP) were all positive for PVY, i.e., similar to the positive control (data not shown). The inhibitory action amounted to 67, 33, and 17% for MSP, MCP, and 11S, respectively. This result confirms the previous ones using DAS-ELISA.

Analyzing ten representative samples of the different treatments by dot-blot hybridization technique showed that six plants out of 10 were free of the virus in case of MSP treatment against five and four plants in the case of MCP and 11S-treated plants, respectively (Fig. 4B). Based on these results, the antiviral action of MSP, MCP, and 11S against PVY amounts to 60, 50 and 40%, respectively. This result confirms the same trend observed with the RT-PCR technique.

Exposing PVY and PVX viruses to the action of methylated chickpea protein (500 µg $ml^{-1}$) for 2 h and analyzing by transmission electron microscopy (TEM) produced the micrographs in Fig. 5. Some structural deformations are noticed in the micrographs of the two viruses being in contact with tested material.

The experimentally proven antiviral activity of the tested substance on the two viruses (PVY and PVX) was also evaluated on the growth and yield of potato plants cultivated

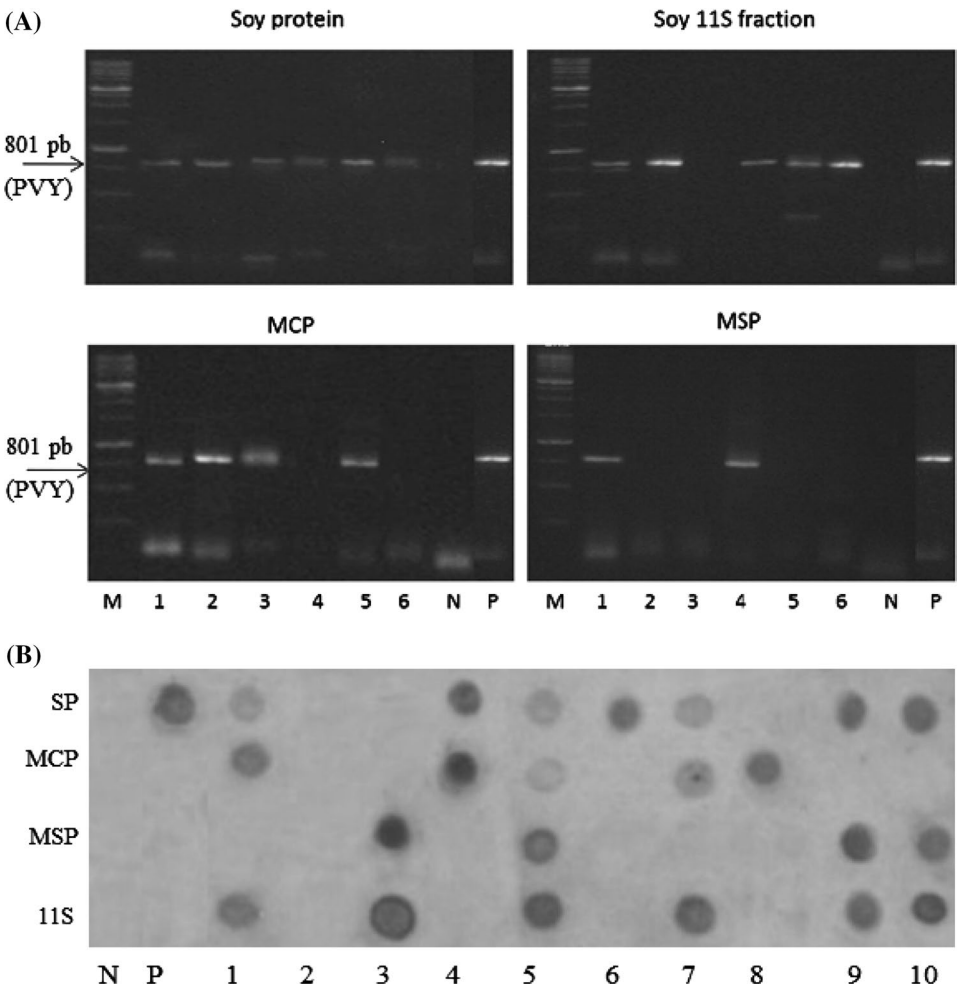


**Fig. 4** Detection of PVY by one-step RT-PCR and visualized by gel electrophoresis using Verso TM one step RT-PCR kit (Thermo scientific) (**A**) and dot-blot hybridization film (**B**) in the leaves of potato plants grown under open field conditions. Plants were mechanically infected with PVY and treated twice with 500 µg $ml^{-1}$ (10 and 20 days after the viral infection) of native soy protein (SP), soy 11S fraction, or methylated soy protein and chickpea protein, (MSP and MCP). Six representative plant samples out of a total of 15 plants/treatment were used for RT-PCR analysis, taking the numbers 1–6 while 10 plants samples out of 15 plants were analyzed for dot-blot hybridization taking the numbers 1–10. Samples N and P are the negative and positive controls, respectively

under open field conditions for two successive seasons for each virus, as shown in Tables 1 and 2.

