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RESEARCH

In-vitro management of Potato Leafroll Virus (PLRV) by using active material of different natural products

Tahsin S. Shoala[1], Ahmed K. El-Attar[2], Ahmed I. Abd El Maksoud[3]

ABSTRACT

Background: Potato Leafroll Virus (PLRV) is considered as one of the most important potato pathogenic virus worldwide. PLRV belongs to genus *Potyvirus*, transmitted by aphids and widespread in most potato growing areas globally, that causes huge losses in potato crop production. PLRV management became a challenge due to the economic importance of potato *(Solanum tuberosum* L.), the fourth largest staple crop in the world. Our research was aiming to manage PLRV *In-Vitro*.
Methods: Infected potato plants (Solanum tuberosum L. var. slany) were externally sterilized and cultured in Murashige and Skoog media (MS media). Four weeks later, potato plants were tested for PLRV using RT-PCR technique, and all of the plants were PLRV positive. MS media were prepared, autoclaved, and active natural materials (Glycyrrhizic acid ammonium salt (GAS), Glycyrrhizic Acid (GA), Curcumin (CU) and Rosmarinic Acid (RA) were sterilized and added to MS media at different concentrations.
Results: Potato plants those treated with Glycyrrhizic acid ammonium salt (2ng, 4ng, and 8 ng/ml MS media) showed that the presence of PLRV at concentration 2ng/ ml MS media while they were negatively diagnosed for PLRV at the other concentrations. However, Treated potato plantlets with curcumin showed that PLRV present in concentrations 2ng/ ml in the MS media but treated potato plants with concentration 4ng and8 ng/ml in the MS media were negative to PLRV. Moreover, treated potato plants with Rosmarinic acid and Glycyrrhizic acid showed that the presence of PLRV in all concentrations (2ng, 4ng, 8ng/ ml in the MS media).
Conclusion: *In-Vitro* application of Glycyrrhizic acid ammonium salt and curcumin at higher concentrations suppressed or decreased PLRV compared to low concentrations and control.

Key words: Potato, PLRV, Natural compounds, Tissue culture, Glycyrrhizic acid, Glycyrrhizic acid ammonium salt, Rosamrinic acid, Curcumin

BACKGROUND

Potato (*Solanum tuberosum*) is one of the most important crops worldwide, and is considered as the fourth important food source after wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) (Rashid *et al*., 2013; Zhang *et al*., 2016). Potato plants can be infected by many viruses, over 40 viruses and viroids (Jeffries *et al*., 2006). PLRV represent genus Polerovirus, family Luteoviridae, and can be transmitted by Aphids especially green peach aphid (*Myzus persicae*). Aphid insects feed on phloem tissues of the infected potato plants by using sap sucking mouth parts and transmit the virus via circulative and non-propagative ways (D'Arcy *et al*., 2005).

Potato leafroll virus (PLRV) is one of the most important, destructive and economical potato pathogenic viral diseases. Potato leafroll virus causes severe damage and great yield

Correspondence: Tahsinshoala2000@gmail.com
Full list of author information is available at the end of the article

losses above 50%, which affect the global yield loss of about 20 million tonnes (Wales *et al*., 2008). Potato crop production losses could vary according to the resistance and susceptibility of potato cultivars in response to PLRV. Potato crop production may decrease by 90% and quality reduced, especially in the highly susceptible potato cultivars to PLRV (Solomon-Blackburn and baker, 2001).

Glycyrrhizic acid (GA) is one of the major bioactive constituents in licorice, which obtained from rhizomes and roots of *Glycyrrhiza glabra* (IHIDMA, 2002). Licorice has been widely applied as herbal medicine against many diseases, such as anti-virus (Pompei *et al*., 1979), anti-bacterial (Ates and Turgay, 2003), antifungal (Alonso, 2004), antioxidant (Fu *et al*., 2013), antitumor (Amirghofran, 2010), antiulcer (Krausse *et al*., 2004), anti-inflammatory (Li *et a*l., 2017), anti-allergic (Han *et al*., 2017), and hepatoprotective (Kim *et al*., 2009). Also, Glycyrrhizic acid can be used in the form of Glycyrrhizic acid ammonium salt. Nowadays, most of research studies are looking for environmentally friendly compound to sort out specific problems without harming the human being and the environment. Therefore, GA as environment-friendly extraction from licorice is essentially required.

Rosmarinic acid (RA) is a phenolic compound synthesized from phenylalanine through the esterification of caffeic acid and tyrosine through 3,4- dihydroxyphenyllactic acid (Ellis and Towers 1970). RA is frequently originated in the species of Boraginaceae and Nepetoideae subfamily of the Lamiaceae. RA, as a pure compound, was firstly isolated from *Rosmarinus officinalis* (Scarpati and Oriente 1958). RA and its derivatives lithospermic acid, yunnaneic acid, salvianolic acid, and melitric acid have various range of biological activities such as anti-viral, anti-microbial, anti-oxidant, anti-inflammatory and anti-tumor (Bulgakov *et al*., 2012; Wu and Wang, 2012).

Curcumin is a hydrophobic polyphenol produced from the rhizome (turmeric) of the herb *Curcuma longa*. Curcumin has been recognized as the active source of turmeric which is chemically structured as a bis-a,b-unsaturated b-diketone that displays keto-enol tautomerism. Curcumin has various biological function activities such as antimicrobial, antioxidant, anti-inflammatory and ant-carcinogenic. Also, curcumin is extremely safe when applied in the highest dosage in different animal models and human studies (Aggarwal *et al*., 2003; Goel *et al*., 2008). Although its efficiency and safety, curcumin has not yet been permitted as a therapeutic agent. Curcumin has many major problems such as poor aqueous solubility, fairly low bioavailability, and concentrated staining colour.

