EFFECTS OF *Satureja hortensis* OIL TREATMENTS AND EXOGENOUS H_2O_2 ON POTATO VIRUS Y (PVY) INFECTED *Solanum tuberosum* L. PLANTS UNDER DROUGHT CONDITIONS

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Abstract. Effects of treatments with *Satureja hortensis* essential oils and H_2O_2 were evaluated in plants testing positive after Potato virus Y (PVY) infection, under drought conditions. *In vitro* PVY infected and uninfected plants were transfered to a green-house, injected with a *Satureja hortensis* essential oils suspension and sprayed twice a week with H_2O_2 (1 mM, pH 5.6). The treatments enhanced minitubers weight and starch content in PVY infected plants. Under drought conditions, minitubers produced by infected and treated plants had significantly more starch than the controls. The treatments had positive effects on infected minitubers, such as weight, reduction of number, starch content, sprouting and tolerance to drought. A signal role for *Satureja hortensis* essential oils and hydrogen peroxide (H_2O_2) in lessenning symptoms is suggested.

Keywords: *Satureja Hortensis essential oil, hydrogen peroxide, potato virus Y, drought stress*

Abbreviations: ROS reactive oxygen species, SH *Satureja hortensis*, Eos essential oils, PVY potato virus Y, DAT days after transplanting, SD standard deviation

INTRODUCTION

Distributed worldwide, Potato virus Y (PVY, *potyvirus* genus, family *Potyviridae*) causes losses in solanaceous crops such potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicum esculentum)* [10, 23, 32, 49]. In case of the potato, the virus not only leads to yield reduction by up to 90%, but also causes tuber necrosis in certain cultivars upon infection with the tuber necrosis strain of PVY (PVY^{NTN}) [32, 49]. So, PVY is one of the most economically important viruses affecting the potato crop in all the world. Thus, efforts to control PVY are essential when producing potatoes for market or seed [3-5, 23].

Satureja hortensis L. (summer savory – Family *Lamiaceae,* order *Lamiales*) is renowned for its aromatic and seasoning properties in food products but also for the antispastic and disinfecting properties. The essential oils is known for its antiseptic (antimicrobial, antifungal and antiviral) properties. It inhibits mould formation. This oil contains hydro-carbonated and oxygenated compounds like α and β pinene, α tujene, camphene, sabinene, myrcene, α phelandren, terpinene, limonene, cymene, 1,8 cineol, β phelandrene, linalol, caryophillene. The main compounds are carvacrol (about 35%) – wich imprints the characteristic smell – tymol and p-cymene [9]. They are also insect-repellent and antimicrobial, antiviral wich could protect the plants [46]. Maybe, one of these compounds or other could be implicated in the processus signaling against stress, in healthy and infected potato plants [53]. Plant cells have defensive responses to pathogen attack associated with changes in oxidative metabolism [22, 24]. 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 [47]. 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 [30, 31] and the other activates plant defense responses, including induction of phytoalexins [2], second messengers or signaling intermediates, antioxidant enzymes and cell wall reinforcement [31]. For example, exogenous application of H_2O_2 induced tolerance to high temperature [29] and to chilling [40] 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 [47, 55, 56].

Water stress is one of the most important environmental factors that limits the growth, yield and quality of potato crops [19, 54]. Potato plants are very susceptible to water deficit, wich causes a severe reduction in leaf area, fresh weight and stolon development [25]. Plants under drought conditions show an increase in reactive oxigen species (ROS) wich leads to expression of genes associated with antioxidant functions for scavenging ROS, resulting in tolerance to drought stress [35]. Similar mechanism are triggered in plants during biotic and abiotic stress. A common response is the production of ROS including superoxide, singlet oxygen , hydroxyl and hydrogen peroxide oxygen. These ROS can be detrimental and promote deleterious effects in the most sensitives biological macromolecues [27, 52], leading to electrolyte leakage, changes in ion fluxes, lipid peroxydation, protein oxidation and imbalances in the oxidative systems at the subcelullar level. Under intense stress, different target molecules are damaged, resulting in cell death [1, 21]. To minimise ROS damaging effects, aerobic organisms envolved both

