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Different methods of grafting and activity of antioxidant enzymes in tomato

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ABSTRACT

Mechanical injuries caused during grafting can induce oxidative stress, causing deleterious effects on the cells. This study aimed to evaluate the damage caused by grafting methods in tomato plants by assessing the enzymatic activity of superoxide dismutase, catalase, peroxidase and polyphenoloxidase and assessing phenol content. Three grafting methods were applied to the 'Guardião'® rootstock (bevel contact, cleft and approach grafting), as well as the 'Pizzadoro'® tomato free-standing plant and five sampling periods (0, 3, 6, 9 and 12 days after grafting). The grafting methods employed did not induce changes in catalase activities. There was alteration in peroxidase activity in response to bevel contact and cleft grafting. Plants showed higher polyphenol oxidase activity after 12 days of grafting in all grafting methods. Total content of phenols in grafted plants did not differ from the free-standing. The results suggest compatibility between rootstock and graft. The survival rate indicates that the methods employed were appropriate. The most recommended grafting methods for tomato are bevel contact and cleft grafting, depending on the survival rate.

Key words: phenolic compounds, peroxidase, polyphenol oxidase, *Solanum lycopersicum*, superoxide dismutase

Diferentes métodos de enxertia e atividade de enzimas antioxidantes em tomateiro

RESUMO

As injúrias mecânicas causadas durante a enxertia podem induzir estresse oxidativo, causando efeito deletério às células. Este estudo objetivou avaliar os danos causados por métodos de enxertia em tomateiro, através da atividade das enzimas superóxido dismutase, catalase, peroxidase e polifenoloxidase e o teor de fenóis. Utilizaram-se três métodos de enxertia sobre o porta-enxerto 'Guardião'® (contato em bisel, fenda garfagem e encostia), além do pé-franco do tomateiro 'Pizzadoro'® e cinco épocas de amostragem (0, 3, 6, 9 e 12 dias após a enxertia). Os métodos de enxertia empregados não induziram alterações nas atividades da catalase. Houve alteração na atividade da peroxidase em resposta aos métodos contato em bisel e fenda garfagem. Em todos os métodos de enxertia, as plantas apresentaram maiores atividades da polifenol oxidase após 12 dias da enxertia. O conteúdo de fenóis totais nas plantas enxertadas não diferenciou do pé-franco. Os resultados sugerem compatibilidade entre porta-enxerto e enxerto. A taxa de sobrevivência indica que os métodos empregados, foram apropriados. Os métodos de enxertia mais recomendados para tomateiro são contato em bisel e fenda garfagem, em função da taxa de sobrevivência encontrada.

Palavras-chave: compostos fenólicos, peroxidase, polifenol oxidase, *Solanum lycopersicum*, superóxido dismutase

Introduction

Grafting is a common practice in the production of quality plants and is widely used in horticulture due to reduced pathogen infection (Lee et al., 2010) and susceptibility to root diseases, and increased production due to higher vigor. Several methods are used for the grafting of tomato, mainly differentiated by the type of cut. Cut shape influences the healing process, and because it is a mechanism that causes injuries, it can induce oxidative stress in cells and tissues.

In plants stressed by injuries or pathogens, or environmental abiotic factors (water, saline, osmotic or temperature stress, luminosity, herbicide effect), the formation of reactive oxygen species may occur (ROS). The superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical (OH^{\cdot}) of these reactive species, as for example hydrogen peroxide (H_2O_2), oxidize important cellular constituents, such as nucleic acids, membrane lipids and proteins, which can lead cells to death (Baxter et al., 2013). Possibly, increased tissue damage and reestablishment may be related to changes in the activity of enzymes or other molecules with antioxidant potential.