Our current research study is aiming to manage Potato leafroll virus (PLRV) using natural materials Glycyrrhizic acid ammonium salt (GAS), Glycyrrhizic Acid (GA), Curcumin (CU) and Rosmarinic Acid (RA).

MATERIALS AND METHODS

Sample collection

Tissue cultured potato plants infected with Potato Leaf Roll Virus were kindly provided by Dr. Ahmed Abd Al-Maksud, Genetic engineer institute, Sadat University, Egypt. Parts from the infected tissue cultured plants harvested and excised under sterilized condition in vitro cultured. Sterilization occurred by immersion potato plantlets for several seconds in 70% ethanol followed by rinsing three times in sterile distilled water under laminar airflow cabinet (Danci *et al*., 2011).

Media preparation

The regeneration medium consisted of: Murashige and Skoog (1962) (MS) salts, $NaH_2PO_4.2H_2O$ (221 mg/l), thiamine HC1 (0.4 mg/l), inositol (100 mg/l), sucrose (30 g/l), agar (8 g/l), pH adjusted to 5.8 then The medium is sterilized by autoclaving for about 20 minutes at 15 psi, 121^{o}C. Autoclaved MS medium was cooled down to 45-50°C and Screw cap jars filled out with 30 ml of the MS medium. Culture glass screw cap jars were then incubated at 20-21^{o}C in 16h photoperiod with 2000 lux intensity. Potato plantlets were regenerated and multiplied through stem cuttings on MS basal medium in Culture glass screw cap jars (Danci *et al*., 2008). Natural materials were prepared at different concentrations, then sterilized by using 0.2µm Millipore and added to MS medium after autoclaving and cooling down to 40-50 ^{o}C.

Glycyrrhizic acid preparation

Glycyrrhizic acid was purchased from Sigma-Adrich Company (CAS Number: 1405-86-3), 0.2 mg of Glycyrrhizic acid was dissolved in 1 ml absolute ethanol at room temperature (20-25°C), then stored in the fridge (4°C).

Glycyrrhizic acid ammonium salt Preparation

Glycyrrhizic acid ammonium salt was purchased from Sigma-Adrich company (CAS number : 53956-04-0), 0.1 mg of Glycyrrhizic acid Ammonium salt was dissolved in 1 ml absolute ethanol at room temperature (20-25°C), then stored in the fridge (4°C)..

Rosmarinic acid preparation

Rosmarinic acid was purchased from Sigma-Adrich Company (CAS Number: 20283-92-5), 0.1 mg of Rosmarinic acid was dissolved in 1 ml absolute ethanol at room temperature (20-25°C), then stored in the fridge (4°C).

Curcumin preparation

Curcumin was purchased from Sigma-Adrich Company (CAS Number: 458-37-7), Stock of curcumin solution (5 mg/ml) was prepared by dissolving curcumin powder in absolute ethanol and stirring for an hour at room temperature (20-25°C), then stored in the fridge (4°C).

RNA extraction

The total RNA has been extracted from infected tissue cultured potato plantlets using the EZ-10 Spin Column Total RNA Minipreps Super kits, then RNA purified according to the protocol of Thermo Scientific GeneJET Plant RNA Purification Mini Kit. The extracted RNA has been applied as a template for cDNA synthesis.

RT-PCR Detection

Molecular diagnosis used to detect the presence or absence of PLRV, according to the instruction manual of the, Verso^{TM} one step RT-PCR kit (Thermo scientific, cat. No. AB 1454/V6/1107). The extracted RNA was used as a template for one tube RT-PCR amplification reaction using Verso TM one step RT- PCR kit utilizing specific primers for the PLRV. PLRV-F: 5'AATAGAATTCTAATGAGTACGGTCGTGGTTARAGG 3' and PLRV-R:5'AAAACCATGGCTATYTGGGGTTYTGCARAGCYAC 3'. RT-PCR was performed in 25 µl total volume containing 4.75 µl of nuclease - free water, 3ng/µl of total RNA, 12.5 µl of one step PCR master mix,3 µl of 10 µM of each primer,0.5 µl Verso enzyme mix and 1.25 µl RT-Enhancer. RT reaction started with incubation at 50°C for 15 min, followed by denaturation at 95°C for 5min. The amplification reaction was performed through 35 cycles in T-Gradient thermal cycler (Biometra, Germany) starting with denaturation at 94°C for 30 sec, primer

annealing at 52°C for 30 sec and extension at 72°C for 2 min. Final extension at the end of the 35th cycle was performed at 72°C for7 minutes. A fragment of PLRV with expected size of (~548 bp) has been amplified (Shojaei, 2009).

Gel electrophoresis preparation

Gel electrophoresis analysis: Seven-micro liter of RT-PCR product were analyzed on 1% agarose gel in TBE buffer (89 mMTris-HCL, pH 8.5) at 120 volt.100 bp sharp DNA ladder marker (RBC) was used to determine the size of RT-PCR products. Gels were stained with ethidium bromide 10 µg/ml and visualized using gel-documentation system (Bio-Rad, GelDoc XR) (Sambrook *et al*., 1989).

