EFFECT OF *BACILLUS SUBTILIS* ON THE ANTIOXIDANT ENZYME ACTIVITY ON GRAFTING OF TOMATO PLANTS

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Abstract

Grafting generally means stress to a plant and this triggers antioxidant defense systems. An imbalance in reactive oxygen species may negatively affect the grafting success. Several research projects have studied the association with plant growth-promoting rhizobacteria (PGPR) and it has been documented that they enhance nutrient acquisition, regulate hormone levels, and influence the antioxidant response in crops. However, little is known about the strategy of inoculating grafted herbaceous plants with PGPR and its effect on the antioxidant response.

The effects of inoculating a strain of *Bacillus subtilis* on the antioxidant metabolism of grafted tomato were evaluated. In this study, two different rootstocks were used for tomato (*Solanum lycopersicum* L. var. Rio Grande (RG)): [*S. lycopersicum* L. var. cerasiforme (Ch)] and eggplant [(*Solanum melanogena* L. (Ber)] to establish a compatible graft (RGCh) and a semi-compatible graft (RGBer). Enzyme activities involved in the antioxidant defense system: superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD), and total phenols were measured during 4 weeks after grafting.

The results show that for RGCh, regardless of the day when it was measured, the tendency was a decrease of the enzyme activity for SOD, CAT, PAL when inoculated with *B. subtilis*; while in the semi-compatible graft RGBer, PPO and PAL decreased their activity after inoculation~~.~~ For both combinations, the quantity of total phenols decreased. These findings~~, give~~ indicate~~ions~~ that *B. subtilis* induced antioxidant mechanisms in grafted plants and suggest that inoculation with this growth~~-~~-promoting bacterium can represent a biotechnological approach to improve success in tomato grafting~~-~~.

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~~These findings, together with the *in vitro* assays performed on *B. subtilis* regarding its scavenging properties, give indications that *B. subtilis* promote grafting influencesalterates antioxidant mechanisms in grafted plants. Thus, inoculation with this growth-promoting bacterium could provide a biotechnological way to improve grafting success and to put in evidence, as well, the properties of this bacterium in promoting grafting.~~

Introduction

Grafting is a horticultural technique that has been practiced since ancient times (Mudge *et al*., 2009). It is very important for woody plants but, in the last century, grafting has become important in the Cucurbitaceae (i.e. watermelon, melon, cucumber) and Solanaceae family (i.e., tomato, eggplant, and pepper) (Bletsos *et al*., 2008). Grafting is also widely used in tomatoes to confer resistance to biotic and abiotic stresses (Singh *et al.,* 2017). Successful grafting may be influenced by factors such as time of grafting, hormonal application, compatibility of the species (Gainza *et al*., 2015), as well as level of the mechanical damage. The latter factor, in particular, can generate an antioxidant response due to the formation of ROS (Reactive Oxygen Species) (Suzuki *et al*., 2012). Superoxide radical (O^{-2}) and hydroxyl radical (OH^{-}) of these reactive species are free radicals that can oxidize important cellular components and cause alterations in DNA, protein, lipids, and carbohydrates or inactivation of enzymes which can lead to cell death (Baxter *et al*., 2014). Therefore, the control of tissue damage and, consequently, the success of the grafting may be related to variation in the activity of enzymes or content of other non-enzymatic molecules related to the antioxidant metabolism. The enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) can be biochemical markers of oxidative damage and their level of activity ~~higher concentrations~~ could be a sign of resistance to stress (Gill & Tuteja, 2010; Maksimovic *et al*., 2013). However, there are other non-proteic substances, such as polyphenols, that are involved in the scavenging of ROS (Foyer & Noctor, 2013). Phenolic compounds are products of the secondary metabolism of plants. Some enzymes such as polyphenol oxidase (PPO) 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). POD, PPO as well as p~~P~~henylalanine ammonia lyase (PAL), the first enzyme involved in the phenyl propanoid pathway and, therefore, in the biosynthesis of the polyphenol compounds, also ~~and polyphenoloxidase (PPO) activities~~ play a relevant role in ~~are also related to~~ plant resistance to stress (Finger, 1994; Soares *et al*., 2005)

A considerable number of bacterial species, mostly associated with the plant rhizosphere, have been tested and found to be beneficial for plant growth, yield, and crop quality. They have been called "plant growth-promoting rhizobacteria (PGPR)" and include strains of the genus *Bacillus* (Rodríguez & Fraga, 1999; Sturz and Nowak, 2000; Sudhakar *et al*., 2000; Ruzzi & Aroca, 2015). Microbial cells have several antioxidant defense mechanisms. *Bacillus* species and many other bacteria exert antioxidant activity producing a range of enzymes (Kaizu *et al*., 1993; Ahotupa *et al*., 1996; Amanatidou *et al*., 2000, Lin & Chang, 2000). Among these, *Bacillus subtilis* produces