In order to eliminate the effect of ROS, cells activate the anti-oxidative metabolism, which may be of enzymatic or non-enzymatic origin. The enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are part of the enzymatic antioxidant system. Higher activity of antioxidant enzymes can be considered a potential biochemical marker for resistance to oxidative damage (Gill & Tuteja, 2010; Maksimovic et al., 2013). On the other hand, non-protein source substances, such as polyphenols, contribute to elimination of reactive oxygen species (Foyer & Noctor, 2013) as part of the non-enzymatic ROS elimination mechanism. Phenolic compounds are products of secondary metabolism of plants and are essential for growth and reproduction, as well as acting as an antipathogenic agent and contributing to pigmentation (Nacz & Shahidi, 2004). Some enzymes such as polyphenoloxidase (PPO) (catechol oxidase, EC 1.10.3.2) and peroxidase (POD) are related to the oxidation of phenolic compounds, catalyzing the oxidation of phenols into quinones, which can spontaneously polymerize to form dark pigments (Constabel & Barbehenn, 2008). Quinones have antimicrobial activity because they form irreversible complexes with proteins (Bruneton, 1995).

Since grafting can cause metabolic stress and tissues respond through the induction of antioxidant metabolism, the objective of this work was to evaluate if the grafting methods employed in tomato alter the activity of antioxidant enzymes (SOD, CAT and POD) and the total content of phenols.

Material and Method

The experiment was conducted in a greenhouse with temperature adjusted to $29 \pm 2^\circ\text{C}$ and relative humidity of 70%, in the Department of Horticulture of the Faculty of Agronomic Sciences (FCA), São Paulo State University (UNESP), Botucatu, São Paulo ($22^\circ 51'S$, $48^\circ 26'W$ and 786 meters a.s.l.).

A completely randomized 4 (grafting methods) x 5 (analysis times) factorial design with four replications, in triplicate, was used. Grafting methods were: bevel contact, cleft and

approach grafting, in addition to the free-standing (control). The experiment was conducted at five collection times: 0, 3, 6, 9 and 12 days after grafting. Each experimental unit was composed of four seedlings. Grafts were performed according to Goto et al. (2003), using the 'Guardião'® (Takii do Brasil) rootstock, the 'Pizzadoro'® (Nunhems) graft and blades for cutting the seedlings.

Samples were represented by whole stems of seedlings. After each collection, frozen in liquid nitrogen and conditioned in freezer at -80°C for further determination of enzyme activity and total phenol content.

The activity of superoxide dismutase (SOD) (Beauchamp & Fridovich, 1973) was determined by the enzyme's ability to inhibit the photo-reduction of blue nitrotriazolium (BNT) in a reaction medium composed of 5 mmol L^{-1} methionine, 0.66 mmol L^{-1} of EDTA, 33 $\mu\text{mol } L^{-1}$ of BNT, 0.00165 mmol L^{-1} of riboflavin, and 3.0 mL of 50 mM potassium phosphate (pH 7.8). The production of blue formazan, resulting from the photo-reduction of BNT was determined by the absorption at 560 nm (Pharmacia Biotech, Ultrospec 2000). A unit of SOD was defined as the amount of enzyme required for 50% inhibition of BNT photo-reduction. The enzymatic activity was expressed in $U\ g^{-1}$ of the protein.

Catalase activity (CAT) (EC 1.11.1.6) was determined by adapting the method of Kar & Mishra (1976). The assay was composed of 150 μL of sample, which was subjected to protein extraction in potassium phosphate buffer + EDTA + DTT + PVPP 100 mmol L^{-1} pH 7.5. 1,950 μL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) was used as determination buffer and 750 μL of 50 mM hydrogen peroxide solution was used as enzymatic substrate. Readings were made at 240 nm (Pharmacia Biotech, Ultrospec 2000). Enzymatic activity was expressed in $\mu\text{mol } H_2O_2\ min^{-1}\ mg^{-1}$ protein.