RESULTS

The average number of leaves of infected tissue cultured potato plantlets increased gradually (8, 17, 34 and 42) after (1, 2, 3 and 4 weeks) respectively as showed in Figure (1). While, the infected tissue cultured potato plantlets with PLRV treated with 2ng/ml Glycyrrhizic Acid (GA) recorded 24 leaves compared to (22 and 21 leaves) in response to (4 and 8 ng/ml) respectively in week 3. In week 4, the treatment of infected potato plants with PLRV with 4 ng/ml (GA) recorder 34 leaves compared to 33 and 30 leaves in response to 2 ng/ml and 8 ng/ ml respectively. The average number of potato plantlets decreased gradually to 24, 22 and 21 leaves in response to (2, 4 and 8 ng/ml) (GA), three weeks after treatment. The treatments of infected tissue cultured potato plantlets with 2, 4 and 8 ng/ml affect negatively the number of leaves and growth rate compared to untreated plants (Fig. 1).

RT-PCR, utilizing specific PCR primers for detecting the presence or absence of PLRV, showed that the treatments of infected tissue cultured potato plantlets with different concentrations of (GA) had no impact on PLRV and all tested samples showed positive results (Fig. 2).

The average number of leaves in infected tissue cultured potato plantlets was (9, 8 and 8 leaves) in response to Glycyrrhizic Acid ammonium salt (GAS) concentrations (2, 4 and 8 ng/ml) respectively compared to 8 leaves in the control, one week after treatment. However, treated potato plantlets with (2, 4 and 8 ng/ ml) Glycyrrhizic Acid ammonium salt (GAS) scored 18, 19 and 13 leaves respectively, two weeks after treatment. Additionally, untreated potato plantlets showed an increase in the number of leaves to 34 and 42 leaves in weeks 3 and 4 respectively. While, the average number of leaves in the 3rd week reached 16, 30 and 31 leaves in response to GAS concentrations 8, 4 and 2 ng/ml respectively. However, in the 4th week number of leaves in treated potato plantlets recorded 40, 36 and 19 leaves in response to GAS concentrations 2, 4 and 8ng/ml respectively (Fig. 3).

Detecting the presence or absence of PLRV using PCR primers showed that the treatments of infected tissue cultured potato plantlets with 8 ng/ ml (GAS) showed no band, however GAS at the concentration of 4 ng/ml showed faint band and suppressed the virus. Moreover, the lowest applied concentration of GAS (2 ng/ml) had no impact on the presence of PLRV (Fig. 4).

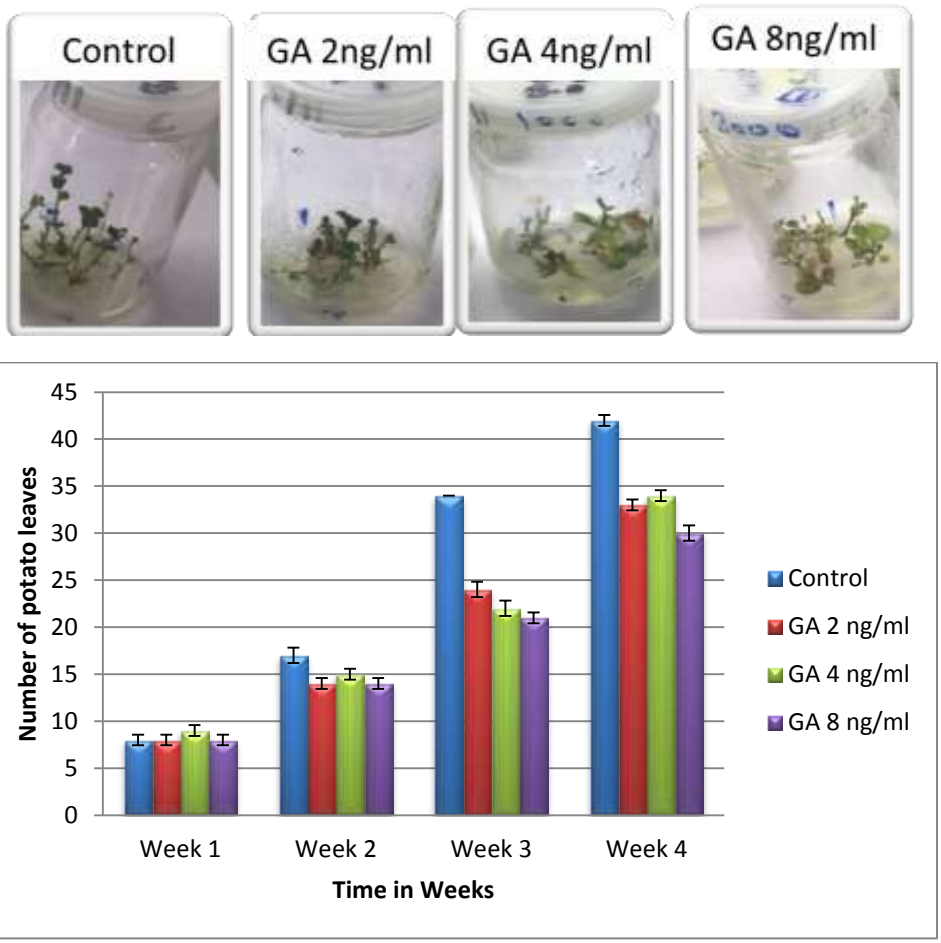

Fig. 1: The average number of leaves in infected tissue cultured potato plantlets S*olanum tuberosum* L. selena with PLRV in response to natural product Glycyrrhizic Acid (GA) (2, 4 and 8 ng/ml) compared to positive control after 1, 2, 3 and 4 weeks.

