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Effects of salicylic acid and heat acclimation on thermotolerance and withanolide accumulation under high temperature stress in the Cape gooseberry (*Physalis peruviana* L.)

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Abstract: The adverse effects of high temperature stress can be alleviated by thermotolerance induced by exogenous application of plant growth regulators or by gradual application of temperature stress. *Physalis peruviana* L., commonly known as the Cape gooseberry, is a source of a variety of phytocompounds such as withanolides (withanone, withaferin A, and withanolide A). These withanolides are potentially high-value drug candidates because of their various pharmacological properties. The production of withanolides via traditional agriculture is commercially inadequate. In the present study, elicitation strategies were employed to improve the crop's thermotolerance and accumulation of withanolides. For these purposes, the effects of heat acclimation (45 °C HA) or salicylic acid (150 mM SA) treatments in inducing withanolide production and thermotolerance were tested in leaves of *P. peruviana* L. grown under high temperature stress (55 °C). Considerable increases in the production of withanolides (up to 86.83 mg g^{-1} dry weight, dw) were observed when the cultures were exposed for 5 h to high temperature stress after pretreatment with SA. SA application and heat acclimation increased the activity of superoxide dismutase (SOD; EC 1.15.1.1) and decreased the catalase activity (CAT; EC 1.11.1.6). Both SA and heat acclimation caused a significant increase in endogenous H_2O_2 and proline content. Changes in related antioxidants paralleling heat acclimation or SA treatment suggest that common mechanisms might be involved in thermotolerance induced by SA and heat acclimation.

Key words: Heat acclimation, high temperature stress, *Physalis peruviana*, salicylic acid

1. Introduction

Withanolides are predominantly secondary metabolites found in the family Solanaceae, including *Physalis* species and especially *Physalis peruviana*, commonly known as the Cape gooseberry or goldenberry. *P. peruviana* originated in tropical South America and then spread throughout the world due to its nutritional and pharmaceutical value, such as high levels of vitamins, phytosterols, withanolides, physalins, carotenoids, cinnamic acid-derived volatiles, antioxidants, antiinflammatory compounds, several essential minerals, and polyunsaturated fatty acids (Hassanien, 2011). On the other hand, the production of various secondary metabolites in plants, including withanolides, results from the plants' interaction with environmental factors (Praveen and Murthy, 2010). Withanolides exhibit significant pharmacological activities, including a hepatoprotective effect against CCl_4-induced hepatotoxicity, and antibacterial, antiinflammatory, antitumor, insect-repellent, immunomodulatory, and cytotoxic activity (Sivanandhan et al., 2012). Because of the significant pharmacological activities of withanolides, medicinal clinicians and chemists have become interested in their chemical synthesis. However, the synthetic production of these substances is not an easy task because of their chiral centers, high energy epoxy ring, stereochemical structure, and rigid translactone groups. Therefore, synthetic production of withanolides is economically impractical because of the production of limited quantities at high expense. On the other hand, in vitro culture of *Physalis* spp., which is more susceptible to environmental conditions, has other problems such as low growth rate, long germination time, and a complex accumulation pattern. Additional biotechnological strategies such as elicitor interventions have been widely used at different developmental stages to enhance secondary metabolite production. Among the various elicitor interventions, salicylic acid (SA) is a potent signaling molecule that provides protection in plants against abiotic stresses by changing the plants' physiological processes (Raskin et al., 1990; Khan et al., 2015); it has been widely used to increase the accumulation of secondary metabolites in plant cell and tissue cultures. SA has also induced plant tolerance mechanisms against major abiotic stress factors such as salinity, drought, metal, osmotic, and heat stresses

