Does grafting provide tomato plants an advantage against H$_2$O$_2$ production under conditions of thermal shock?

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Non-grafted tomato plants (Lycopersicon esculentum L. cv. Tmknvf2) and grafted tomato plants (L.esculentum L. cv. RX-335) were grown for 30 days at three different temperatures (10°C, 25°C and 35°C). In the leaves of these plants, the enzymatic activities of SOD, GPX, CAT, APX, DHAR and GR were analysed, as were the concentrations of total H$_2$O$_2$, ascorbate and glutathione as well as foliar DW. Regardless of whether the plant was grafted or not, our results indicate that the thermal stress occurred mainly at 35°C, with the following effects: (1) high SOD activity; (2) H$_2$O$_2$ accumulation; (3) foliar-biomass reduction; (4) low GPX, CAT, APX, DHAR and GR activities; and (5) high concentrations of ascorbate and glutathione. In addition, our data show these effects to be much weaker in grafted than in non-grafted plants, directly reflected in greater biomass production. Therefore, the use of grafted plants under excessively high temperatures may offer an advantage over non-grafted plants in terms of resistance against thermal shock.

Introduction

Many crops are cultivated in areas where the climatic conditions are not always ideal and where temperatures may periodically fall far below or rise substantially above optimal levels. Such conditions can trigger oxidative metabolism in plants, brought on by an overproduction of active oxygen species (AOS), such as superoxide radicals (O$_2^-$), hydroxyl (OH), singlet oxygen (¹O$_2$), and hydrogen peroxide (H$_2$O$_2$) (Elstner and Oswald 1994, Prasad et al. 1994, Queiroz et al. 1998). For their chemical properties, the AOS are highly reactive and can damage proteins, chlorophylls, membrane lipids and nucleic acids, upsetting the homeostasis of the organism (Shaaltiel and Gressel 1986, Scandalios 1993). To prevent or alleviate these damages, plants have developed various mechanisms based on the production of defence antioxidants (Jahnke et al. 1991, Walker and McKersie 1993, Hodges et al. 1997).

The electrons generated by the activity of PSII give rise to the formation of different AOS, the radical O$_2^-$ being one of the most important (Salin 1989, Bowler et al. 1992, Scebba et al. 1998). The enzyme SOD brings about the dismutation of the radical O$_2^-$ to H$_2$O$_2$, for which it is commonly considered as the first line of cell defence (Halliwell and Gutteridge 1989). In fact, H$_2$O$_2$ can be detoxified from the different cell compartments by different enzymes, either on the one hand by the activity of GPX or CAT, which bring about the automatic catalysis of H$_2$O$_2$ to H$_2$O (Peters et al. 1989, Takahama and Oniki 1992), or on the other hand, by an oxidation-reduction system of antioxidant metabolites (ascorbate and glutathione) which involves other enzymes (APX, DHAR and GR) called the ascorbate/glutathione cycle or route of Halliwell-Asada (Asada and Takahashi 1987, Halliwell and Gutteridge 1989, Salin 1989, Ushimaru et al. 2000).

The exposure of plants to stress situations generally triggers antioxidant defence systems, since the regulation of the coding genes for these enzymes appears to be highly sensitive to the rise in cellular levels of AOS produced under these conditions (del Rı´o et al. 1991, Palma...
et al. 1991, Scandalios 1993, Allen 1995, Rao et al. 1996). In this way, higher cellular levels of H$_2$O$_2$ under thermal stress indicate a clear rise in AOS production (Lafuente and Martínez-Téllez 1997, Paolacci et al. 1997). Both high as well as low temperatures can prompt H$_2$O$_2$ accumulation in different plant tissues resulting from stronger SOD activity and by a partial inhibition of the enzymes in charge of detoxification (Yamakawa 1983, Campa 1990, Gaspar et al. 1991). In this way, H$_2$O$_2$ accumulation in plants subjected to thermal stress is considered the prime cause for the reduction in plant biomass (Queiroz et al. 1998).

The cultivation of grafted plants began in late 1920 primarily to counteract or diminish attacks by soil pathogens such as *Fusarium oxysporum* (Yamakawa 1983, Lee 1994). However, the current applications of grafting involve virtually all fields of plant physiology. For example, grafted plants were used to induce resistance against low root temperatures (Bulder et al. 1990), against iron chlorosis in calcareous soils (Romera et al. 1991, Shi et al. 1993), greater nutrient uptake (Bavaresco et al. 1991, Ruiz et al. 1996, 1997), increased synthesis of endogenous hormones (Proebsting et al. 1992), greater growth and productivity of the aerial part (Hussein and Slack 1994, Ruiz and Romero 1999) and improved fruit quality (Autio 1991).

