STUDIES REGARDING THE EFFECTS OF *Rosmarinus officinalis* OIL TREATMENTS IN HEALTHY AND POTATO VIRUS Y (PVY) INFECTED PLANTS *Solanum tuberosum* L.

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Abstract. The potato virus Y cause loss in yield and quality of tubers. Hydrogen peroxide, ascorbic acid and antioxidants such as rosmarinic acid present in oils extracted from Rosmarinus officinalis plants are implicated in signaling against stress. The effects of these chemicals on tuber yield and pigments content were evaluated in plants testing positive after virus mechanical infection. Without chemical treatment, positive plants showed significant reductions in leaf pigments content and tuber weights compared to uninfected controls. Hydrogen peroxide, ascorbic acid and oil treatments of PVY infected plants significantly reduced the number of minitubers, enhancing their weights, while leaf pigment content also increased. This research demonstrates potential benefits of treatments with oils extracted from Rosmarinus officinalis plants and hydrogen peroxide or ascorbic acid in enhancing the yield and quality of tubers.

Keywords: *Rosmarinus officinalis* oil, potato virus Y, carotenoides, chlorophyll

Abbreviations. AA ascorbic acid, DHA dehydroascorbate, ROS reactive oxygen species, RA rosmarinic acid, RO *Rosmarinus officinalis*, PVY potato virus Y, SD standard deviation

INTRODUCTION

Potato virus Y (PVY) (*Potyviride*) is one of the most important viruses of potato (*Solanum tuberosum* L.) [30]. High PVY level can cause stand loss, reduced yields, undersized tubers and reduced quality [6, 15]. Over the past 20 years, PVY has become an increasingly serious constraint to seed potato production in the world [9, 22]. Thus, efforts to control PVY are essential when producing potatoes for market or seed [2-4].

Rosmarinic acid (RA), $C_{18}H_{16}O_8$ is a phenolic compound and well-known constituent of *Rosmarinus officinalis* plants (rosemary–Family *Lamiaceae,* order *Lamiales*). It has antioxidant activity and pharmaceutical properties such as the ability to reduce pollinosis and allergies [28]. RA is also insect-repellent and antimicrobial, antiviral wich protects the plants. Oils extracted from *Rosmarinus officinalis* introduced in healthy and infected potato plants could be implicated in the processus signaling against stress [32]. Plant cells have defensive responses to pathogen attack associated with changes in oxidative metabolism [16]. One of the consequences of stress is an increase in the cellular concentration of reactive oxygen species (ROS), which are subsequently converted to hydrogen peroxide (H_2O_2). These ROS, particularly H_2O_2, play versatile roles in normal plant physiological processes and in resistance to stresses. H_2O_2 produced in excess is harmful, but lower concentrations are beneficial [29]. H_2O_2 is believed to play two distinct roles in pathogenesis. One involves the oxidative burst in the hypersensitive response, which restricts pathogen growth [21] and the other activates plant defense responses, including induction of phytoalexins [1], second messengers or signaling intermediates, antioxidant enzymes and cell wall reinforcement [21]. For example, exogenous application of H_2O_2 induced tolerance to high temperature [19] and to chilling [23] in microplants of *Solanum tuberosum*. Genetic and physiological evidence suggests that H_2O_2 acts as a signaling second messenger, mediating the acquisition of tolerance to both biotic and abiotic stresses and providing information about changes in the external environment [29].

Another molecule that participates in response to both biotic and abiotic stresses is ascorbic acid (AA), which acts as an antioxidant, protecting the cell against oxidative stress caused by environmental factors and pathogens. As a direct scavenger of ROS, protecting or regenerating carotenoids or tocopherols, AA is the major redox buffer in plants, and is present at high concentrations in most plant cell compartments, including the apoplast [25]. AA is a cofactor of many enzymes, such as ascorbate peroxidase, which converts H_2O_2 to water, and violaxanthin de-epoxidase, which is required for dissipation of excess excitation energy during nonphotochemical quenching of chlorophyll a fluorescence, [31] AA is oxidized in many of its functions, producing the monodehydroascorbate radical, which can be reduced to ascorbate by monodehydroascorbate reductase or undergo dismutation to produce dehydroascorbate, which can be reduced back with glutathione as the reducing substrate [14, 25]. Changes in AA content can modulate PR gene expression and systemic acquired resistance, acting as a signal transducing molecule [12, 27]. Moreover, AA is a regulator of cell division, cell elongation and growth [17]. Considering that RA from *Rosmarinus officinalis* oils has antiviral and antioxidant activity [5, 32] and that H_2O_2, AA have been implicated in signaling gene expression against biotic and abiotic stresses [12, 26], the objectives of this work were to evaluate the effects of treatments with oils extracted from *Rosmarinus officinalis* plants, hydrogen peroxide and AA on photosynthetic pigments and on the tuber yield in potato healthy plants and mechanical inoculated plants with potato virus Y (PVY).