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(Fayez and Bazaid, 2014; Khan et al., 2014; Zhang et al., 2015). It has been proposed that at least one of the mechanisms of SA effect is through upregulation of levels of active oxygen species such as H_2O_2 (Chen et al., 1993). *Arabidopsis* and tobacco leaves treated with SA have been shown to accumulate H_2O_2 (Chen et al., 1993; Rao et al., 1997). Due to climate change, high temperatures have become a potential threat for the growth, development, and yield of plants. High temperature stress affects various plant regulatory mechanisms that allow adaptive responses and tolerance (Larkindale et al., 2005; Khan et al., 2015). It is well known that when a plant is exposed to high temperatures, exogenously supplied SA provides distinct benefits to the plant (He et al., 2002; Larkindale and Knight, 2002; Clarke et al., 2004), as well as improved thermotolerance (He et al., 2002; Wang et al., 2010). These reports have encouraged the author to undertake a systematic investigation of the influence of SA and HA on *P. peruviana* growth and withanolide accumulation under in vitro conditions.

In the current study, the hypothesis was that optimizing SA and HA can increase plant biomass and withanolide production in *P. peruviana*. Thus, the aims of the study included (i) induction of thermotolerance in *P. peruviana* shoot cultures when exposed to SA pretreatment or nonlethal heat acclimation, (ii) development of an efficient plant growth system using SA pretreatment or heat acclimation, (iii) analysis of the variations in withanolide (withanolide A, withaferin A, and withanone) accumulation, and (iv) examination of the influence of SA pretreatment or heat acclimation on H_2O_2 content and antioxidants, especially SOD, CAT, and proline.

2. Materials and methods

2.1. Plant materials

Seeds of *P. peruviana* were purchased from commercial sellers (NutriBoost®) in 2016.

2.2. Seed sterilization and germination

Surface sterilization of seeds and germination were performed according to a method previously published by Yücesan et al. (2015). Nodal segments were used as explants from 30-day germinated seedlings for culture initiation. These explants were cultured on MS medium containing 0.8% agar, 3% sucrose, and 0.5 mg/L TDZ for direct shoot induction for 30 days. Plants grown in vitro were exposed to a nonlethal temperature (45 °C) for 1 h for acclimation treatments (HA). For SA treatment, the plants were subjected to foliar spraying with a 150 mM SA solution (Sigma, St. Louis, MO, USA) (Sivanandhan et al., 2012), while distilled water was used in the experiment as a control. After 1 h of incubation for acclimation and elicitation, the cultures were exposed to 55 °C for 5 h in order to generate high temperature stress (Dat et al., 1998). Both cultures (treated and control) were then transferred into a culture room with a controlled environment (23 ± 2 °C under a 16-h light/8-h dark photoperiod). Every 2 weeks, the cultures were transferred into fresh MS medium with the same composition.

2.2. Extraction of withanolides and HPLC analysis

The extraction and quantitative analyses of withanolide from leaf samples were performed according to a protocol previously published by Takshak et al. (2014). Briefly, 0.1 g of powdered leaf material was sonicated with 5 mL of methanol (HPLC grade) in Eppendorf tubes at 4 °C for 15 min. After 15 min, the Eppendorf tubes were transferred into a refrigerator and kept at 4 °C for 24 h to allow complete extraction. Then the crude extract was centrifuged for 5 min at 3000 × *g*, the supernatant was removed, and the pellet was preconcentrated with nitrogen gas. Later the pellet was dissolved in methanol (HPLC grade) and then filtered using PTFE Millipore syringe filters (0.22 µm). Next, 2 mg of each standard compound (withanolide A, withanolide, and withaferin A) was dissolved in 5 mL of methanol (HPLC grade) in a volumetric flask, and calibration curves (ranging from 0 to 100 ppm) were prepared (Figure 1) using pure standards purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. Quantification of withanolide via HPLC

The separation and quantification of withanolides were performed using HPLC. Briefly, an aliquot of 10 µL of each sample (n: 3 samples for each injection) solution was injected into the HPLC system at a flow rate of 0.6 mL/min (binary pump, LPG 3400SD Dionex, Sunnyvale, CA, USA) at 30 °C (column oven system, TCC3000SD, Dionex), using an Inertsil ODS-3 (GL Sciences Inc., Tokyo, Japan) column (150 × 4.6 mm) along with an autosampler (WPS-3000-SL Dionex Semi Prep). All samples were evaluated with a UV-DAD detector (MWD-3100 Dionex UV-VIS Detector) with a selected wavelength of 229 nm. The isocratic flow was performed using methanol/water, each containing 1% trifluoroacetic acid (w/v) in a gradient fashion (45:55–65:35) over 15 min (Grover et al., 2013).