non-enzymatic and enzimatic antioxidants. Purely enzymatic defenses, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POXs) directly scavenge superoxyde radicals and H_2O_2, converting them to less reactive species [50]. POXs have been asociated with an ever-increasing number of physiological processes, especially detoxyfying H_2O_2 [37]. In plant pathogen relationships, ROS are involved in induction of defense genes, antioxidant enzymes such as CAT and POX and acumulation of secondary metabolites [44]. Genetic and physiological evidence suggests that H_2O_2 acts as a signaling second messenger, mediating the aquisition of the tolerance to both biotic and abiotic stresses and providing information about changes in the external environment [11, 20, 47]. The roles of H_2O_2 in the signaling of mechanisms that induce tolerance responses to biotic [12, 38, 57] and abiotic [17, 39, 40] stresses have been widely documented.

There are limited informations about occurence of symptoms with interaction between PVY and abiotic stress. Xu et al. (2008) showed in their papers that potato viruse infection improve drought tolerance [59]. In this research we studied the effect of the virus – water stress interaction on the occurence of symptoms in virus infected potato plants under essential oils treatments and H_2O_2 – mediated greenhouse conditions.

MATERIALS AND METHODS

Plant material. *Solanum tuberosum* L. microplants cv Roclas, testing virusfree, were obtained from the Biotechnology Department (from the in vitro germoplasm collection of the Institut for Research and Development of Potato and Sugar Beet Braşov, Romania). Potato microplants were obtained from a previous selection under green house conditions, before inclusion in the in vitro collection. For obtaining positive material, a part of the plants have been mechanicaly inoculated, using a PVY secondary infected plant from Record variety. The infection of the material was confirmed by ELISA tests. Single node cuttings were *in vitro* propagated in test tubes on Murashige and Skoog [41] medium, at 20±1°C under a 16 h photoperiod (fluorescent lights, 400–700 nm), in sterile conditions. Forty PVY infected microplants and forty healthy microplants were transplanted to pots (17 x 14 cm) containing peat-moss under greenhouse conditions 30 days after the single-node subculture step. These plants were maintained under greenhouse conditions for 90 days after transplanting (DAT) and each pot was allocated to an experimental unit, with ten plants per treatment. Before the treatments and after 45 DAT the presence of PVY was tested by ELISA.

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 [16]. The analysis was performed following essentially the protocol described by Clark and Adams (1977) [15] (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 Tecan reader (Magellan softwere). The samples having A_{405} values exceeding the cut-off (two times the average of healthy controls) were considered virus infected.

Stress and chemical treatments. All experiments were performed in triplicate. Microplants were transplanted to pots and after 7, 14 and 21 days, all the plants (excepting the controls) were injected with *Satureja hortensis* oil (1/100) 10units 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 1 mM H_2O_2 at pH 5.6 and the earth of the pots with 10mL essential oils suspension (1/1000). The fertilization was made every 15 days and the plants were watered twice a week. Ten infected plants and ten negative plants for each treatment were sprayed with H_2O_2 in randomized arrays and subjected to drought conditions. Drought stress (suppressed water) or well watered conditions were applied from 75 DAT up to harvest. Minitubers number, weight and starch were recorded at 90 DAT as a productivity estimation. Controls and plants untreated were sprayed with distilled water. Six virus infected (positive) and healthy (negative) plants were sprayed in randomized arrays for each chemical treatment, and each treatment was performed in three independent experiments.

Minitubers starch content Starch content was determined by spectrophotometric assay by antrone reaction [45]. For each treatment, a composite 1g sample of pith from three minitubers was used. Tissue was ground in a mortar with 10ml 80% (v/v) ethanol [45]. The analyses were performed the day after harvesting.

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

RESULTS

Effects of treatments with *Satureja hortensis* essential oils and H_2O_2, were compared on tuber harvest parameters (weight, starch, number, sprouting) of both healthy and virus infected (PVY) plants cv Roclas plants.