Some amelioration in the growth parameters (fresh weight, dry weight, No. of shoots) was remarked in the PVY- and PVX-infected plants after treatment with the methylated products (MSP and MCP) as compared to the positive and negative control and also to the native protein treatments (SP and CP). More evidently, the tuber yield of PVY-infected treated by MSP, MCP, and 11S increased by 68, 42 and 36% over the positive control, respectively and to lesser respective extents over the negative control; 34, 13 and 9%. Similar results were obtained with the PVX-infected plants giving yield increases about 52, 41 and 30 over the positive control and 30, 21 and 11% over the negative control in response to MSP, MCP and 11S applied twice after 10 and 20 days from infection as a foliar spray at (500 µg $ml^{-1}$). The pictures in Fig. 6. present the morphological appearance of potato plants before and after inoculation with PVY, then treated with MCP at 500 µg $ml^{-1}$. The images were taken 14 days after the second foliar spray treatment. The PVY-infected plants (positive control) showed different signs of viral infections, e.g., a mild mosaic with slight leaf crinkling, leaf mottling, vein-banding and clearing, and distortion. Foliar spray treatment with MCP at 500 µg $ml^{-1}$ could considerably reduce the visible viral infection symptoms as compared to the positive viral control.

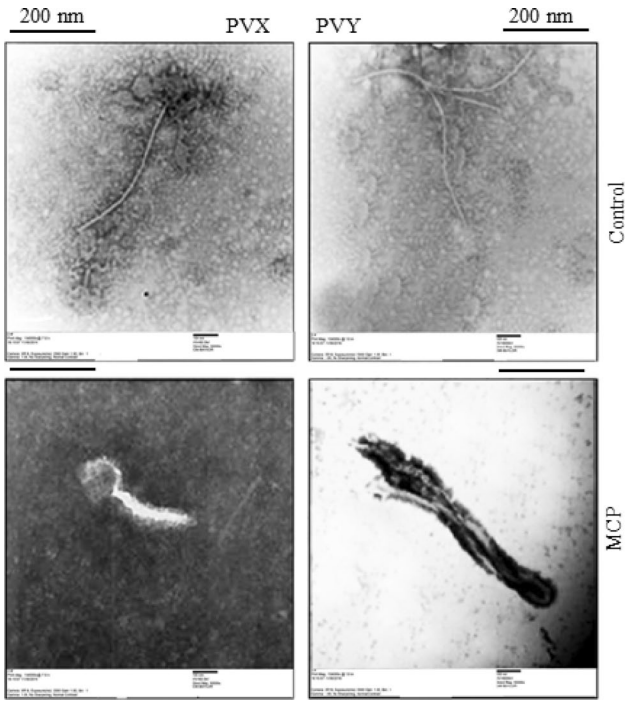


**Fig. 5** Transmission electron microscopy (TEM) images (100 nm/50,000 ×) of potato virus Y and X (PVY and PVX, respectively) in their native forms (control) and after subjection to 500 µg $ml^{-1}$ methylated chickpea protein at room temperature for 2 h