2.4. Enzyme extraction and protein determination

Enzyme extraction and protein determination for analyses of CAT and SOD activities were performed as previously described by Cingoz et al. (2014). Briefly, CAT activity was determined spectrophotometrically at 240 nm using a specific absorption coefficient at 0.0392 cm^3 μmol^{-1} H_2O_2, and it was calculated as µmol H_2O_2/mg protein/min. SOD activity involving inhibition of nitroblue tetrazolium (NBT) reduction was determined spectrophotometrically at 560 nm. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

2.5. Measurement of H_2O_2 content

H_2O_2 concentration was determined spectrophotometrically according to a protocol previously published by Brennan

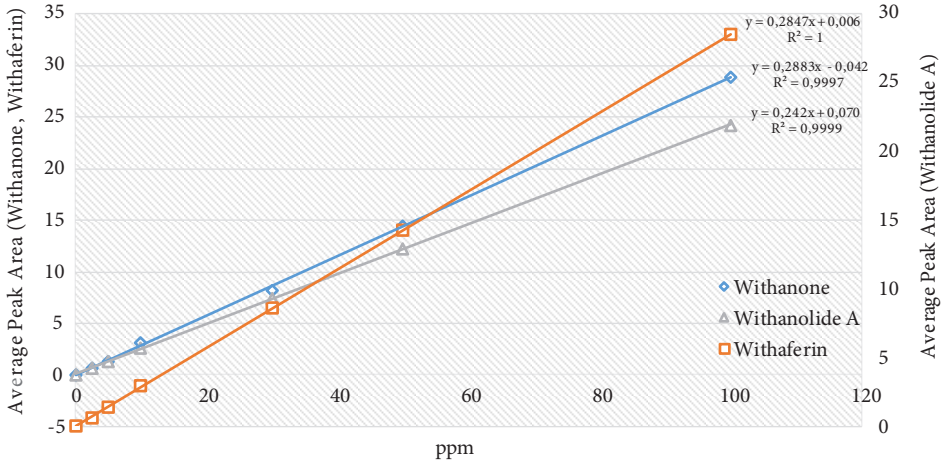

Figure 1. Calibration curves and R^2 values for each standard sample (withaferin A, withanone, and withanolide A).

and Frenkel (1977), in which 200 mg of leaf material was ground in liquid nitrogen, then transferred to centrifuge tubes containing 2 mL of prechilled acetone. The centrifuge tubes were sonicated at 4 °C for 15 min to allow complete extraction. After 15 min of sonication, the crude extract was centrifuged for 5 min at 13,000 × *g* at 4 °C; 1 mL of the supernatant was then added to the new tubes containing 0.1 mL 5% $TiSO_4$ in 98% H_2SO_4 and 0.2 mL NH_4OH. After centrifugation at 3000 × *g* for 10 min, the orange-yellow–colored deposit was collected and rinsed with cold acetone 3 times to reduce the interference of plant pigments. After the addition of 5 mL of 2 M H_2SO_4, the absorbance was measured at 415 nm. The H_2O_2 content was calculated as μmol H_2O_2 mg^{-1} protein.