Weight. Minituber weight of PVY positive plants was significantly diminished compared to the control unifected plants. The treatments significantly affected minituber weight (fig 1A) PVY infected plants treated with essential oils and H_2O_2 had significantly ($P<0.05$) increased minituber weight to similar vallues as the uninjected and unsprayed control. Minitubers produced by uninfected plants that were treated also significantly ($P<0.05$) increased their weight in all treatments (fig 1A). Minitubers produced under drought conditions by negative plants had significantly ($P<0.05$) reduced weight by 36% compared to the uninfected plants under irrigation. Minitubers produced by treated and

PVY inoculated plants under drought showed the highest weight of all the treatments under drought (fig 1A). Very interesting, the treatments with *S. hortensis* essential oils and H_2O_2 significantly increased ($P<0.05$) the minitubers weight in infected plants compared to the uninfected plants treated with *S. hortensis* essential oils and H_2O_2.

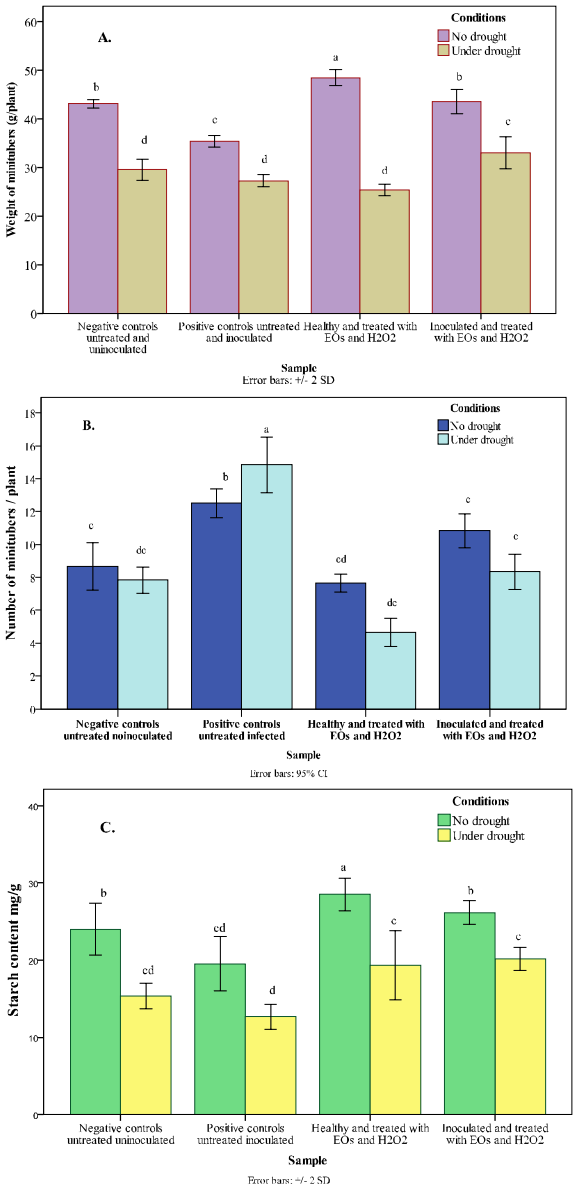

Figure 1. Tuber weight (A), minituber number (B) and starch content (C) of healthy plants and potato virus Y (PVY) infected plants, under drought conditions (■ dark colour of the bars) and not drought conditions (□ light colour of the bars), following treatments with *Satureja hortensis* (SH) essential oil and H_2O_2 (1mM) or water (controls), twice weekly from 30-75 DAT. Watering was withheld at 75 DAT. Data are means ± SD of 3 experiments (n=3). Bars with different letters differ significantly by ANOVA and Duncan's test ($P<0.05$).

Number of minitubers. PVY inoculated and infected plants produced a higher number of minitubers but with less weight than the unifected control plants. The treatments significantly reduced the minitubers number compared to the unifected control to similar values as the unifected control (fig 1B). Similary, under drought conditions, the infected control plants had significantly more minitubers than the healthy plants (negative controls). Interestingly, the treatments significantly reduced by 55% (compared to infected control) or to similar value (compared to irrigated and uninfected controls) the number of minitubers in infected plants (fig 1B). In another research work we observed that the treatments with some essential oils, H_2O_2 and ascorbic acid significantly reduced minituber number compared to infected controls and significantly enhanced minituber weight [6-8]. In the present research we observed these effects in all conditions: under drought and well-watered conditions.