MATERIALS AND METHODS

Plant material. *Solanum tuberosum* L. microplants cv Roclas, testing virusfree, were obtained from the Biotechnology Department of N.I.R.D.P.S.B* . Single node cuttings were propagated in test tubes on Murashige and Skoog [24] medium (prepared in the same Biotechnology Department), at 20±1°C under a 16 h photoperiod (fluorescent lights, 400–700 nm), in sterile conditions. The microplants were transferred to greenhouse conditions 30 days after the single-node subculture step. For obtaining positive material, a part of these plants have been mechanicaly inoculated, using a PVY secondary infected plant from Record variety. The infection of the material was confirmed by ELISA tests.

ELISA test. A press with smooth roles was used for preparation leaf samples. The antiserum and conjugated used for viruses detection were obtained in our laboratory [8]. The analysis was performed following essentially the protocol described by Clark and Adams (1977) [7] (100 µl from each reactives solutions). Microplates were filled with substrate solution (p-nitrophenylphosphate) incubated 1 hour and the absorbance values were estimated at 405 nm (A_{405}) on PR1100 reader. The samples having A_{405} values exceeding the cut-off (two times the average of healthy controls) were considered virus infected

Chemical treatments. Microplants were transplanted to pots and after 10, 20 and 30 days, all the plants (excepting the controls) were injected with *Rosmarinus officinalis* oil (dilution 1/1000) 10 units (100µl) each plant. From 7 days later from the first injection, the plants were sprayed twice weekly for the next 2 months with 10 mL per plant of either 1 mM H_2O_2 or 3 mM AA at pH 5.6. Controls and plants treated only with natural oil were sprayed with distilled water. Four virus infected (positive) and healthy (negative) plants were sprayed in randomized arrays for each chemical treatment, and each treatment was performed in four independent experiments. Number and weight of tubers per plant, were recorded 60 and 90 days after transplanting.

Pigment analysis. Measurements were performed for each experiment on plants, 80 days after transplanting. Five leaf discs (about 1.5 cm diameter) per plant were taken from mid-shoot leaves of three plants per treatment. Samples for each assay comprised 15 discs, homogenized in 4 mL of 80% acetone at 4°C. Insoluble materials were removed by centrifugation at 2500 rpm for 10 min. Chlorophylls a and b, and carotenoids, were analyzed spectrophotometrically according to the method of Lichtenthaler and Wellburn (1983) [18].

Statistical analysis. Data were analyzed by ANOVA and Duncan's Multiple Range Test and scored as significant if $P<0.05$. In the aim to illustrate the precision of the mean we used the confidence interval (CI).

RESULTS

Effects of treatments with *Rosmarinus officinalis* oil and H_2O_2 or AA, were compared on pigment contents and tuber harvest parameters of both healthy and virus infected (PVY) plants cv Roclas plants.

Photosynthetic pigment analysis

Changes in photosynthetic pigment contents were evaluated 80 days after transplanting (Fig. 1A,B & Fig. 2A,B). Without chemical treatments, the positive leaves showed significant reductions, compared to uninfected leaves, in chlorophyll a (by 29%), chlorophyll b (44%), total chlorophyll (30%), and carotenoids (57%). Treatments with RO (*Rosmarinus officinalis* oil) and H_2O_2 or AA significantly increased pigment contents of virus PVY infected plant leaves to levels similar to uninfected plants (with the exception of the oil treatments on chlorophyll a and AA effects on carotenoids). No significant differences were induced by these treatments in the uninfected plants (Fig. 1A,B & Fig. 2A,B).