Given that plant species differ in sensitivity to heat fluctuations, grafting onto root bases more resistant to these changes could encourage growth and development of the above-ground part and thereby improve plant adaptation to thermal stress. In this light, the aim of the present work was to compare the advantage of using grafted tomato plants under conditions of thermal stress as an available tool for improving the plant resistance to stress in these plants.

**Materials and methods**

**Experimental design**

Two varieties of tomato plants (*Lycopersicon esculentum* L.) were used: cv. Tmknvf2, and cv. RX-335. Seeds of both varieties were germinated and grown for 30 days in a growth chamber under controlled conditions of humidity and photoperiod, at a constant temperature of 23–26°C (optimal growth temperature for these plants) (Maroto 1995). Afterwards, the two varieties were grafted together (by needle graft), using RX-335 as the rootstock and Tmknvf2 as the scion.

After 30 days the grafts were inspected to confirm proper healing of the joint, and the experiments were begun. Three different experiments were conducted in which the only variable was temperature. In each experiment, 12 non-grafted tomato plants (*L. esculentum* L. cv. Tmknvf2) and 12 grafted ones (*L. esculentum* L. cv. Tmknvf2 × *L. esculentum* L. cv. RX-335) were grown in a growth chamber. The first experiment was conducted at 10°C (day/night), the second at 25°C (day/night) and the third at 35°C (day/night). Temperature was measured by CR21X sensors (Campbell Scientific) placed in the middle and upper shoot zone of the plants as well as in the root zone at 12 cm in depth. In all cases, the temperature difference between shoot and the root zone was ±2°C, a margin of error small enough to be disregarded in the interpretation of the results.

Each experiment lasted 30 days; that is, from day 60–90 after germination. In all cases, the growth-chamber conditions were maintained constant with a relative humidity of 60–80% and 16 h or photoperiod (PPDF of 350 μmol m$^{-2}$ s$^{-1}$ measured in the highest part of the plants with a 190 SB quantum sensor, LI-COR, Inc., NE, USA).

All plants were grown in individual pots (25 cm in diameter and 25 cm in height) completely filled with vermiculite. For 90 days, all the plants received a complete nutrient solution composed of: 2 mM KNO$_3$, 4 mM (NO$_3$)$_2$Ca, 1.5 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$, 3 mM SO$_4$K$_3$, 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$_4$)$_6$Mo$_7$O$_2$$_4$ and 2.5 μM H$_2$BO$_3$ (Van Zinderen 1986). This solution was renewed every 3 days and the pH was maintained at between 6.0 and 6.1.

**Plant sampling**

Plants were sampled on day 60 after sowing, all sampled leaves being in the mature state. The material was rinsed three times in H$_2$O after disinfecting with 1% non-ionic detergent (Decon 90) (Wolf 1982), and then blotted on filter paper. Of each treatment, half of the plants were used for the analysis of SOD, CAT, GPX, APX, DHAR, GR, H$_2$O$_2$, AsA, DHA, total ascorbate, GSH, GSSG and total glutathione (triplicate assays for each extraction). The other half of the treated plants were used to determine shoot DW. Leaves of these plants were dried in a force-air oven at 70°C for 24 h. DW was recorded and expressed as g DW shoot$^{-1}$.

**Metabolite assays**

The methods used to extraction and of total H$_2$O$_2$ were those of McNervin and Uron (1953) and Brennan and Frenkel (1977). Hydroperoxides form a specific complex with titanium (Ti$^{4+}$), which can be measured by colourimetry at 415 nm. The concentration of peroxide in the extracts was determined by comparing the absorbance against a standard curve representing a titanium-H$_2$O$_2$ complex from 0.1 to 1 mM. The hydroperoxides represent the total peroxides.