Starch. PVY infection leads to a low starch content. The starch content in infected plants was significantly ($P<0.05$) lower than in unifected plants. However, the treatments with *S. hortensis* essential oils and H_2O_2 significantly ($P<0.05$) enhanced the starch content in both infected (22% increase) and unifected (16% increase) plants compared with their controls (fig 1C). Under drought stress, minitubers of uninfected and PVY inoculated plants significantly decreased in starch content by 33% and 39% respectively, compared to the irrigated uninfected controls. The treatments applied on PVY infected plants induced significantly higher levels in the minitubers than those of the infected and untreated control plants (fig 1C).

Sprouting. Minitubers from infected plants significantly reduced percentage of sprouting. Multiple sprouting (more than one sprout /minituber) was significantly ($P<0.05\%$) enhanced by 12.4% by the treatments of PVY inoculated plants compared to the positive control plants (PVY inoculated and untreated) (table 1). No significant effect of the treatment on minitubers sprouting was observed on unifected minitubers. The effect of essential oils and H_2O_2 treatments on sprouting was accentuated by drought stress. Infected minitubers had significantly 11.4% reduced total sprouting compared to the healthy minitubers. Essential oils and H_2O_2 significantly induced more sprouting in minitubers from inoculated plants, compared to the positive control. The treatments with essential oils and H_2O_2 increased multiple sprouting in infected and uninfected minitubers compared to the controls (Table 1).

DISCUSSION

In potato seed programs was implemented a model based on the *in vitro*-to-green house system. We used this model for investigate the effect of the interaction between potato virus Y (PVY)- water stress on the occurence of symptoms in infected plants treated with *S. hortensis* essential oils and H_2O_2 in mediated greenhouse conditions. Under green house conditions, the PVY infected plants exhibited specific symptoms such as mosaic in the foliage, reduced plant weight, stem tickening, internod shortening and reduced minitubers production (sometimes if the strain is very virulent systemic shock reaction and / plant death). Known symptoms usually for this kind of virus were

Table 1. Effect of the treatments on tuber sprouting. Sprouted tubers 140 days after harvest in potato healthy plants (virus free) and PVY inoculated (infected) and treated (injected with *S. hortensis* essential oils suspension and sprayed with 1mM H_2O_2 from 30-75 DAT). Watering was withheld at 75 DAT. Data are means ± SD of three experiments. Means labeled with different letters differ significantly by ANOVA and Duncan's test ($P<0.05$)

Sample Tubers from:	Conditions	Number of tubers	Single sprouting (%)	Multiple sprouting (%)	No sprouting (%)	Total sprouting (%)
Negative Control	No drought	220-340	84.6±1.4 (a)	15.4 ± 1.4 (c)	0.0±0.0	100 (a)
Positive Control		340	77.4 ±1.5 (bc)	16.4 ± 1.2 (c)	7.2 ±0.8 (b)	93.8 (b)
Negative Control	Under drought	190-220	81.6 ± 2.1 (b)	17.0±1.9 (b)	1.4 ± 0.2 (d)	98.6 (a)
Positive Control		320-410	68.9 ± 2.4 (cd)	18.4 ± 1.0 (b)	12.7±2.3(a)	87.3 (c)
Healthy and treated plants	No drought	190-220	84.7 ±2.1 (a)	15.3 ± 2.1 (c)	0.0±0.0	100(a)
Infected and treated plants		220-240	68.3 ±3.9 (cd)	27.9 ±4.9 (a)	3.8 ±2.0 (c)	96.2 (ab)
Healthy and treated plants	Under drought	190-220	70.6±2.7 (cd)	29.4 ±2.7 (a)	0.0±0.0	100 (a)
Infected and treated plants		220-340	69.2 ±4.8 (cd)	30.8 ±4.8 (a)	0.0±0.0	100 (a)

absent under drought stress in the conditions of our experimetns. In a green house the environmental stress was likely more stable with gradual changes compared to the field conditions where environmental changes can abruptly occur. In other cases, for another pathogen (for example phytoplasma) the impact of climatic diferences on phenotiype expanssion has previously been reported [26].