2.6. Proline analysis

Proline was determined according to the method of Bates et al. (1973); 500 mg of leaf material was extracted with 5mL of 3% sulfosalicylic acid and centrifuged at 3000 × *g* for 20 min. Two mL of the supernatant was then mixed with 2 mL of ninhydrin and 2 mL of glacial acetic acid. After 1 h at 100 °C, the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene and measured spectrophotometrically at 520 nm against standard proline. Proline content is shown in μmol g^{-1} dw.

2.7. Statistical analysis

The data were statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test at $P \leq 0.05$. Three replicates per treatment were used, and each replicate involved 3 measurements.

3. Results and discussion

Plants can exhibit heat tolerance when the surrounding environmental temperature increases above nonlethal levels. In this process, metabolic changes in the plant in response to high temperatures minimize heat injury and provide adaptation under adverse conditions (Levitt, 1980). Recent reports show that exogenous SA application or gradual application of temperature stress alleviates high temperature stress effects and induces thermotolerance in several plant species (Khan et al., 2015).

Based on this information, in the present study, we investigated variations in withanolides and antioxidant metabolites in leaves of *P. peruviana* under high temperature stress with thermotolerance induced by foliar spraying of SA and simultaneous heat acclimation for 1 h. It was shown that heat acclimation and SA pretreatment had a significant effect on withanolide accumulation in *P. peruviana* leaves under high temperature stress (Table). The highest amounts of withaferin A (44.47 ± 1.27 mg g^{-1} dw), withanone (17.31 ± 0.65 mg g^{-1} dw), and withanolide A (25.04 ± 0.94 mg g^{-1} dw) were obtained when cultures were combined with both SA and high temperature for 5 h. Withanolide A content exhibited 5-fold higher production under SA + 55 °C treatment. In addition, HA with high temperature also significantly increased the concentrations of withaferin A, withanone, and withanolide A compared to the control treatments (17.83 ± 0.17 mg g^{-1} dw of withaferin A, 4.70 ± 0.27 mg g^{-1} dw of withanone, and 5.65 ± 0.30 mg g^{-1} dw of withanolide vs. 28.51 ± 1.74 mg g^{-1} dw of withaferin A, 9.13 ± 0.02 mg g^{-1} dw of withanone, and 22.24 ± 1.97 mg g^{-1} dw of withanolide for HA + 55 °C treatment). Moreover, the total withanolides remarkably increased to a 3-fold higher accumulation under SA + 55 °C treatment. Our findings are directly in line with previous findings reported by Sivanandhan et al. (2014), who concluded that SA as an elicitor is sufficient for the production of withanolide A, withanone, and withaferin A. Our results also revealed

Table. Withanolide content of *P. peruviana* leaves treated by high temperature following salicylic acid or heat acclimation applications. Data represented are means of 3 separate experiments ± SD. Values followed by different letters in the same column are significantly different ($P < 0.05$).

	Amounts of withanolides (mg g^{-1} dw)			
Types of elicitors	Withaferin A	Withanone	Withanolide A	Total
Control	$17.83^{d} \pm 0.17$	$4.70^{d} \pm 0.27$	$5.65^{e} \pm 0.30$	28.18
HA	$17.50^{d} \pm 0.51$	$4.95^{d} \pm 0.13$	$10.08^{d} \pm 2.13$	32.54
SA	$22.27^{c} \pm 1.07$	$6.83^{c} \pm 0.22$	$14.74^{c} \pm 0.64$	43.85
HA + 55 °C	$28.51^{b} \pm 1.74$	$9.13^{b} \pm 0.02$	$22.24^{b} \pm 1.97$	59.89
SA + 55 °C	$44.47^{a} \pm 1.27$	$17.31^{a} \pm 0.65$	$25.04^{a} \pm 0.94$	86.83

that SA-only treatment induced a higher accumulation of withanolides in cultures, while no significant change occurred in withaferin A and withanone accumulation at nonlethal temperatures. Our results are consistent with those of previous studies on SA influence on secondary metabolite production (Kiddle et al., 1994; Ali et al., 2007; Awate and Gaikwad, 2014; Cingoz and Gurel, 2016).