Polyphenoloxidase (PPO) activity was determined through the method adapted from Kar & Mishra (1976), by measuring the conversion of catechol to quinone. The substrate used was composed of 0.1 M catechol in sodium acetate buffer (pH 5.0). The reaction occurred at 30°C for 30 minutes and the readings were made at 395 nm (Pharmacia Biotech, Ultrospec 2000). Enzymatic activity was obtained in μmol catechol oxidized $min^{-1}\ \mu g^{-1}$ of protein.

Peroxidase activity (POD) was determined by the method of Lima et al. (1999), with modifications. The assay was performed in 5.0 mL of 0.1 M sodium acetate buffer (pH 5.0). The substrate used was composed of 0.5 mL of 30% hydrogen peroxide in 0.2 M potassium phosphate buffer (pH 6.7) and 0.5 mL of phenol and aminoantipyrine solution. The reaction started with addition of the crude extract for five minutes. Enzymatic activity was read at 505 nm (Pharmacia Biotech, Ultrospec 2000) and the result was expressed as $\mu\text{mol } H_2O_2$ decomposed $min^{-1}\ mg^{-1}$ of protein.

Analysis of total phenols was performed using Folin-Ciocalteu's reagent (Singleton & Rossi Jr., 1965). After extraction in 50% (v/v) acetone solution, samples were incubated in an ultrasonic bath (Sonica S3) for 20 minutes and centrifuged at $6,000 \times g$ (Hettich Zentrifugen, Mikro220R) for 10 minutes. Two extractions were performed and the supernatants were then combined. The Folin-Ciocalteu

reagent was added, and after three minutes at 25°C, saturated Na₂CO₃ solution was added and after 1 h, the absorbance was measured at 760 nm (Pharmacia Biotech, Ultrospec 2000) and the results were expressed as mg phenols G⁻¹ of fresh mass (FM), in gallic acid equivalents.

Statistical analysis consisted of analysis of variance (test F) at 5% of probability (Table 1) and comparison of means by Tukey test (5%).

Results and Discussion

The types of grafting employed did not induce significant changes in CAT activity (Table 1). The highest levels of activity occurred at 0 and 12 days after grafting, but grafting methods did not differ from the free standing at these times of analysis (Table 2). Similar results were described by He et al. (2009), where there were no differences in CAT activity when comparing grafted, rootstock and free-standing tomato plants. According to Fernández-García et al. (2004), catalase is considered an enzyme involved in the cell defense process against high H₂O₂ production that takes place after grafting process, generated during lignification, in tomato plants. Thus, grafting methods used in this study did not induce significant stress to affect the catalase enzyme activity.

Analysis of SOD activity and total phenol content was significantly different among the grafting methods employed

Table 1. Summary of F test of the analyses of activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO) and phenol content according to grafting method and days after grafting

FV	SOD	CAT	POD	PPO	PHENOL
Grafting method (F1)	10.56*	1.92 ^{ns}	0.26 ^{ns}	2.13 ^{ns}	5.05*
Time after grafting (F2)	34.39*	7.59 *	11.56*	458.13*	4.68*
Interaction F1x F2	7.90*	1.72 ^{ns}	3.03*	3.12*	4.89*
Treatments	13.90*	2.99*	4.39*	98.76*	4.86*
CV (%)	0.87	13.97	7.74	3.24	3.75

Significant F test (*) and non-significant (ns) (p < 0.05).

Table 3. Activity of the enzymes superoxide dismutase (SOD, U g⁻¹ protein), peroxidase (POD, μmol H₂O₂ decomposed min⁻¹ mg⁻¹ protein), polyphenol oxidase (PPO, μmol catechol oxidized min⁻¹ mg⁻¹ protein) and total phenol content (mg g⁻¹ fresh mass) according to grafting method and days after grafting