## Discussion

The properties of MSP and MCP prepared for the present study were confirmed to be similar to previously prepared and published ones (Sitohy et al. 2013). The high esterification extents of MSP and MCP (80% and 83%) were associated with a common iso-electric point (PI) of both around 8, following (Sitohy and Osman 2010). Grafting methyl groups on the protein-free carboxylate groups during esterification may simultaneously enhance hydrophobicity. This relatively higher PI, alongside an augmented hydrophobicity,

**Table 1** Effect of native chickpea protein (CP), methylated chickpea protein (MCP), native soybean protein (SP), methylated soybean protein MSP) and 11S soybean globulin (500 µg $ml^{-1}$) on the growth and yield traits of potato plants infected with potato virus Y (PVY)

| Treatment | FW (g)† | DW (g) | No. of shoots | Plant height (cm) | No. of tubers/plant | Weight (g) of tuber/plant | Total tuber yield (kg/ha.) | % Yield increase/PC | % Yield increase/NC |
|---|---|---|---|---|---|---|---|---|---|
| NC* | $390 \pm 2.83^{e}$ | $73.5 \pm 4.95^{d}$ | $4.10 \pm 0.14^{e}$ | $53.7 \pm 1.84^{c}$ | $7.50 \pm 0.71^{ab}$ | $447 \pm 4.24^{c}$ | $20{,}477 \pm 673^{bc}$ | – | – |
| PC** | $378 \pm 2.83^{f}$ | $66.0 \pm 4.24^{e}$ | $4.40 \pm 0.14^{d}$ | $54.6 \pm 2.05^{bc}$ | $6.50 \pm 0.71^{b}$ | $352 \pm 4.24^{e}$ | $16{,}310 \pm 504^{c}$ | – | – |
| CP | $552 \pm 4.95^{d}$ | $104 \pm 4.95^{c}$ | $5.50 \pm 0.28^{b}$ | $53.7 \pm 1.84^{c}$ | $7.10 \pm 0.57^{ab}$ | $375 \pm 3.54^{d}$ | $19{,}643 \pm 2525^{bc}$ | 20 | −4 |
| MCP | $590 \pm 4.24^{b}$ | $111 \pm 4.95^{b}$ | $5.75 \pm 0.21^{a}$ | $56.2 \pm 1.13^{ab}$ | $7.55 \pm 0.64^{ab}$ | $520 \pm 3.54^{a}$ | $23{,}096 \pm 673^{b}$ | 42 | 13 |
| SP | $570 \pm 6.36^{c}$ | $109 \pm 4.95^{b}$ | $5.10 \pm 0.14^{c}$ | $56.2 \pm 2.55^{ab}$ | $7.75 \pm 0.07^{a}$ | $435 \pm 3.54^{c}$ | $21{,}072 \pm 505^{b}$ | 29 | 3 |
| MSP | $622 \pm 2.62^{a}$ | $118 \pm 3.54^{a}$ | $4.15 \pm 0.21^{e}$ | $54.4 \pm 2.26^{bc}$ | $7.40 \pm 0.14^{ab}$ | $522 \pm 2.83^{a}$ | $27{,}382 \pm 1684^{a}$ | 68 | 34 |
| 11S | $586 \pm 4.95^{b}$ | $110 \pm 3.54^{b}$ | $4.40 \pm 0.14^{d}$ | $57.1 \pm 1.41^{a}$ | $6.85 \pm 0.21^{ab}$ | $478 \pm 3.54^{b}$ | $22{,}262 \pm 842^{b}$ | 36 | 9 |
| *P*-value | < 0.001 | < 0.001 | < 0.001 | 0.0019 | 0.0367 | < 0.001 | < 0.001 | | |

Data are the means of two successive seasons ± SD. Different letters in the same column indicate significant difference. The variation was assessed by one-way ANOVA and the differences between experimental groups were calculated by Tukey's test

*FW* fresh weight, *DW* dry weight, No

*Negative control (received neither viral infection nor treatment)