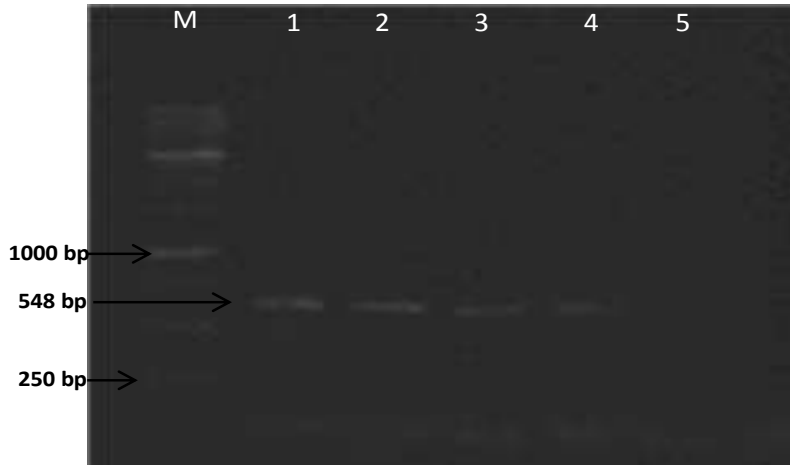

Fig. 2: Gel electrophoresis analysis for potato plantlets S*olanum tuberosum* L. selena tested for PLRV using RT-PCR utilizing specific PCR primers. Lane M 1Kb DNA Marker, lane 2 positive control infected with PLRV (~548bp). Lanes (2, 3 and 4) infected tissue cultured potato plantlets treated with different concentration of Glycyrrhizic Acid (GA) (2, 4 and 8 ng/ml) respectively. Lane No. 5: Healthy plant control.

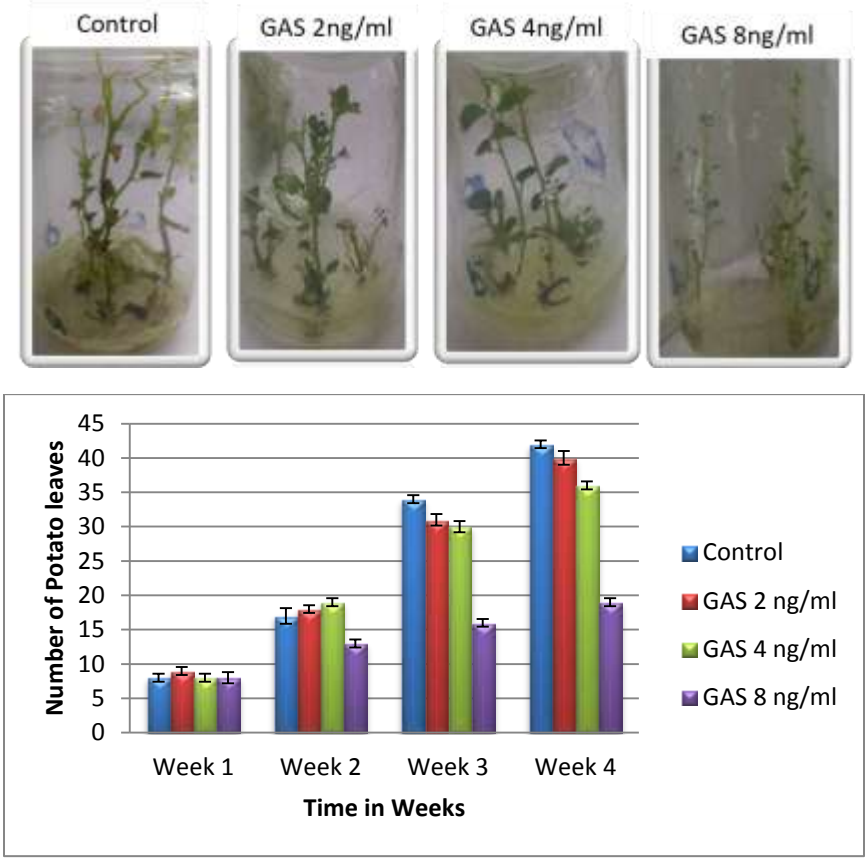

Fig. 3: The average number of leaves in infected tissue cultured potato plantlets S*olanum tuberosum* L. selena with PLRV in response to natural product Glycyrrhizic Acid ammonium salt (GAS) (2, 4 and 8 ng/ml) compared to positive control after 1, 2, 3 and 4 weeks.

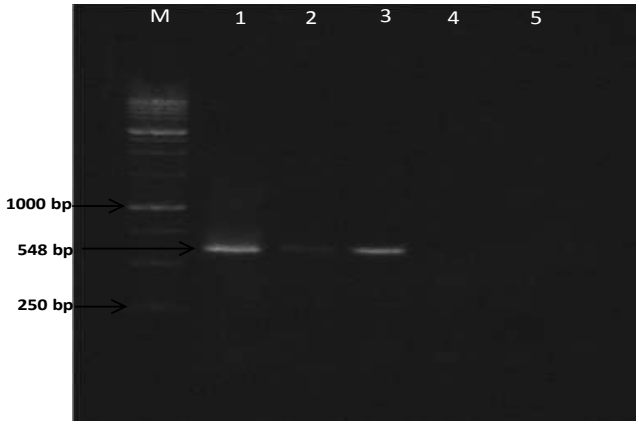

Fig. 4: Gel electrophoresis analysis for potato plantlets S*olanum tuberosum* L. selena tested for PLRV using RT-PCR utilizing specific PCR primers. Lane M 1Kb DNA Marker; Lane 1 positive control infected with PLRV (~548bp); Lanes 2, 4 infected tissue cultured potato plantlets treated with (4 and 8 ng/ml) Glycyrrhizic Acid ammonium salt (GAS) respectively; Lane 3, infected tissue cultured potato plantlets treated with 2 ng/ml Glycyrrhizic Acid ammonium salt (GAS); Lane No. 5: Healthy plant control.