Grafting methods	0	3	6	9	12
Days after grafting (DAG) - SOD					
Contact beveled	1.20 aB	1.22 aA	1.19 aB	1.16 aC	1.18 aB
Cleft grafting	1.20 aB	1.22 aA	1.17 bC	1.16 aC	1.18 aBC
Supported grafting	1.20 aA	1.17 bB	1.17 bB	1.16 aB	1.18 aB
Free-standing	1.19 aA	1.16 bBC	1.17 bBC	1.16 aC	1.18 aAB
CV (%) 0.87					
Days after grafting (DAG) - POD					
Contact beveled	0.13 aAB	0.11 bB	0.13 aAB	0.14 aA	0.15 aA
Cleft grafting	0.13 aAB	0.11 bC	0.12 aBC	0.14 aAB	0.15 aA
Supported grafting	0.14 aA	0.13 aA	0.13 aA	0.12 aA	0.14 aA
Free-standing	0.14 aA	0.14 aA	0.13 aA	0.12 aA	0.14 aA
CV (%) 7.74					
Days after grafting (DAG) - PPO					
Contact beveled	128.08 aB	97.02 bcD	100.51 aD	109.70 aC	145.15 aA
Cleft grafting	129.09 aB	96.19 cD	105.58 aC	108.36 abC	149.96 aA
Supported grafting	128.98 aB	103.67 abC	106.24 aC	104.43 abC	149.52 aA
Free-standing	127.08 aB	105.71 aC	99.50 aC	102.50 bC	145.97 aA
CV (%) 3.24					
Days after grafting (DAG) - Total phenols					
Contact beveled	0.66 aAB	0.68 aAB	0.67 aAB	0.70 aA	0.63 bB
Cleft grafting	0.65 aB	0.66 aAB	0.65 aB	0.70 aA	0.70 aA
Supported grafting	0.64 aB	0.65 aAB	0.64 aB	0.63 bB	0.70 aA
Free-standing	0.62 aB	0.69 aA	0.64 aB	0.64 bB	0.63 bB
CV (%) 3.75					

* Means followed by the same lowercase letters in the columns and uppercase letters in the lines do not differ statistically according to Tukey test (p < 0.05).

Table 2. Catalase activity (CAT, μmol H₂O₂ min⁻¹ mg⁻¹ protein) according to grafting method in tomato

Factors	Means
Grafting methods	
Contact beveled	2.0 x 10 ⁻⁰⁹ a*
Cleft grafting	1.8 x 10 ⁻⁰⁹ a
Supported grafting	1.8 x 10 ⁻⁰⁹ a
Free-standing	2.0 x 10 ⁻⁰⁹ a
Days after grafting (DAG)	
0	2.2 x 10 ⁻⁰⁹ a
3	1.8 x 10 ⁻⁰⁹ b
6	1.6 x 10 ⁻⁰⁹ b
9	1.6 x 10 ⁻⁰⁹ b
12	2.3 x 10 ⁻⁰⁹ a

* Original data transformed to 1/√X. Means followed by the same letter do not differ statistically from each other. *Means followed by the same lowercase letters in the columns and uppercase letters in the lines do not differ statistically according to Tukey test (p < 0.05).

(Table 1). SOD activity was higher at three days after grafting (DAG) in the case of bevel and cleft grafting methods (Table 2). On the day of the grafting (time 0), none of the methods induced significant changes in the activity of this enzyme, indicating that the plants, at that time, were all under the same conditions in relation to the production of oxidizing compounds.

When cells present increased SOD activity, this can be an indication of damage by reactive oxygen species (ROS). SOD is the first line of cellular defense. It eliminates radicals O⁻² and triggers H₂O₂ production (Sánchez-Rodríguez et al. 2010). However, in this study it was observed that, despite statistical significance (Table 1), SOD (Table 3) did not show changes that could indicate that the methods used induced biochemical damage.