**Positive control (received viral infection but not treatment)

**Table 2** Effect native chickpea protein (CP), methylated chickpea protein (MCP), native soybean protein (SP), methylated soybean protein (MSP), and soy 11S globulin (500 µg $ml^{-1}$) on the growth and yield traits of potato plants artificially infected with potato virus X (PVX)

| Treatment | FW (g) | DW (g) | No. of shoots | Plant height (cm) | No. of tubers/plant | Weight (g) of tuber/plant | Total tuber yield (kg/ha.) | % Yield increase/PC | % Yield increase/NC |
|---|---|---|---|---|---|---|---|---|---|
| NC* | $480 \pm 4.24^{b}$ | $90.0 \pm 2.83^{b}$ | $4.15 \pm 0.21^{d}$ | $52.1 \pm 1.34$cd | $9.05 \pm 0.07^{a}$ | $456 \pm 1.41^{d}$ | $20{,}596 \pm 168^{d}$ | – | – |
| PC** | $351 \pm 4.95^{f}$ | $69.5 \pm 2.12^{e}$ | $4.85 \pm 0.21^{c}$ | $48.9 \pm 1.63^{e}$ | $6.75 \pm 0.35^{b}$ | $401 \pm 1.06$g | $17{,}619 \pm 337^{e}$ | – | – |
| CP | $399 \pm 2.83^{e}$ | $78.5 \pm 2.12^{d}$ | $5.15 \pm 0.21^{b}$ | $51.1 \pm 1.34^{d}$ | $6.95 \pm 0.49^{b}$ | $446 \pm 1.41^{e}$ | $21{,}310 \pm 168$cd | 21 | 3 |
| MCP | $421 \pm 4.95^{d}$ | $82.5 \pm 3.54^{c}$ | $4.90 \pm 0.14^{c}$ | $53.6 \pm 1.98^{bc}$ | $7.40 \pm 0.42^{b}$ | $496 \pm 1.41^{b}$ | $24{,}881 \pm 1179^{b}$ | 41 | 21 |
| SP | $401 \pm 1.41^{e}$ | $89.5 \pm 2.12^{b}$ | $4.10 \pm 0.28^{d}$ | $58.7 \pm 1.91^{a}$ | $6.80 \pm 0.28^{b}$ | $426 \pm 1.41^{f}$ | $21{,}191 \pm 337$cd | 20 | 3 |
| MSP | $553 \pm 4.24^{a}$ | $95.5 \pm 2.12^{a}$ | $5.40 \pm 0.14^{a}$ | $54.3 \pm 2.40^{b}$ | $7.40 \pm 0.85^{b}$ | $619 \pm 1.58^{a}$ | $26{,}843 \pm 774^{a}$ | 52 | 30 |
| 11S | $448 \pm 4.24^{c}$ | $89.5 \pm 0.71^{b}$ | $5.50 \pm 0.28^{a}$ | $51.7 \pm 1.91^{d}$ | $7.20 \pm 0.42^{b}$ | $488 \pm 3.54^{c}$ | $22{,}858 \pm 337^{c}$ | 30 | 11 |
| *P*-value | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.0017 | < 0.001 | < 0.001 | | |

Data are the means of two successive seasons ± SD. Different letters in the same column indicate significant difference. The variation was assessed by one-way ANOVA and the differences between experimental groups were calculated by Tukey's test

*FW* fresh weight, *DW* dry weight, No

* Negative control (received neither viral infection nor treatment)

** Positive control (received viral infection but not treatment)

maybe two main reasons for enhancing MSP and MCP's bio-reactivity. Similarly, the protein fraction 11S globulin was characterized by a relatively higher PI (6.5) than the total protein (4.5) and molecular mass of 21 and 34 KD corresponding to the basic and acidic subunit, enabling it to be reactive against microorganisms, as previously found (Abdel-Shafi et al. 2019; Amer et al. 2020; Mahgoub et al. 2016; Omar et al. 2018; Osman et al. 2013, 2020, 2018; Sitohy et al. 2012).