The average number of leaves in the infected tissue cultured potato plantlets recorded the highest number (50 leaves), 4 weeks after treatment compared to 46 and 43 in response to

Rosmarinic acid (RA) concentrations 4 and 2 ng/ ml respectively. However, treated potato plantlets with (2, 4 and 8 ng/ ml) Rosmarinic acid (RA) recorded 31, 35 and 36 leaves respectively compared to 34 leaves in the untreated potato plantlets , three weeks after treatment. Number of leaves in infected tissue cultured potato plantlets showed same number 17 leaves in response to concentrations 2 and 8 ng/ml of RA compared to 18 leaves in response to 4 ng/ml and 15 leaves in the untreated plantlets (Fig. 5).

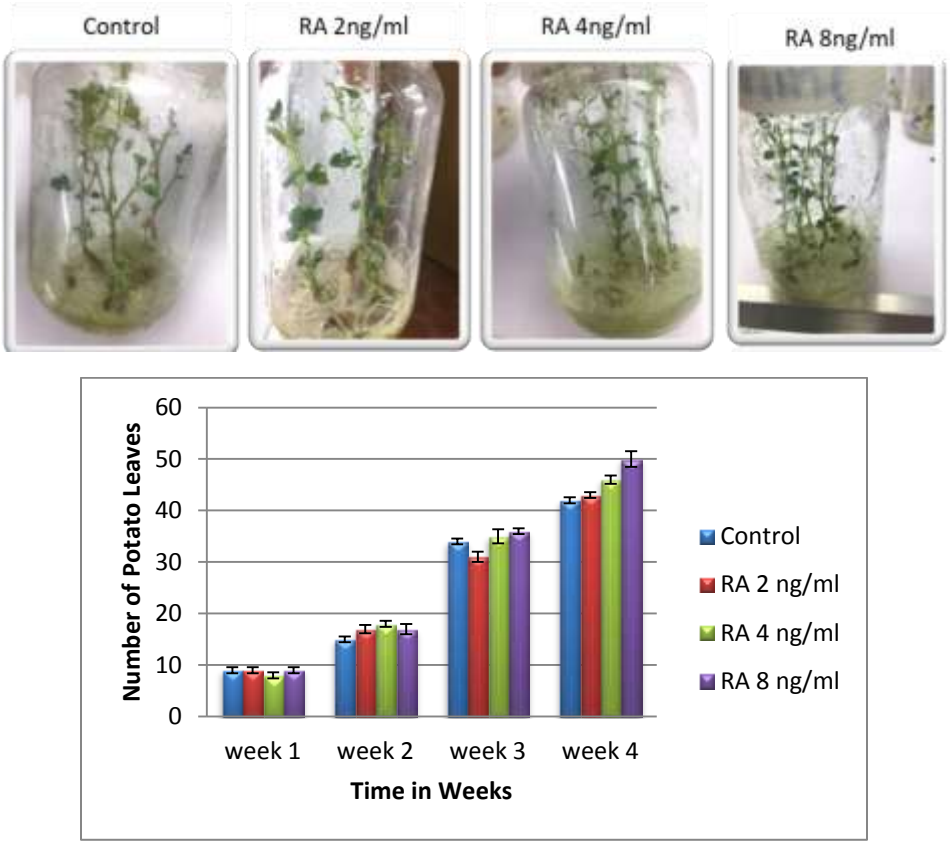

Fig. 5: The average number of leaves in infected tissue cultured potato plantlets S*olanum tuberosum* L. selena with PLRV in response to natural product Rosmarinic Acid (RA) (2, 4 and 8 ng/ml) compared to positive control after 1, 2, 3 and 4 weeks.

Detecting the presence or absence of PLRV using PCR primers revealed that the treatments of infected tissue cultured potato plantlets with 4 and 8 ng/ ml (RA) showed faint bands in response to treatment; however RA at the concentration of 2 ng/ml showed strong band of PLRV. Moreover, the lowest applied concentration of RA (2 ng/ml) had no impact on the presence of PLRV (Fig. 6).

Infected tissue cultured potato plantlets *Solanum tuberosum* L. selena with PLRV, showed that the average number of leaves increased significantly and scored 75 and 70 leaves in response to 4 and 2 ng/ ml of Curcumin (CU) respectively compared to 41 leaves in treated plantlets with 8 ng/ml and 42 leaves in the control, 4 weeks after treatment. The average number of potato plantlets scored the highest number of leaves (55) followed by 50 and 33 leaves in response to 4, 2 and 8 ng/ml respectively compared to 34 leaves in the control, 3 weeks post treatment (Fig. 7).