In the present study, antioxidant molecules were determined in the leaves of *P. peruviana* shoot cultures to the subsequent induced thermotolerance (Figure 2). Heat acclimation or exogenous SA application increased SOD activities, while it reduced CAT activity in cultures under high temperature stress. Decreases in CAT and increases in SOD activity were noticeable when both high temperature and SA were applied to the cultures when compared to the control group. Similarly, increasing SOD and decreasing CAT activities were reported in grape leaves when cultures were treated with heat acclimation and SA application (Wang and Li, 2006). Lopez-Delgado et al. (1998) showed that exogenous H_2O_2 application increased thermotolerance in potato microplants. It is also known that the accumulation of H_2O_2 to some extent has a signaling role in plants during acclimation to abiotic stress (Dat et al., 1998; Van Camp et al., 1998). Accumulation of H_2O_2 following exogenous SA application has also been observed in various plant species; it has been concluded that SA directly inhibits CAT activity via CAT binding properties (Conrath et al., 1997; Rao et al., 1997; Kawano et al., 1998). Because of the reduced CAT activity in our study, the endogenous H_2O_2 content was also measured in the leaves of *P. peruviana* shoot cultures until induced thermotolerance. Both heat acclimation and SA pretreatment caused a significant increase in endogenous H_2O_2 content and reduced CAT activity in cultures under high temperature stress. SOD catalyzes $O_2^{\cdot -}$ to form H_2O_2, but CAT utilizes H_2O_2 as its substrate. According to the information given above, increased activity of SOD for $O_2^{\cdot -}$ conversion and decreased activity of CAT in the cultures under high temperature stress following pretreatment could be associated with H_2O_2 accumulation and induced thermotolerance in our study. It is widely accepted that H_2O_2 and other ROS are also important signaling molecules in the activation of defense genes in response to biotic and abiotic stress factors (Foyer and Noctor, 2009; Bartoli et al., 2012). Possibly by redox changes, H_2O_2 might directly or indirectly activate unknown signaling components, such as transcription factors, to regulate the transcription of proline biosynthesis genes. Proline is one such antioxidant which accumulates in response to biotic and abiotic stresses, including water stress (Zhang et al., 1995), salt stress (Fedina et al., 2006), extreme temperatures (Ruiz et al., 2003), and heavy metal toxicity (Chen et al., 2001). The relationship between proline accumulation and environmental stress suggests that proline could have some protective function. It was reported by Tari et al. (2002) that SA enhanced the accumulation of certain osmolytes such as proline in tomato plants. Our previous report also showed that SA and/or high temperature treatment caused a higher accumulation of proline in *D. trojana* Ivanina (Cingoz and Gürel, 2016). The connection between ROS and proline in response to biotic stress has already been highlighted by Fabro et al. (2004). Ben-Rejeb et al. (2015) reported that H_2O_2 production in response to NaCl or mannitol stress caused an increase in endogenous proline accumulation in *Arabidopsis thaliana*. In the present study, both SA and heat acclimation caused a significant increase in proline and H_2O_2 content in cultures under high temperature stress. The present data has suggested that H_2O_2 could act as a secondary messenger involved in triggering proline biosynthesis.

In conclusion, this study shows the effects of SA and HA on the production of withanolides and induced thermotolerance in shoot cultures of *P. peruviana*. A positive correlation between elicitor treatment and withanolide accumulation has been established for the first time. The production of withanolides was found to be dependent on heat acclimation or exogenous SA application combined with heat stress. When both SA and HA were applied to the cultures, a better elicitor influence was achieved for withanolide production. We provide an effective

