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Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants

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Abstract

Tomato plants, *Lycopersicon esculentum* L. cv. Tmknvf$_2$, and watermelon plants, *Citrullus lanatus* [Thomb.] Mansf. cv. Dulce maravilla, were grown for 30 days at different temperatures (15, 25 and 35°C). We analysed soluble phenolics, enzymatic activities (phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase), and dry weight. The impact of the three temperatures was different in tomato and watermelon. Our results indicate that heat stress in tomato plants occurred at 35°C, while chilling stress occurred in watermelon plants at 15°C. Thermal stress in both plants caused: (1) decreased shoot weight; (2) accumulation of soluble phenolics; (3) highest phenylalanine ammonia-lyase activity; and (4) lowest peroxidase and polyphenol oxidase activity. These results indicate that thermal stress induces the accumulation of phenolics in the plant by activating their biosynthesis as well as inhibiting their oxidation. This could be considered an acclimation mechanism of the plant against thermal stress.

Keywords: *Lycopersicon esculentum*; *Citrullus lanatus*; Phenolic compounds

1. Introduction

During the normal processes of growth and development, plants are subjected to different types of stress, such as drought, heat, ultraviolet light, air pollution, and pathogen attack [1–3]. Most plants suffer from both physiological and biochemical damage by exposure to temperatures higher or lower than optimal for growth [4,5]. The results of these injuries, which are reflected in most metabolic processes [6–8], may be a reduced growth capacity of the crops and therefore lower commercial yield [9]. It has been demonstrated that thermal stress induces the production of phenolic compounds, such as flavonoids and phenylpropanoids [10–13].

Phenylalanine ammonia-lyase (PAL) is considered to be the principal enzyme of the phenylpropanoid pathway [14], catalysing the transformation, by deamination, of L-Phenyalanine into *trans*-cinnamic acid, which is the prime intermediary in the biosynthesis of phenolics [15,16]. This enzyme increases in activity in response to thermal stress and is considered by most authors to be one of the main lines of cell acclimation against stress in plants [14,16,17].

Phenols are oxidised by peroxidase (POD) and primarily by polyphenol oxidase (PPO), this latter enzyme catalysing the oxidation of the *o*-diphenols to *o*-diquinones, as well as hydroxylation of monophenols [18–21]. These activities of enzymes increase in response to different types of stress, both biotic and abiotic [22–26]. More specifically, both enzymes have been related to the appearance

Abbreviations: dw, Dry weight; DTT, 1,4-dithio-DL-threitol; ETDA, Ethylenediamine tetraacetic acid; PAL, Phenyalanine ammonia-lyase; PMSF, Phenylmethanesulfonyl fluoride; POD, Peroxidase; PPO, Polyphenol oxidase; PVP, Polyvinylpyrrolidone; RNA, Ribonucleic acid; SDS, Sodium dodecyl sulfate; TFA, Trifluoroacetic acid.

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of physiological injuries caused in plants by thermal stress [4,5,17,21,27,28].

Although many works relate phenol metabolism (synthesis and oxidation), as well as the content of these compounds, to the induction of some type of stress in plants, few works relate the increases of phenols with thermal stress. Therefore, the aim of the present work is to analyse the role of phenol metabolism in response to the different temperatures applied (15, 25 and 35°C) in tomato and watermelon plants. The temperatures used in our experiments (15, 25 and 35°C) enabled us to observe the metabolic response of phenols to heat stress, exemplified by the tomato, for which optimal growth temperatures of the cultivar used were between 15–22°C [29,30]. In addition, we observed the response to chilling stress, exemplified by the watermelon plants, for which the optimal growth temperature is around 35°C [30,31].

2. Materials and methods

2.1. Crop design

Tomato (*Lycopersicon esculentum* L. cv. Tmnkvf$_2$) and watermelon (*Citrullus lanatus* [Thomb.] Mansf. cv. Dulce maravilla) plants were germinated and grown for 30 days in growth chamber at optimal growth temperatures for each species: 22–26°C for the tomato [29,30] and 33–37°C for the watermelon [30,31]. Afterwards, 12 plants per species were transferred to a cultivation chamber set at 15°C (day/night), another 12 plants to a chamber set at 25°C (day/night) and the remaining 12 to a third chamber set at 35°C (day/night). Each thermal-stress experiments was realised during a period of 30 days, since 30 days after sowing to 60 days after sowing. The growth chamber in all cases were maintained at a relative humidity of 60–80% and 16 h of photoperiod at a PPFD of 350 μmol m^{-2} s^{-1} (measured at the top of the plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, EN, USA).