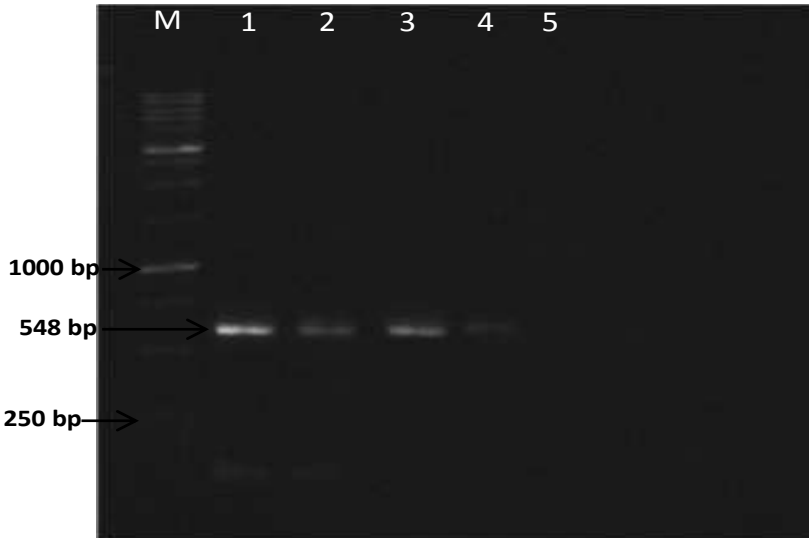

Fig. 6: Gel electrophoresis analysis for potato plantlets S*olanum tuberosum* L. selena tested for PLRV using RT-PCR utilizing specific PCR primers. Lane M 1Kb DNA Marker; Lane 1 positive control infected with PLRV (~548bp); Lanes 2 and 4 infected tissue cultured potato plantlets treated with (4 and 8 ng/ml) Rosmarinic Acid (RA) respectively; Lane 3, infected tissue cultured potato plantlets treated with 2ng/ml Rosmarinic Acid (RA); Lane No. 5: Healthy plant control.

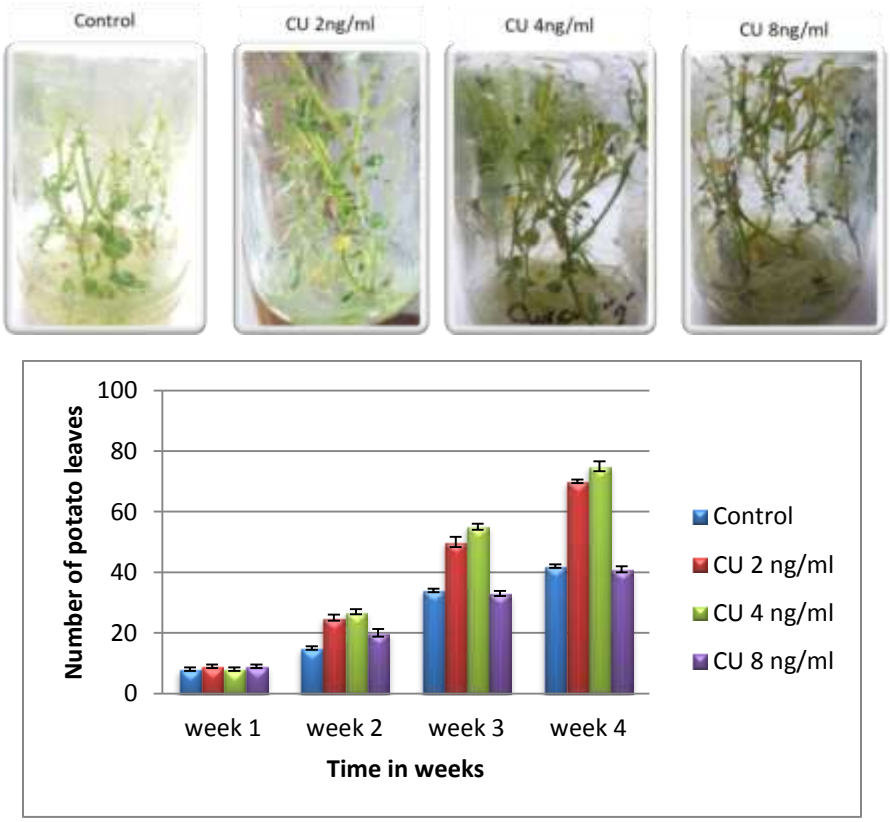

Fig. 7: The average number of leaves in infected tissue cultured potato plantlets S*olanum tuberosum* L. selena with PLRV in response to natural product Curcumin (CU) (2, 4 and 8 ng/ml) compared to positive control after 1, 2, 3 and 4 weeks.

Detecting the presence or absence of PLRV using PCR primers showed that the treatments of infected tissue cultured potato plantlets with 2, 4 and 8 ng/ ml of Curcumin (CU) showed faint bands which means suppression of PLRV virus in response to (CU) treatments (Fig. 8).