Reduced ascorbate (AsA), dehydroascorbate (DHA), and total ascorbate (AsA + DHA) were determined following Gosset et al. 1994). From the same extract, AsA and total ascorbate were assayed. Ascorbate standards of between 0.001 and 0.5 μmol ml$^{-1}$ ascorbate in m-phosphoric acid were analysed in the same manner as extracts. For each sample, DHA was estimated from the difference of total ascorbate and AsA.
The GSSG, GSH and total glutathione (GSSG + GSH) amounts were determined following Gosset et al. (1994). From the same extract, GSSG and total glutathione were assayed. A standard curve was developed by preparing solutions of 0.002–0.0001 g ml⁻¹ GSH in 60 ml⁻¹ m-phosphoric acid (pH2.8) containing 1 mM EDTA, diluting 1:2000 with 50 ml⁻¹ Na₂PO₄, and analysing in the same manner as the extracts. Levels of GSH were estimated as the difference between total glutathione and GSSG.

Enzyme assays

SOD activity was assayed by monitoring the inhibition of photochemical reduction of NBT, according to the methods of Giannopolitis and Ries (1977) and Beyer and Fridovich (1987), with some modifications (Yu et al. 1998). A 5-ml reaction mixture was used, containing 50 mM HEPES (pH7.6), 0.1 mM EDTA, 50 mM Na₂CO₃ (pH 10.0), 13 mM methionine, 0.025% (v/v) Triton X-100, 63 μM NTB, 1.3 μM riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 15 min; PPFD was 380 μmol m⁻² s⁻¹. Identical reaction mixtures that not were illuminated were used to correct for background absorbance. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NTB as monitored at 560 nm.

CAT activity was determined as described Bandiani et al. (1990) by following the consumption of H₂O₂ (extinction coefficient, 26.6 mM⁻¹ cm⁻¹) at 240 nm for 3 minutes.

GPX activity was determined as described Kalir et al. (1984) and Ruiz et al. (1998) by the oxidation of guaiacol in the presence of H₂O₂ (extinction coefficient, 26.6 mM⁻¹ cm⁻¹) at 470 nm.

APX activity was determined according to Gossett et al. (1994) by following the decrease in A₂₉₀ of an assay mixture containing 0.5 mM ascorbate (extinction coefficient, 2.8 mM⁻¹ cm⁻¹). Corrections were made for non-enzymatic rates and for interfering oxidations.

DHAR activity was determined following Ushimaru et al. (2000) and GR activity was assayed as described by Ushimaru et al. (2000).

In our enzyme assays activities rates were determined at substrate saturation. All of the activities were expressed as a function of the oxidized or reduced substrate per milligram of protein per minute. The protein concentration was determined by the method of Bradford (1976) using BSA as standard.

Statistical analysis

Analysis of variance was used to assess the significance of treatment. Results shown are mean values ± se. A correlation analysis was also conducted to determine the relationship between the different variables. Levels of significance are represented by at *P < 0.05, **P < 0.01, ***P < 0.001 and NS, not significant by ANOVA at P = 0.05. Differences between the two temperatures and separated ANOVAS were tested for each variable. The t-test for each variable was then carried out and a Bonferroni correction was made.

Results and discussion

Plants subjected to thermal stress tend to overproduce AOS in different plant tissues (Levine et al. 1994), and in response the enzyme SOD constitutes the first line of cellular defence, detoxifying O₂⁻ radicals and giving rise to H₂O₂ production. Figure 1 shows that SOD activity significantly differed with respect to the three temperatures applied (P < 0.001), regardless of the whether the plant was grafted or not. The SOD activity proved highest at 35°C (superoptimal temperature), where values were 262% higher in grafted plants and 310% higher in non-grafted plants than at 25°C (the optimal temperature for tomato plants) (Maroto 1995), the temperature that gave the lowest SOD values. When the plants were grown at 10°C, SOD activity was higher than at 25°C, although less notably than when plants were grown at 35°C. Some works indicate that thermal stress can induce SOD activity by an overproduction of AOS under such conditions (Smirnoff 1993). Figure 1 reflects lower SOD activity in grafted plants than in non-grafted ones, irrespective of the growth temperature. However, the greatest differences in SOD activity were found between grafted and non-grafted plants at 35°C (P < 0.001), for which, in principle, we might assume that the production of AOS under thermal-stress conditions (in our case 35°C) remained lower in grafted than in non-grafted plants.

To test the above hypothesis we determined the concentration of the foliar H₂O₂, this being the first compound resulting from the detoxification of the O₂⁻ rad-
Table 1. Effect of temperature on H$_2$O$_2$ concentrations and DW in grafted and non-grafted tomato plants. H$_2$O$_2$ expressed as μmol of H$_2$O$_2$ (g FW)$^{-1}$. Shoot DW expressed g per plant.