**Fig. 6** Morphological appearance of potato plants before and after inoculation with PVY, then treated with MCP at 500 µg $ml^{-1}$. The images were taken 14 days after the second foliar spray treatment

The results derived from the preliminary greenhouse experiment, showing a concentration-dependent antiviral activity against both the esterified legume proteins (MSP and MCP) agree with previous reports on some esterified proteins on different viruses (Abdelbacki et al. 2010; Taha et al. 2010). The supremacy of the esterified proteins' antiviral action over the native proteins is in accordance with findings by (Sitohy et al. 2011). The results, showing antiviral activity even at the lowest used concentration (100 µg $ml^{-1}$) while the maximum was at 500 µg$ml^{-1}$, confirm the antiviral action of MSP, MCP, and 11S and the adequacy of the second concentration for combating the virus. Practically, this is a relatively low concentration, particularly when the antiviral agent is submitted by foliar spray, where a considerable part is lost during the application. The native forms seemed nearly void of antiviral activity, while the antiviral potencies of MSP and MCP seem nearly equal.

The results indicating the supremacy of double spray over single spray treatment may indicate the single application's insufficiency to produce the maximal antiviral action. In such a case, applying a second treatment of the substance may suppress the potential recovery of potato viruses after the first foliar application's effect has faded or vanished. This may also confirm the specificity of the applied substance against the infecting virus. The greenhouse experiment showed the antiviral action's stability since there were only slight changes in the virus level after 7 and 21 days of the substance application. However, the same result may indicate a gradual diminishing of the antiviral actions, probably

due to the antiviral substance's consumption through the interaction with the virus or with different plant substances. The results obtained in the greenhouse conditions gave the basis of the design of the open field experiment, where the substance concentration was always 500 µg $ml^{-1}$, and the frequency of application was double spray 10 and 20 days after application, as well as the timing of analytical measurements, was always after 21 days of the last application, unless specified.

The open-field experiment confirmed and substantiated the initial results obtained in the greenhouse experiments. Thus, the tested substances have considerable capacity to protect potato plants grown under open field conditions against the viral economic damages caused by PVY and PVX during plant growth and harvest. Using the technique, DAS-ELISA in assessing the relative viral load in potato plants has confirmed the reduction of PVX or PVY propagation in the virus-infected plants by the treatment with 500 µg $ml^{-1}$ of MSP, MCP, 11S after 10 and 20 days of infection, confirming the results of the greenhouse experiments. These results also showed that the action against PVY was more evident than against PVX, while the native protein showed the least antiviral action depicting the following order of effect; MSP > MCP > 11S > SP. The higher biological activity of the modified proteins than the native ones and also the supremacy of MSP over MCP is in line with previous studies. It may be due to differences in the magnitude of the positive charges and hydrophobicity on the molecule surface (Sitohy and Osman 2011).

Using the RT-PCR in determining the viral load in the same samples has indicated inhibitory actions amounting to 67, 33, and 17% against PVY for MSP, MCP, and 11S, respectively, following the previous order derived from DAS-ELISA results. However, the magnitude of inhibition was not at the same level. Likewise, using the dot-blot hybridization technique showed antiviral inhibitory actions of 60, 50, and 40% for MSP, MCP, and 11S against PVY, respectively. These results are closer to RT-PCR than DAS-ELISA, although the three techniques were all in the same trend. Collectively the results confirm the considerable antiviral action of the studied proteins, particularly MSP, MCP, 11S against PVY, and PVX. This action was further visualized by Transmission electron microscopy (TEM), which indicated physical deformations in the viral particles of both PVY and PVX when in contact with methylated chickpea protein (MCP), evidencing the direct action of the tested, modified protein on the viral particles. This action may arise from the potentiality of the interaction between the positively charged proteins with the negatively charged nucleic acid backbone, as previously evidenced (Sitohy et al. 2002).