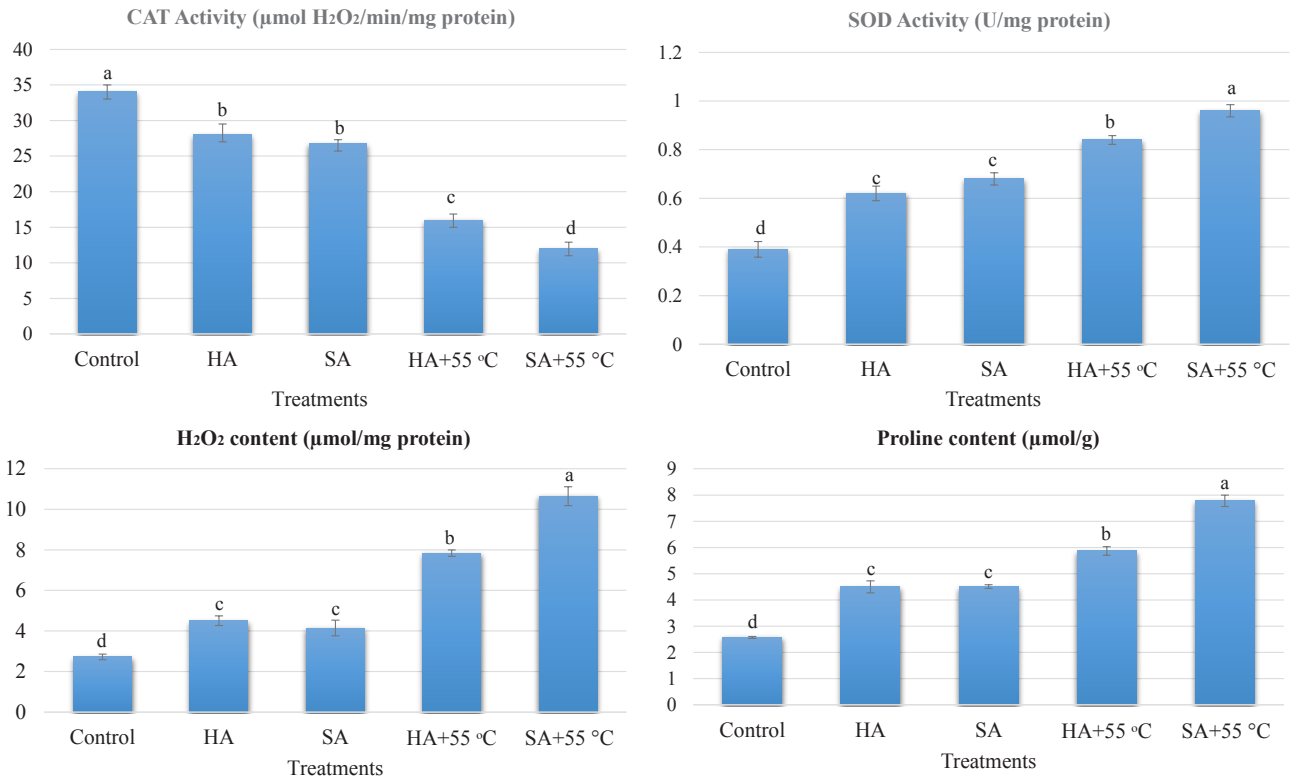

Figure 2. Catalase (CAT) and superoxide dismutase (SOD) activity, H_2O_2 and proline content in leaves of *P. peruviana* subjected to high temperature stress (55 °C) following either spraying with 150 µm of SA solution or a 1-h HA treatment (45 °C). Data represented are means of 3 separate experiments ± SD; columns showing different letters mean the results are statistically different.

protocol here for increasing withanolide biosynthesis for commercial biotechnology applications. In addition, the effects of SA and HA were investigated for induced thermotolerance. According to these results, changes in related antioxidants paralleling both heat acclimation and SA application suggest that the same mechanisms might be involved in induced thermotolerance, and SA confers better tolerance than heat acclimation. SA-pretreated and heat-acclimated cultures better adapt themselves to resisting high temperature stress by changes in the H_2O_2 metabolizing antioxidative system.

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