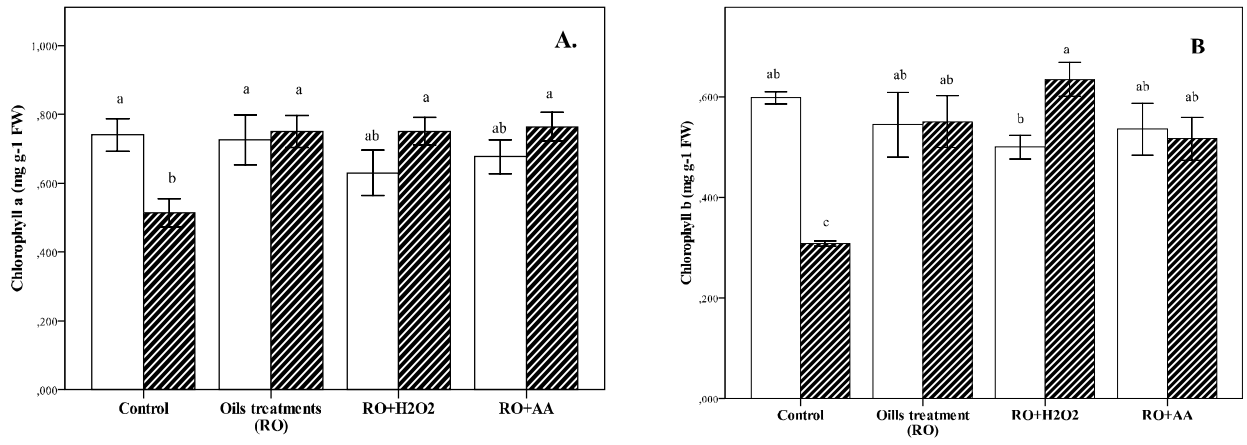

Figure 1. Chlorophyll a (A) and chlorophyll b (B) of leaves of healthy plants (□) and potato virus Y (PVY) infected plants (■), following treatments with *Rosmarinus officinalis* oil (RO) and spray with H_2O_2 (1mM) or AA (3mM) or water (controls), twice weekly for 60 days. Data are means ± SD of four experiments (n=4). Bars with different letters differ significantly by ANOVA and Duncan's test ($P<0.05$).

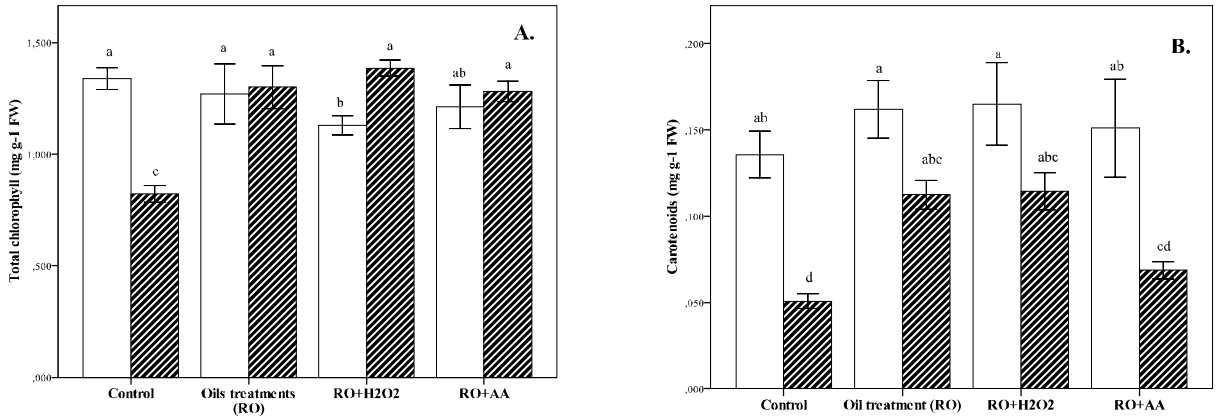

Figure 2. Photosynthetic pigments. A) total chlorophyll, and B) carotenoids of leaves of healthy plants (□) and PVY infected plants (■), following *Rosmarinus officinalis* oil (RO) and H_2O_2 (1 mM) or AA (3 mM) treatments or water (controls), twice weekly. Data are means ± SD of four experiments (n=4). Errors bars are 95% CI of means. Bars with different letters differ significantly by ANOVA and Duncan's test (P<0.05).