Other enzymes that catalyze the oxidation of hydrogen peroxide, such as peroxidases, may have increased activity contributing to the elimination of free radicals formed, and also in the lignification process that takes place after grafting (Fernández-García et al., 2004). In this study, POD showed

no differences between the grafting methods (Table 1), but difference over time after grafting was observed in the bevel and cleft grafting methods (Table 3), especially in the last days of analysis. This can be attributed to the healing process. Despite these changes, it is not possible to state that the grafting methods used induced significant changes in POD activity, as could be observed in the last days of analysis, where no significant differences between grafting types or time of analysis (plant growth) were seen. Other studies showed that the low variation of peroxidase activity may be an indicator of compatibility (Telles et al., 2009) and survival, as found in this study.

Seedlings clearly showed similar behavior to free-standing plants, even after the grafting, thus showing good health. In tomato and other vegetables and fruits, POD is one of the enzymes involved in the last stage of lignification (Nicholson & Hammerschmidt, 1992) that occurs in response to stress caused by injury or by pathogen attack. In addition, this enzyme is one of the responsible for the production of dark compounds, demonstrating the oxidation of phenolic compounds. This oxidative process is related to the decrease in the content of phenolic compounds, important for lignification and survival rate of the seedlings (Telles et al., 2009).

Darkening of the area (formation of melanic compounds) is common after grafting due to oxidation of phenolic compounds to quinones and semiquinones caused by the activity of enzymes such as polyphenol oxidase and peroxidase (Pourcel et al., 2006; Constabel & Barbehenn, 2008). In the present study, this effect was not demonstrated because the graft region did not present dark areas. Highest PPO activities were seen 12 days after grafting (Table 3) in all grafting methods, including free-standing plants, but without significant difference. The formation of these melanic compounds may be a response to the presence of pathogens or oxidative damage. PPO and POD, the enzymes responsible for this oxidation (Pourcel et al., 2006), presented close values (analyzed within each enzyme), mainly in the last day of analysis. This result may be indicative of compatibility between the rootstock and the graft and the survival rate found. According to Telles et al. (2009), high peroxidase activity and high concentration of phenolic compounds can be biochemical indicators of seedling compatibility and survival, as found in this study for tomato.

Phenolic compounds presented a difference in bevel and cleft grafting 9 days after grafting, and in cleft and approach grafting 12 days after grafting (Table 3). Phenols are important components when evaluating the grafting process, since they are also related to stress (Yin et al., 2013) and tissue lignification (Telles et al., 2009). These components act in the defense, developing a toxic action during pathogen attacks or in the deposition in the cellular wall (Yin et al., 2013). Mng'omba et al. (2007) reported that higher levels of phenolic compounds and the appearance of necrotic areas may be related to incompatibility between graft and rootstock, causing a failure in the grafting process. On the other hand, Telles et al. (2009) state that higher content of phenolic compounds and high peroxidase activity favor the union of grafts. The levels of total soluble phenols found in tomato seedlings did not differ from free-standing plants, which may be a positive factor,

showing that the methods used, namely, grafts and rootstocks, were appropriate.

The tomato plants submitted to the cleft grafting method had greater survival rates after grafting (95.8%), followed by the plants grafted by the bevel contact (89.6%). The lowest survival rate was found in plants produced by the approach method (78.5%). According to Goto et al. (2003), cleft grafting is widely used in crops of Solanaceae family, such as tomato and peppermint. However, some nurseries and producers have also adopted the bevel contact method. This facilitates, above all, the rapidity of grafting. However, the approach method, which is rarely used in Solanaceae, was confirmed as not recommendable for tomatoes, because seedlings presented the lowest survival rate after grafting, and also, there was a low activity of the enzymes evaluated.

The observed survival rate is related to the activity of the enzymes and the total phenol content, demonstrating that, besides the adequate health of seedlings, the methods employed do not induce sufficient oxidative stress that may cause irreversible damages in these plants.

Conclusions

The grafting methods used for tomato did not induce oxidative damage, as evidenced by the SOD, CAT, POD and PPO enzymatic activity, in addition to the total phenol levels. These evaluations allowed verifying the health and the high survival rate of seedlings.

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