During all experiments the seedlings were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) filled with vermiculite, and the plants received a nutrient solution of: 2 mM KNO_3, 4 mM $(NO_3)_2Ca$, 1.5 mM NaH_2PO_4, 2 mM $CaCl_2$, 3 mM K_2SO_4, 1.25 mM $MgSO_4$, 5 μM Fe-EDTA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$ and 2.5 μM H_3BO_3 [43]. The nutrient solution (pH 6–6.1) was renewed every 3 days.

2.2. Plant sampling

Plants were sampled on day 60 after sowing, all sampled leaves being the mature state. The material was rinsed three times in H_2O after disinfecting with 1% non-ionic detergent (Decon 90) [32], and then blotted on filter paper. A subsample of leaves was used fresh for the analysis of PAL, POD, PPO and total phenols, performing triplicate assays for each extraction. A subsample of shoots of the plants was dried in a forced-air oven at 70°C for 24 h. Dry weight was recorded and expressed as mg dry wt per shoot.

2.3. Plant analysis

2.3.1. Extraction and assay of PAL (EC 4.3.1.5)

The extraction was carried out following the method proposed by Lister et al. [33]. Fresh plant material was ground at 4°C in buffer composed of 5 ml of 50 mM Na_2HPO_4/KH_2PO_4, pH 7.0, 5% polyvinylpyrrolidone (PVP) (M_r 44 000), 50 mM Na ascorbate, 18 mM mercaptoethanol, 0.1% (v/v) Triton X-100. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20 000 × *g* for 10 min. $(NH_4)_2SO_4$ was added to the supernatant (to 35% saturation), which was then centrifuged for 20 min at 20 000 × *g* to remove the PVP. More $(NH_4)_2SO_4$ was added to this supernatant to reach a final saturation of 80%. This fraction was centrifuged at 20 000 × *g* for 20 min and the pellet resuspended in extraction buffer (without PVP and Triton). This solution was used for PAL assays. Protein was estimated by the method of Bradford [34] using BSA as a standard.

PAL activity was assayed by an adaptation of the methods of Zucker [35] and McCallum and Walker [36]. The assay mixture consisted of 0.06 M Na borate buffer, pH 8.8, and crude enzyme. The reaction was started by the addition of 11 mM L-phenylalanine. Tubes were incubated at 30°C for 60 min and the reaction stopped by the addition of 35% (w/v) trifluoroacetic acid (TFA). Tubes were then centrifuged for 5 min at 5000 × *g* to pellet the denatured protein. PAL activity was determined from the yield of cynnamic acid, estimated from absorbance at A_{290} in the presence and

absence of phenylalanine. To determine whether the reaction was enzymatic, a sample extract was boiled and assayed.

2.3.2. Extraction and assay of PPO (EC 1.14.18.1)

The extraction method used was that proposed by Thypyapong et al. [20] with some modifications. Leaves were ground to a fine powder with a pestle and extracted at a ratio of 150 mg fresh weight to 1 ml extraction buffer (100 mM Tris–HCl, pH 7.0, 100 mM KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 3% [w/v] PVP) containing SDS at 0, 0.5, 1, 2 or 4 (w/v) each. The homogenates were centrifuged at $12\,000 \times g$ for 15 min, and the supernatant was used to measure the protein concentration by the method of Bradford [34] using BSA as standard. PPO was also assayed. All these procedures were carried out at 0–4°C.

The PPO activity was assayed as described by Nicoli et al. [37] with some modifications. Optimum activity was reached using SDS at 2% (data not shown). The assay mixture consisted of 30 mM caffeic acid in 100 mM buffer (Na_2HPO_4/KH_2PO_4), pH 7.0, through which air was bubbled for 5 min. Catalase (420 units) from bovine liver (EC 1.11.1.6) (Fluka) was added in 0.1 ml H_2O to prevent peroxidation of the substrate. The assay was initiated by the addition of enzyme extract. PPO activity was measured by the change in A_{370} of the assay mixture (30°C) based on the measurement of the disappearance of caffeic acid by enzymatic oxidation. To determine whether the reaction was enzymatic, the sample extract was boiled and assayed.