Tubers harvest

Final harvests were carried out at 60 or 90 days after transplanting. At 60 days no significant differences were observed in the number of tubers in positive or uninfected control-treatments (Fig. 3A). However, at the same date, positive plants treated only with *Rosmarinus officinalis* oil produced significantly more tubers (by 47%) than the positive controls. None of the treatments induced significant differences in the number of tubers in negative plants (Fig. 3A). At 90 days after transplanting, the number of tubers produced by positive control plants was significantly higher than the uninfected control (by 65%) (Fig. 3B). In uninfected plants no significant differences were obtained by the treatments relative to their controls (Fig. 3B). However, all the treatments significantly reduced the number of tubers produced per plant (by 25, 29 and 25% respectively) in the positive plants compared to their control (Fig. 3B). Interestingly, this reduced number of tubers was similar to that produced by uninfected plants subjected to any of the treatments (Fig. 3B).

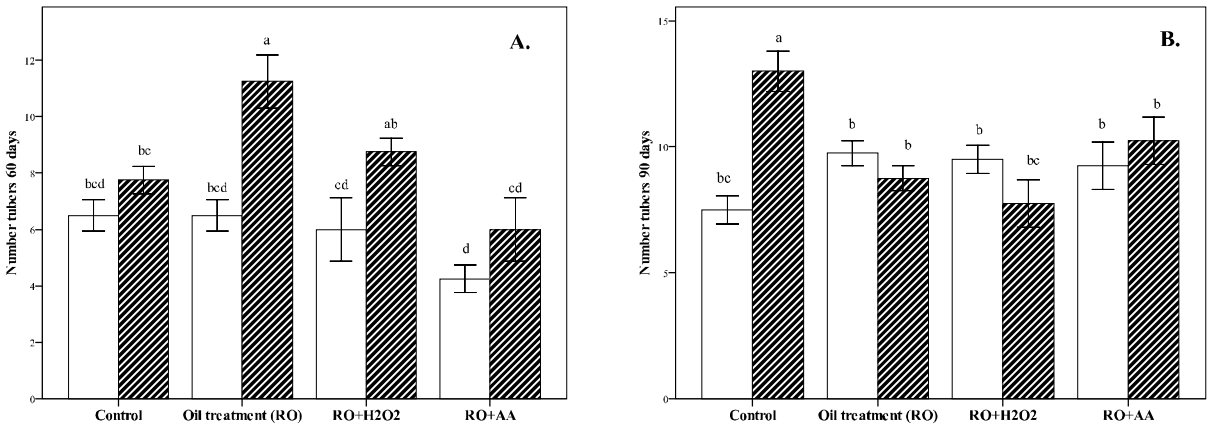

Figure 3. Number of tubers produced by plants healthy (□) or positive-infected plants with potato virus Y(PVY) (■), following injections with *Rosmarinus officinalis* oil (RO) and spray treatments with H_2O_2 (1 mM), AA (3 mM) or water(controls), twice weekly for 60 days. Data are means ± SD of four experiments (n=4). Bars with different letters differ significantly by ANOVA and Duncan's test (P<0.05).

Tuber weights of the uninfected control plants were significantly higher (by 80 and 64%) than the positive control by 60 and 90 days respectively (Fig. 4A and B). However, H_2O_2 and *Rosmarinus officinalis* oil treatments significantly enhanced the weight of tubers at 60 days (by 95% and 116% respectively) in the positive plants compared to their control (Fig. 4A). Furthermore, this response was maintained at 90 days after transplanting (107% and 78% respectively), when the AA treatment also registered a significant (47%) increase (Fig. 4B). The chemical treatments of positive plants resulted in tuber weights that were either not significantly different to, or greater than (in the H_2O_2 treatment at 90 days), those of uninfected controls (Fig. 4 A,B). Significant reduction by the chemical treatments of the weight of tubers harvested was observed in the uninfected plants compared with their control at 60 days, this effect remaining significant at 90 days for the plants treated only with *Rosmarinus officinalis* oil (Fig. 4).