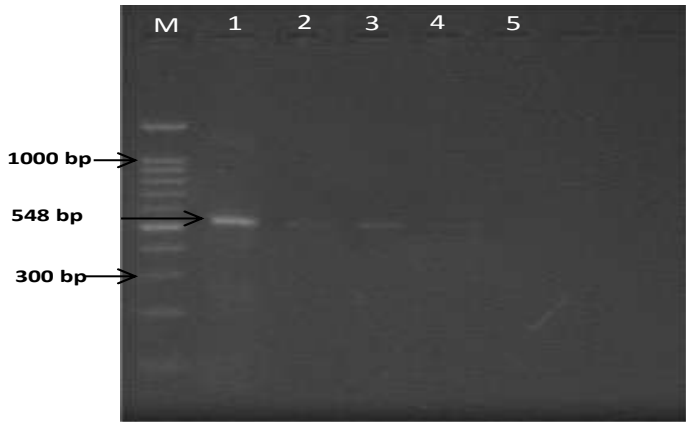

Fig. 8: Gel electrophoresis analysis for potato plantlets S*olanum tuberosum* L. selena tested for PLRV using RT-PCR utilizing specific PCR primers. Lane M 1Kb DNA Marker; Lane 1 positive control infected with PLRV (~548bp); Lanes 2 and 4 infected tissue cultured potato plantlets treated with (4 and 8 ng/ml) Curcumin (CU) respectively; Lane 3, infected tissue cultured potato plantlets treated with 2ng/ml Curcumin (CU); Lane No. 5: Healthy plant control.

DISCUSSION

Potato leafroll virus is considered as one of the most imperative, damaging and economical potato pathogenic virus. Potato Leafroll Virus causes severe damage and great yield losses above 50% (Wales *et al*., 2008). The best solution to cure infected potato plants with PLRV is to get rid of the plants using safe quarantine protocol. On the other hand, Shoot tip meristem technique could be applied to produce potato plants free from viruses but without any management to the plants infected with PLRV. Tissue culture technique could be applied successfully to eliminate or decrease the presence of PLRV by using Thermotherapy protocol according to the physical features of the virus or Chemotherapy protocols.

Our research aiming to use natural products to manage, decrease or eliminate the presence of PLRV in potato plants *Solanum tuberosum* L. selena. Active natural materials Glycyrrhizic Acid (GA), Glycyrrhizic Acid ammonium salt (GAS), Rosmarinic acid (RA) and Curcumin (CU), were applied at concentrations (2, 4 and 8ng/ml) in the MS medium to study the impact of those natural materials on potato plantlets infected with PLRV. Different applied natural products exist in all plants at different concentrations and could be increased or decreased according to induction of specific pathways and production of certain secondary metabolites. Application of natural materials could be positively or negatively impacts at certain concentrations on the growth rate and immunity of the plants according to induced different pathways.

Rosmarinic acid is a phenolic compound and ester of caffeic acid, which is naturally present in numerous plants of the Lamiaceae family, basil (*Ocimum tenuiflorum*).

Application of different concentrations of Rosmarinic acid (RA) on infected potato plantlets *Solanum tuberosum* L. selena with PLRV showed that gradual increase in the average number of leaves in potato plantlets compared to untreated plantlets. Also, highest concentrations of Rosmarinic acid 8ng/ml MS medium suppressed the PLRV, while the low concentration of

Rosmarinic acid had no effect on the PLRV virus. RA in the highest concentration induced different signaling pathways which impacted positively in the growth of potato plantlets and suppressed the presence of PLRV virus.

Curcumin as strong active natural product at different concentrations 2 and 4 ng/ml increased the number of potato plantlets compared to concentration 8ng/ml and untreated plants. Curcumin suppressed the presence of PLRV in the plants by inducing different signaling pathways MAPK genes and increased the quantity of terpenoids, curcumin as strong secondary metabolites which impact positively in the growth of potato plantlets and negatively impacted on the presence of PLRV virus.

Glycyrrhizic Acid ammonium salt (GAS) at different concentrations had no great impact on the growth of potato plantlets, but at high concentrations 4 and 8 ng/ml suppressed the presence of PLRV virus. However, Glycyrrhizic Acid (GA) at different concentrations had no positive impact neither in the growth of potato plantlets nor the presence of PLRV virus. Accordingly, Glycyrrhizic Acid ammonium salt (GAS) is the soluble form of Glycyrrhizic Acid (GA) so that affected positively in the growth of the potato plantlets and negatively on the PLRV. The treatment of infected tissue cultured potato plantlets with Glycyrrhizic Acid ammonium salt (GAS) induced many signaling pathways and increased the end products of secondary metabolites terpenoids and glycyrrhizin through various signaling pathways which increased the resistant levels of potato platelets in response to PLRV (Fig 9).

Reactive Oxygen species (ROS) genes are the first defense system which normally induced within few seconds in response both biotic and abiotic stress, also could function as protectants against different stresses and signals activator (Jiang *et al.,* 2007). Induction of ROS required for full activation of Mitogen-activated protein kinases (MAPKs) genes, which are considered as well characterized families for signaling molecules in higher plants. Also, MAPKs regulate wide range of important and critical cellular processes including cell division, stress responses, metabolism, different developmental processes regulated by hormones and biologically active compounds. Oxidative signal Inducible1 (Oxi1) is a serine/ threonine kinase necessary, a member of AGC of protein kinase family and protein kinase linking ROS accumulation to plant resistance to different stimuli and oxidative burst-mediated signaling in plant roots (Rentel et al., 2004). Also MAPKs genes very important key player in calcium signaling pathways which control different pathways like stomata opening and closure, callose deposition and seeds germination (Peterson et al., 2009). MAPKs cascades also can control leaf extension and root length in response to different stimuli and according to our research the applied natural products stimulate MAPKs cascades and enhanced plant growth which resulted from increasing the photosynthesis pathways growth hormones (Fig 9). Also, stimulate of MAPKs cascades led to an increase in trichrome glandular density which affected positively in the phytohormone levels. Stimulation of the phytohoromone levels induces the plastidic MEP (Methyl Erythritol Phosphate) and enzymes of cytosolic MVA (mevalonate) pathway. Both MEP and MVA pathways stimulate IPP (Isopentenyl Pyrophosphate) and DMAPP (Dimethylallyl Pyrophosphate) are very important in the induction of Terpene synthesis pathway ended by production of terpenoids like Sabinene, β - phyllandrene and other essential oils which affect positively on the quality and quantity of certain important essential oils and improve the resistance level against different stimuli. Nutrient materials and water uptake enhanced as a result of increasing root length and leaf area. Nitrogen uptake, which is considered as an important fundamental constituents of amino acids and enzymes, its sufficiency would positively affect terpenoid biosynthesis. Nitrogen sustains terpenoid productions by improving electron transport rate and photosynthesis process (Ormeno and Fernandez, 2012) (Fig 9). Nitrogen