The antiviral action of the tested substances (MSP, MCP, and 11S) has apparently been transformed into better growth and yield parameters in the potato plants infected with either PVY or PVX and grown under open field conditions. This antiviral action against potato plant viruses may resemble the action of the methylated proteins against tobacco mosaic virus (Wang et al. 2013) and against tomato yellow leaf curl virus (TYLCV) when using methylated whey proteins (Abdelbacki et al. 2010). This action might have occurred through perturbing the viral replication through protein-RNA reaction or treating protein-viral protein interactions. Previous studies proved the potentiality of DNA-basic protein interaction (Sitohy et al. 2001d). More evidently, the considerably high relative increases in the tuber yield in either the PVY-infected (64, 47 & 36%) or PVX-infected (51,38 and 30) plants over the positive control after treatment with MSP, MCP, and 11S, respectively, indicate that the substances have broad specificity and high activities against potato viruses.

However, the action of these substances (MSP, MCP, and 11S) was not restricted to reducing the viral load, but it was probably extended to promoting the general physiological status of the treated plants since the yield results indicated also yield gains not only over the positive control but also over the negative control within still a significant range of 8–31%. The general promoting action of the tested proteins on plant physiology may go in line with the promoting operation of the salicylic acid application on potato virus X-infected tomato plants through enhancing the expression of particular proteins in plants, offsetting the impact of the viral infection (Cueto-Ginzo et al. 2016). This effect may also agree with the previously observed rises in some bioactive molecules as lycopene and ascorbic acid in plants in response to spray with legume-derived protein hydrolysates (Sitohy et al. 2020b).

## Conclusions

The results derived from the preliminary greenhouse experiment showed a concentration-dependent antiviral activity of both the esterified legume proteins (MSP and MCP), excelling their native forms. Maximum viral inhibition was obtained at a concentration of 500 µg $ml^{-1}$ and was further extended by twice repeating the plant treatment after ten days of the first one. In such a case, applying a second treatment of the substance may suppress the PVX virus's potential recovery after the first foliar application's effect has vanished. The only slight changes in the studied proteins' antiviral action between 7 and 21 days after application indicate their stability. Nevertheless, the gradual and slight diminishing of the antiviral actions may probably arise from consuming the antiviral substance by the interaction with the virus or with different plant substances. So, it is recommended to use 500 µg $ml^{-1}$ of the three materials as double spray 10 and 20 days after infection. The antiviral action of

the tested substance, measured by DAS-ELISA, was in the following order of effect; MSP > MCP > 11S > SP, indicating the modified proteins' higher biological activity than the native ones and also the superiority of MSP over MCP.

Using the RT-PCR technique in determining the viral load in the same samples indicated inhibitory actions amounting to 67, 33, and 17% against PVY for MSP, MCP and 11S. Using the dot blot hybridization technique gave corresponding values of 60, 50 and 40%, indicating the closeness of the results of these two techniques, confirming the prevailing trend of action. The results confirm the studied proteins' considerable antiviral action, particularly MSP, MCP, and 11S against PVY and PVX. This action was further visualized by transmission electron microscopy (TEM), indicating the physical deformations in the viral particles of PVY and PVX by methylated chickpea protein (MCP), evidencing a direct action of the modified protein on the viral particles.

The antiviral action of the tested substances (MSP, MCP, and 11S) has been positively reflected in the growth and yield parameters of potato plants infected with either PVY or PVX. More evidently, the considerably high relative increases in the tuber yield in either the PVY-infected or PVX-infected plants over the positive control by MSP, MCP and 11S, indicate the adequacy and top activities against potato viruses. The action of the studied substances (MSP, MCP, and 11S) was not restricted to reducing the viral load. It is likely to extend to enhancing the general physiological state of plants because their yields not only outperformed the positive control but also the negative one.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s41348-021-00448-9.

**Acknowledgments** This project was financially supported by the Science and Technology Development Fund (STDF), Egypt, Grant No. 371.

**Author Contributions** MS has conceived the research concept. MS, ST and AA have planned for the research actions. AO and MA have prepared the antiviral candidate proteins. AA and AH have conducted the experimental agricultural work and the sample analysis. MS, AO and SO revised the results. All authors participated in interpreting the results. AO wrote the draft manuscript and MS wrote the final manuscript.

**Funding** Science and Technology Development Fund (STDF), Egypt.

**Conflict of interest** The authors declare no conflict of interest.

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