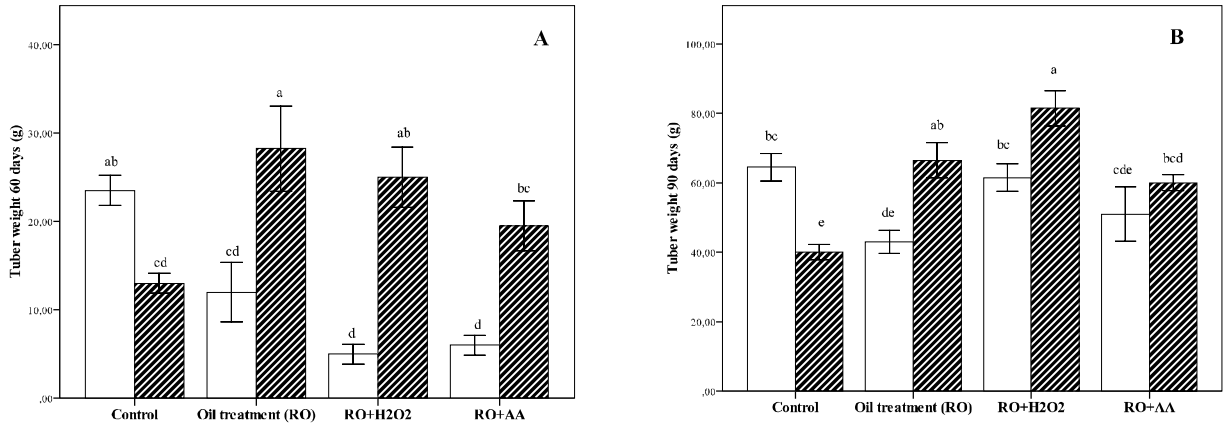

Figure 4. Weight of tubers produced by healthy plants (□) or positive-infected plants with potato virus Y(PVY) - (■), following injections with *Rosmarinus officinalis* oil (RO) and spray treatments with H2O2 (1 mM), AA (3 mM) or water(controls), twice weekly for 60 days. Data are means ± SD of four experiments (n=4). Bars with different letters differ significantly by ANOVA and Duncan's test ($P<0.05$).

DISCUSSIONS

Hydrogen peroxide is a diffusible signal-transducing molecule and its accumulation is perceived by the plant as a signal of environmental change, alerting the cell to both biotic and abiotic threats [25]. It also alters the concentrations and redox status of intracellular antioxidants, such as ascorbate [12].The role of H_2O_2 in the induction of tolerance to stresses in potato plants has been demonstrated. López-Delgado et al. [20] and Mora-Herrera et al. [23] showed that exogenous H_2O_2 induced tolerance to high temperature and freezing in potato plants. Wu et al. [33] [34] observed that transgenic potato plants expressing a fungal gene encoding glucose oxidase, which generates H_2O_2 when glucose is oxidized, exhibited strong resistance to *Erwinia carotovora subsp carotovora*, and to *Phytophthora infestans*. This resistance to soft rot and to potato late blight was apparently mediated by elevated levels of H_2O_2. The results of the present study demonstrated that plants mechanical infected with potato virus Y (PVY) suffered significantly harmful effects on pigment contents and on the number, weight of tubers produced. In general, these effects were reduced by injected the plants with *Rosmarinus officinalis* oil and spraying H_2O_2 or AA. Concerning the changes in the leaves pigment contents, foliar mosaic (alternativ pale green and dark green areas) represents a common symptom of primary infection with potato virus Y (PVY). Our results show that the presence of potato virus Y (PVY) in potato plants significantly reduced the content of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids. *Rosmarinus officinalis* oil injections and H_2O_2 or AA treatments of mechanical infected plants with potato virus Y (PVY) significantly increased the levels of chlorophylls compared with positive control plants, while similarly treated uninfected plants sprayed did not show significant differences in these pigments.

Under greenhouse conditions, 90 days after transplanting, the infected plants produced a higher number of tubers than the uninfected controls, relative to uninfected controls. Increased number and reduced weight of tubers is a characteristic response to stress in potato. The virus also cause an array of symptoms suggestive of disturbances in the normal balance of plant hormones such as cytokinins and auxins [10]. Increased number of tubers could be due to disturbance of plant hormones involved in tuber formation [13].

It has been suggested that a physiological balance of antioxidant components is necessary in order to obtain protection to generalized stress; however, antioxidants are not always accessible to some of the sites where they are most needed in times of stress [11]. Our results agree with this statement since the *Rosmarinus officinalis* oil injections and AA treatments induced significant anti-stress effects only in the tubers from positive plants. Similar affirmation could apply for H_2O_2.

This research presents a novel potential approach for overcoming the most common damage in tubers of potato virus Y (PVY) infected material, using natural compounds that offer the possibility of reduction of biocide usage.

The elucidation of the precise role played by *Rosmarinus officinalis* oil treatments in addition with H_2O_2, AA on potato virus Y (PVY) infected and healthy plants awaits further investigation.

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