2.3.3. Extraction and assay of POD (EC 1.11.1.7)

The method used was a modified version of that proposed by Kalir et al. [38]. Fresh plant material was ground with 50 mM Tris–acetate buffer, pH 7.5, 5 mM 2-mercaptoethanol, 2 mM 1,4-dithio-DL-threitol (DTT), 2 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM PMSF, and 1% (w/v) PVP. The homogenate was filtered through two layers of Miracloth and centrifuged for 30 min at 37 000 *g*. The pellet was discarded and the supernatant used for peroxidase assays and to measure protein concentration by the method of Bradford [34] using BSA as standard.

POD activity was determined following the change of A_{485} due to guaiacol oxidation at 30°C [25,38]. The reaction mixture contained 100 mM Tris–acetate buffer, pH 5.0, 1 mM guaiacol and 0.003 mM H_2O_2. To test whether the reaction was due to POD, control assays contained catalase from bovine liver (EC 1.11.1.6) (Fluka) (420 units in 0.1 ml H_2O). To determine whether the reaction was enzymatic, the sample extract was boiled and assayed.

2.3.4. Extraction and quantification of phenolics

Phenolics of plant material were extracted with methanol. Total phenolic content was assayed quantitatively by A_{765} with Folin–Ciocalteau reagent [39,40]. The results obtained were expressed as mg of caffeic acid per (g fresh weight [FW]).

2.4. Statistical analysis

The data shown are mean values $\pm$ SE. Levels of significance are represented by at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns: not significant.

3. Results

3.1. Phenolic metabolism in tomato plants

Our results showed stronger shoot growth at 15 and 25°C, the latter temperature giving the highest growth, which was twice that found at 35°C ($P < 0.001$; Table 1). The lowest biomass production occurred in our tomato plants at 35°C (Table 1), representing a 44% reduction with respect to highest values at 25°C.

In our experiment, PAL activity differed significantly (Table 1) in response to the three temperatures applied. The highest PAL activity was recorded at 35°C, with two-fold increase with respect to the lowest activity, recorded at 25°C (Table 1). The concentration of total phenolic compounds showed significant differences in response to different temperatures applied (Table 1).

The oxidative activities of phenols in our experiment, POD and PPO activities were lowest at 35°C, declining 53 and 76.5%, respectively (POD, $P < 0.001$; PPO, $P < 0.001$) from the highest values recorded at 25°C (Table 1).

3.2. Phenolic metabolism in watermelon pants

Watermelon, which tolerates greater heat than tomato, shows greatest vegetative development at 35°C. In this experiment, the highest values for dw/shoot were registered at 35°C and the lowest at 15°C (Table 2), this representing a 63.36% reduction ($P < 0.001$).

PAL activity was highest at 15°C, with significant differences between the different applied ($P < 0.001$). At 15°C this activity was five-fold greated than the lowest value registered at 35°C (Table 2). The total phenols concentration also showed significant differences ($P < 0.001$) between the three temperatures, and the same trend for PAL activity occurred at 15°C, representing a three-fold increase over the lowest concentration, recorded at 35°C (Table 2).

With respect to oxidative enzymes, POD and PPO, the application of different temperatures gave different results, which were quite significant (POD, $P < 0.001$; PPO, $P < 0.001$) in showing an inverse trend in relation to PAL activity and concentration of total phenols. In our experiment, POD and PPO activities fell with respect to the highest activities recorded at 35°C, by 49 and 73%, respectively (Table 2).

4. Discussion

Because cold and heat stress can stunt in plants [10,13] and because that the lowest biomass production occurred in our tomato plants at 35°C (Table 1) and in our watermelon plants at 15°C (Table 2), it can be stated that at these temperatures respectively the plants undergo thermal stress.

The metabolism of soluble phenolics is regulated by the activity of various enzymes. The first step necessary for the synthesis of the phenylpropanoid skeleton in higher plants is the deamination of the L-phenylalanine, giving rise to *trans*-cinnamic acid and ammonium [14,15]. This reaction is catalysed by the enzyme PAL, which is commonly considered the principal enzyme in the biosynthesis of phenolic compounds [14,41]. PAL activity is affected by a great number of factors, both biotic and abiotic, including light, temperature, growth regulators, inhibitors of RNA and protein synthesis, drought and mineral nutrition [3,22–26,42]. It has been demonstrated that heat and cold stress induced the production of soluble phenolics and thereby increased PAL activity [10–13].

The highest PAL activity in tomato plants was recorded at 35°C (Table 1) and in watermelon plants was recorded at 35°C (Table 2). Therefore, the PAL activity increased probability, in response to heat and cold stress [14,16,17]. These results are consistent with other authors who consider PAL to be one of the prime elements of cell acclimation against thermal stress in plants [10–13].