<table>
<thead>
<tr>
<th>TEMP</th>
<th>H$_2$O$_2$$^a$</th>
<th>DW$^b$</th>
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<tbody>
<tr>
<td></td>
<td>Grafted</td>
<td>Non-grafted</td>
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<tr>
<td>10$^\circ$C</td>
<td>32.09 ± 1.01</td>
<td>42.19 ± 1.02</td>
</tr>
<tr>
<td>25$^\circ$C</td>
<td>29.03 ± 0.98</td>
<td>24.77 ± 0.89</td>
</tr>
<tr>
<td>35$^\circ$C</td>
<td>43.95 ± 1.12</td>
<td>71.83 ± 1.29</td>
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As revealed in Fig. 2A,B, the activities of GPX and CAT showed similar behaviour, responding significantly to the different temperatures applied ($P<0.001$). The highest GPX and CAT activities (Fig. 2A,B, respectively) resulted when the plants were grown at 25$^\circ$C, while at 10$^\circ$C and especially at 35$^\circ$C the activities of both enzymes declined considerably, irrespective of whether the plant was grafted or not. This decline at 35$^\circ$C, with respect to 25$^\circ$C, for GPX activity was 66% (Fig. 2A) in non-grafted plants and 48% in grafted plants. For the CAT activity (Fig. 2B), this reduction was 40% both in grafted plants as well as non-grafted ones. Some researchers hold that conditions of thermal stress can promote enzymatic activity of GPX and CAT (Lafuente and Martínez-Téllez 1997, Paolacci et al. 1997). Nevertheless, our results appear to indicate the contrary, leading us to conclude that excessively high temperatures might partially inhibit these enzymes, giving rise to H$_2$O$_2$ accumulation in different plant tissues. The correlation coefficients between the H$_2$O$_2$ concentration and the activities of GPX and CAT in all cases proved negative (non-grafted: GPX-H$_2$O$_2$, $r = -0.816^{**}$; CAT-H$_2$O$_2$, $r = -0.89^{***}$; grafted: GPX-H$_2$O$_2$, $r = -0.959^{***}$; CAT-H$_2$O$_2$, $r = -0.889^{***}$), implying that H$_2$O$_2$ is not detoxified by either of the two enzymes and that this compound accumulates in the different plant tissues.

As indicated above, the accumulation of foliar H$_2$O$_2$ is lower in grafted plants than in non-grafted, and thus it might be expected that the reduction in foliar biomass would also be lower, as reflected in Table 1. In this sense, the use of grafted plants implies an advantage with respect to non-grafted plants, since under the same conditions greater biomass production resulted in plants grafted over a more resistant rootstock.

The fact that our experiment reflected a lower H$_2$O$_2$ concentration in grafted plants could be due either to lower AOS production or to more efficient detoxification of this compound owing to the activity of a number of enzymes involved in the ascorbate/glutathione cycle (APX, DHAR, GR) which act in the breakdown of H$_2$O$_2$ into H$_2$O and O$_2$, through a system of oxidation/reduction of antioxidant compounds such as ascorbate and glutathione (Queiroz et al. 1998, Ushimaru et al. 2000). Therefore, in this study, we included not only the activities of CAT and GPX, but also of the antioxidant enzymes and compounds of the ascorbate/glutathione cycle.

On the other hand, Table 1 shows that at 35$^\circ$C the H$_2$O$_2$ concentration in non-grafted plants was almost double that in grafted plants, and thus we conjecture that in the latter the AOS production was less than in the non-grafted plants.

Willenkens et al. (1997) demonstrated that an H$_2$O$_2$ accumulation in the different tissues of a plant could result in reduced biomass. In fact, Table 1 shows lower foliar biomass both in grafted and non-grafted plants at 35$^\circ$C, the temperature at which the highest concentrations of H$_2$O$_2$ were found. Furthermore, the relationship between the two parameters proved negative and significant in all cases (non-grafted: DW-H$_2$O$_2$, $r = -0.922^{***}$; grafted: DW-H$_2$O$_2$, $r = -0.924^{***}$), supporting the hypothesis of Willenkens et al. (1997).