sustains terpenoid productions by improving electron transport rate and photosynthesis process (Ormeno and Fernandez 2012).

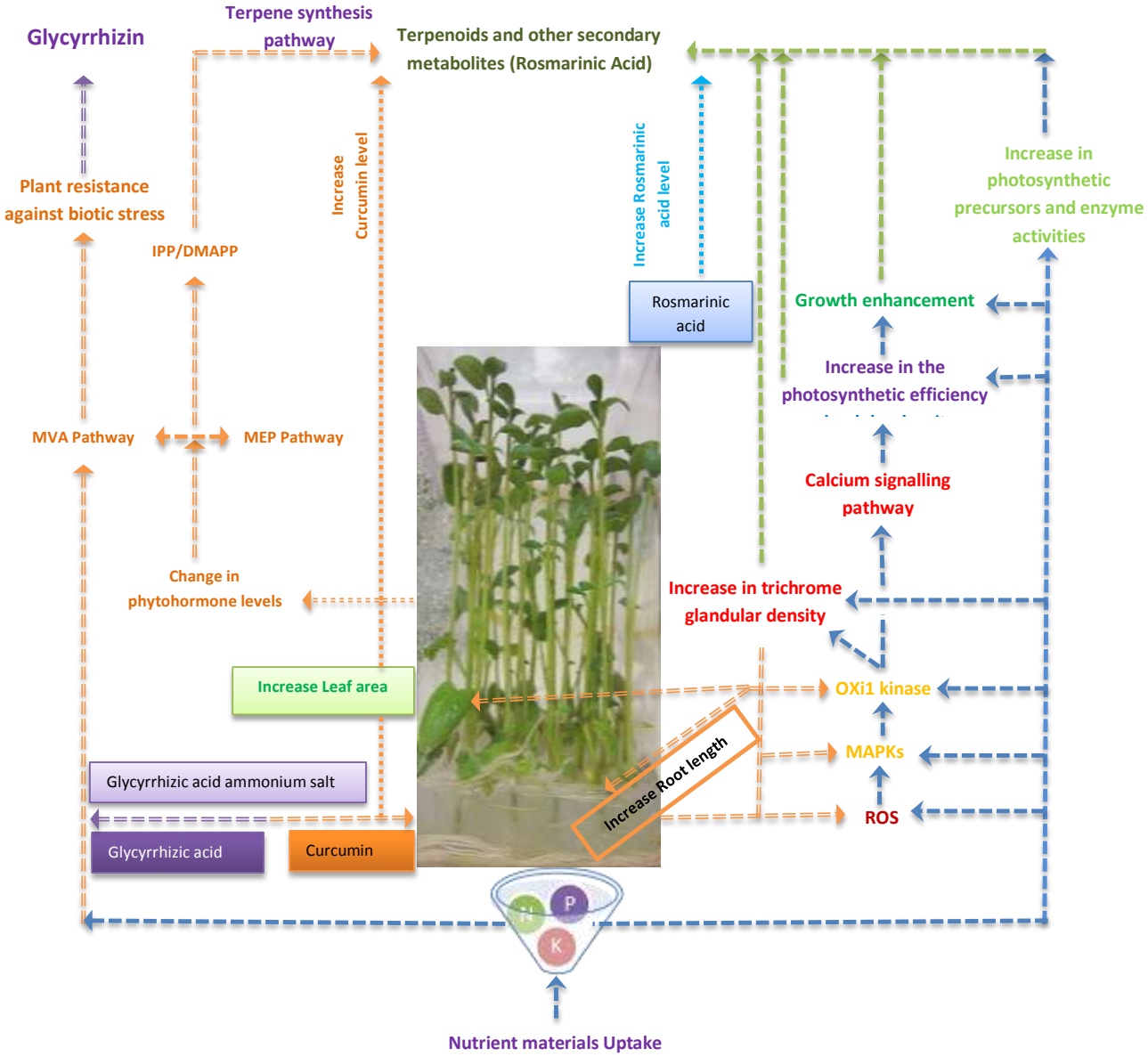

Fig. 9: Schematic diagram for different physiological pathways in infected tissue cultured *Solanum tuberosum* L. selena with PLRV in response to natural products Glycyrrhizic acid, Glycyrrhizic acid ammonium salt, Rosmarinic acid and Curcumin.

AUTHOR DETAILS

[1]College of biotechnology, Misr University for Science and Technology, 6th of October city.
[2]Virology Department, Plant pathology Institute, ARC, Egypt.
[3]Industrial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Menoufia, Egypt

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