The relationship between PAL activity and soluble phenolics concentration in tomato ($r = 0.812$***) and watermelon ($r = 0.91$***) plants could indicate an accumulation of phenolics compounds in the plants in response to heat and cold stress respectively, caused by activation of enzyme PAL, as proposed by other authors [10–14,16,17].

In addition, the metabolism of phenolic compounds also includes the action of oxidative enzymes such as POD and PPO, which catalyse the oxidation of phenols to quinones [20,44]. Some studies have reported that these enzyme activities increase in response to different types of stress,

Table 1
Phenolic metabolism to different temperatures applied in tomato plants[a]

Temperature	PAL activity	Total phenols	POD activity	PPO activity	Shoot dry weight
15°C	50.41 ± 4.47	3060 ± 120	11.42 ± 0.18	16.84 ± 1.25	11.67 ± 0.24
25°C	32.23 ± 2.83	2266 ± 88.5	15.93 ± 0.25	29.49 ± 2.19	13.96 ± 0.19
35°C	69.75 ± 6.13	5520 ± 216	7.53 ± 0.21	6.89 ± 0.51	7.41 ± 0.11
Significance	***[b]	***	***	***	

[a] PAL activity: μmol cinnamic acid per (mg protein) h^{-1}; Total phenols: μg caffeic acid per (g FW); POD activity: μmol guaiacol oxidised per (mg protein) min^{-1}; PPO activity: μmol caffeic acid oxidised per (mg protein) min^{-1}; shoot dry weight (g). Data are means ± SE ($n = 6$).

[b] Levels of significance are represented by at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Table 2
Phenolic metabolism to different temperatures applied in watermelon plants[a]

Temperature	PAL activity	Total phenols	POD activity	PPO activity	Shoot dw
15°C	69.73 ± 4.02	5930 ± 207	9.58 ± 0.32	11.49 ± 1.52	2.37 ± 0.1
25°C	53.28 ± 3.07	3494 ± 222	11.51 ± 0.39	17.55 ± 1.79	5.12 ± 0.08
35°C	15.44 ± 0.89	1958 ± 68	18.72 ± 0.64	43.02 ± 4.44	6.76 ± 0.12
Significance	***[b]	***	***	***	

[a] PAL activity: μmol cinnamic acid per (mg protein) h^{-1}; Total phenols: μg caffeic acid per (g FW); POD activity: μmol guaiacol oxidised per (mg protein) min^{-1}; PPO activity: μmol caffeic acid oxidised per (mg protein) min^{-1}; shoot dry weight (g). Data are means ± SE ($n = 6$).

[b] Levels of significance are represented by at $*P<0.05$, $**P<0.01$ and $***P<0.001$.

both biotic and abiotic [22–26]. More specifically, both enzymes have been related to the appearance of physiological injuries caused by thermal stress [4,5,17,21,27,28]. In contrast to finding of these authors, in our experiments, POD and PPO activities were lowest in tomato plants at 35°C (Table 1) and in watermelon plants at 15°C (Table 2). Moreover, the relationship between POD and PPO activities and soluble phenolics concentration in tomato (total phenols-POD, $r = -0.890$***; total phenols-PPO, $r = -0.820$***) and watermelon (total phenol-POD, $r = -0.841$***; total phenols-PPO, $r = -0.739$***) plants appear to indicate that 35°C (for tomato plants) and 15°C (for watermelon plant) caused heat and cold stress, by subjecting the plants to a superoptimal and suboptimal temperatures respectively. Under stress, PAL was activated and the enzymes that oxidise soluble phenolic compounds, such as POD and PPO, were inhibited. Consequently, soluble phenolic compounds are accumulated perhaps as results of an acclimation mechanism to overcome heat and cold stress in tomato and watermelon plants, respectively.

5. General conclusions

According to these results, we conclude that tomato plants may develop a acclimated mechanism against superoptimal thermal stress caused at 35°C, a temperature well above the optimal growth temperature of 25°C, whereas watermelon plants show a acclimation against suboptimal thermal stress caused at 15°, that is well below their optimal growth temperature of 35°C. This acclimated mechanism in both plants appears to consist of the accumulation of phenolic compounds as a possible form of adapting to this stress. It may be possible, by manipulating factors involved in the bioactivity of phenolic compounds, to trigger acclimated mechanisms in plants under stress caused by temperature.

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