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and especially 35°C, these activities diminished sharply, this decline being far more drastic when the plants were not grafted. According to some researchers, excessively high temperatures partially inhibit the activities of the enzymes APX, DHAR and GR (Lafuente and Martínez-Téllez 1997, Queiroz et al. 1998), conclusions which support our results. Figure 3A,B,C show that the most significant differences between grafted and non-grafted plants appear at 35°C (P<0.001 in all cases). Figure 4 reveals that grafting promoted APX activity up to the point that, at both 10°C and 35°C, the activity of this enzyme was greater than in non-grafted plants grown at 25°C, a situation similar with respect to the activities of the enzymes DHAR (Fig. 3B) and GR (Fig. 3C).

The correlation coefficients between the activities of these three enzymes and the H₂O₂ concentration proved negative in all cases, although the statistical significance was much stronger in non-grafted than in grafted plants (non-grafted: APX-H₂O₂, r = −0.978***; DHAR-H₂O₂, r = −0.878***; GR-H₂O₂, r = −0.928***; grafted: APX-H₂O₂, r = −0.521*; DHAR-H₂O₂, r = −0.672**; GR-H₂O₂, r = −0.716**). This could explain the foliar concentrations of H₂O₂ found in grafted plants, which were far less than in non-grafted ones.

Fig. 2. (A) GPX activity in grafted and non-grafted tomato plants at three temperatures. GPX activity expressed as μmol guaiacol oxidized (mg prot)⁻¹ (min)⁻¹. (B) CAT activity in grafted and non-grafted tomato plants at three temperatures. CAT activity expressed as μmol H₂O₂ reduced (mg prot)⁻¹ (min)⁻¹.

Fig. 3. (A) APX activity in grafted and non-grafted tomato plants at three temperatures. APX activity expressed as μmol ascorbate oxidized (mg prot)⁻¹ (min)⁻¹. (B) DHAR activity in grafted and non-grafted tomato plants at three temperatures. DHAR activity expressed as μmol dehydroascorbate reduced (mg prot)⁻¹ (min)⁻¹. (C) GR activity in grafted and non-grafted tomato plants at three temperatures. GR activity expressed as μmol NADPH oxidized (mg prot)⁻¹ (min)⁻¹.
This indicates that in grafted plants the ascorbate/glutathione detoxification system functions better at extreme temperatures, detoxifying greater amounts of H$_2$O$_2$ and thereby preventing this compound from reaching high toxicity levels by accumulating in plant tissues. In this way, grafted plants achieve greater tolerance to thermal fluctuations apparently by developing a more resistant root zone, which in turn leads to better overall plant development, reflected by increased foliar biomass (Table 1).

Finally, ascorbate and glutathione showed similar behaviour, both differing significantly at each of the three temperatures applied (Table 2). For both compounds, the lowest concentrations were found when the plants were grown at 25°C, whereas in both cases concentrations increased considerably when the temperature was 10°C, and more so at 35°C. Hodges et al. (1997) reported that under stress provoked by excessively high temperatures, plants generally augment synthesis of different forms of ascorbate and glutathione for use as substrates needed for ascorbate/glutathione detoxification of H$_2$O$_2$. Our results indicate that the stress provoked by the high temperatures partially inhibited the enzymes involved in this cycle (APX, DHAR and GR). However, plants grown at 10°C and particularly at 25°C (optimal growing temperature) registered the lowest concentrations of the different forms of ascorbate and glutathione, confirming the conclusions of Salin (1989), stating that under normal conditions of growth and development, the antioxidant defence systems inevitably produce AOS to maintain homeostasis, but also efficiently eliminate them. However, Table 2 shows that the concentrations of these two antioxidant compounds were lower in grafted than in non-grafted plants. Therefore, in grafted plants, the ascorbate/glutathione cycle functions better and the aforementioned two compounds follow a continual oxidation/reduction process, remaining at low levels in plant cells.

In summary, tomato plants grafted to a more vigorous root stock show greater resistance to thermal fluctuations, resulting in greater plant development reflected in greater plant biomass (Table 1). This advantage may be owed to better H$_2$O$_2$ accumulation in the different plant tissues (Table 1), by virtue of stronger enzymatic activity of GPX and CAT (Figs 1 and 2A, respectively) or of optimal functioning of the ascorbate/glutathione oxidation/reduction cycle. Therefore, the use of grafted plants under excessively hot temperatures implies an advantage for the plant under such conditions through an intrinsic mechanism of counteracting excessive AOS production, and thus H$_2$O$_2$ accumulation in the different plant tissues, thereby promoting better growth and development under situations of thermal stress.

**References**


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50