Under drought stress, deprivation of water leads to nutrient deficiency and oxidative stress, similar to responses in PVY inoculated plants under irrigated and drought conditions showed a significant reduction in water potential compared to the control, as also occurs during drought stress [25]. Alterations in the photosyntetic process associated with stomatal conductance are induced by different pathogens [33]. In *Cocos nucifera* reduced stomatal conductance was associated with the occurence of symptoms, increased ABA concentration and a decrease in photosynthetic pigments and water potential [33, 36]. In *Spartium junceum* under water deficit, some pathogens can promote structural changes in xylem, enhancing the hidraulic resistence and stomatal conductance, resulting in the loss of water [34]. Maybe, the remarcable effects of treatments with *S. hortensis* essential oils and H_2O_2 application in unifected plants under drought conditions occured because of the significant reduction in water potential, possibly related to a reduced capacity for stress aclimatization in unifected plants compared to infected plants, wich may strengh\then their drought resistance mechanism. H_2O_2 can induce stress [18] and in our study the induced stress was intensified by the water deficit and by the special oils used. In the following research work we have intention to evaluate the water potential of the plants.

A part of the pathogens (phytoplasma, viruses, viroids) induced an increase in ABA content in infected plants wich could be mediated by H_2O_2 [43, 59]. Likewise, the infection of *Oryza sativa* and *Beta vulgaris* improves drought tolerance by production of osmoprotectants and antioxidants, similar to those produced by water stress [58]. Minitubers weight and starch levels were induced by the treatments under well watered conditions, but under drought stress both were enhanced only in infected plants (fig. 1 A, C). This counteracts the virus –induced reduction in minitubers starch level and weight [8], wich may be linked to obstruction of the sieve elements, a similar observation being made to another pathogen, like phytoplasma [13,14,48]. Additionally, pathogens interfere with carbohydrate metabolism [28, 26] and translocation of assimilates to the tubers. Previously we observed a significant enhancement of chlorophyll content by H_2O_2 treatment of PVY inoculated plants, resulting in the augmentation of minituber weight [6-8]. Ozaki et al. 2009 [42] also demonstrated that increased photosynthates due to H_2O_2 exposure of plants are either converted into starch or exported from the chloroplast to cytosol for soluble sugar synthesis, resulting in an enhanced level of soluble sugar and starch in *melon* leaves, as well as increased shoot and fruit dry weight. It is worth mentioning that the effects of H_2O_2 on starch metabolism and drought resistance were particularly exhibited by PVY inoculated plants, suggesting crosstalk between plant responses to pathogen and biotic stress [1].

The informations on the combined tolerance to biotic and abiotic stress is scarce. This work study demonstrates an ameliorative effect of the essential oils and H_2O_2 on the combined stresses. We demonstrate the effect of PVY-water stress on the occurence of symptoms and the antioxidant response in PVY infected potato plants under H_2O_2 –mediated greenhouse condition. Low H_2O_2 concetration 1mM significantly reduced disease symptoms under drought stress for minituber production and starch accumulation, with repercussions in minituber size augmentation and induced multiple sprouting.

A potential ameliorative effect of another essential oils and H_2O_2, ascorbic acid in PVY inoculated plants has been reported [6-8]. The practical use of these treatments for overcoming damage in non-sed tubers, as demonstrated in the present paper, is a strong justification for continue investigation of the physiology of H_2O_2 and antioxidants, of some compunds from *S. hortensis* essential oils in relation to similar symptoms produced when phloem transport of photosynthates is affected in diseases, in spite of the causal agent [51].

The treatments with *Satureja hortensis* essential oils and H_2O_2 were favorable for diminution in stress-damage symptoms in infected plants. These treatments enhanced sprouting in tubers from PVY inoculated (infected) minitubers and *Satureja hortensis* essential

oils and H_2O_2 -ameliorative damage effects were obsereved on potato plants growing under combined biotic (the virus) and abiotic (the drought) stress conditions.

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