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~~itself~~ two CATs (Lowen & Switala, 1987) and SOD (Murphy *et al*.,1987) as well as other metabolites (Kaspar *et al*., 2019). In detail, *Bacillus* spp. induce in colonized plants~~,~~ antioxidant enzymes, such as SOD, CAT, POD, PPO, PAL, and phenolic acids favoring plant response to stress conditions (Radhakrishnan *et al*., 2017; Rais *et al.,* 2017). The positive impact of *B. subtilis* has also been shown in tomato plants in biocontrol of bacterial wilt caused by *Ralstonia solanacearum*, through a role in increasing activities of PAL, PPO, POD, and SOD (Li *et al*., 2008), as well as in growth stimulation and induction of systemic resistance in tomato against early and late blight by inducing ~~defense~~ defense-related enzymes such as PPO, POD, and SOD (Chowdappa *et al*., 2013). Recent reports suggest that grafting onto suitable rootstocks can alleviate the damage caused by soilborne pathogens and the adverse effects of abiotic stresses besides enhancing the efficiency of water and nutrient use of tomato plants (Singh *et al*. 2017). In addition, grafting tomato on eggplant is a potential tool for improving waterlogging tolerance and related resistance to tomato bacterial wilt disease (Bahadur *et al.* 2015; Kariada Dan & Aribawa, 2017).

Thus, this study was focused on defining the effects of the PGPR, *B. subtilis*, on grafting of tomato plants on tomato (compatible rootstock) and ~~on~~ eggplant (semi compatible rootstock) by assessing modifications~~alterations~~ in the ~~enzyme~~ activity of SOD, CAT, PAL, POD, PPO, and in phenol content. To the aim, a preliminary *in vitro* antioxidant activity of *B. subtilis* was performed, then the scions of a tomato variety were immersed into the bacterial solution of *B. subtilis* and~~,~~ grafted on the ~~different~~ rootstocks. Then activities of enzymes, SOD, CAT, PAL, POD, PPO, total phenols were measured during ~~a period of~~ 4 weeks after grafting, to characterize their involvement in the antioxidant defense system during the plant response to the grafting process.

Materials & Methods

Preparation of inoculum of strains

Eight strains of *B. subtilis* provided by Biotecnología Microbiana S.A. de C.V. were used. Inoculum of the eight strains was prepared for all experiments by harvesting cells from cultures previously grown on potato dextrose (PD) broth ~~cultures grown~~ at 28 °C for 24 h on an orbital shaker at 150 rpm. The concentration of the inoculum was adjusted ~~using a spectrophotometer~~ to 10^6 CFU/mL ≈ 0.1 OD_{535} nm ~~by~~ using a spectrophotometer reading (Thompson *et al*., 1996)

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In vitro antioxidant activity of B. subtilis

Resistance to hydrogen peroxide (H_2O_2)

The method of Kadaikunnan *et al*. (2015) was used with some modifications. 10^6 CFU/mL ≈ 0.1 OD_{535} nm of strains of *Bacillus* cells were grown in 500 mL Erlenmeyer flasks containing 250 mL PD broth supplemented with 0.2, 0.4, 0.6, 0.8, or 1 mM H_2O_2 at 28 °C on an orbital shaker at 150 rpm for 24 h. The control treatment consisted of the growing medium inoculated with *B. subtilis* hydrogen peroxide-free. Cell growth was measured spectrophotometrically at 535 nm every hour and increases in cell growth were measured as increases in optical density (OD)

Hydroxyl radical scavenging activity ($OH^{\cdot}$)

Once the strain growth corresponding to 10^6 CFU/mL ≈ 0.1 OD_{535} nm was achieved, neutralization of the $OH^{\cdot}$ radicals was determined using the Fenton reaction, according to Kadaikunnan *et al*. (2015). Briefly, 1 mL of bright green reagent (0.435 mM), 2 mL~~ of~~ $FeSO_4$ (0.5 mM) and ~~,~~ 1.5 mL ~~of~~ H_2O_2 (3% w/v) were mixed with different volumes of each strain (0.5, 1.0, 1.5, 2.0 and 2.5 mL). The suspension~~y~~ were incubated at room temperature for 15 min, and then the absorbance was spectrophotometrically measured at 624 nm. The ability of the bacteria to scavenge hydroxyl radicals was determined according to the following equation.

$$Scavenging\ activity\ (\%) = \left[\frac{(A_S - A_0)}{(A - A_0)}\right] \times 100$$

where, A_s is the absorbance of the sample, A_0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and the Fenton reaction system.
The change in the absorbance of the reaction mixture indicated the scavenging ability of *B. subtilis* for hydroxyl radicals.

Total antioxidant activity (DPPH free radical scavenging activity)
The total antioxidant activity (TAC) of *B. subtilis* strains was evaluated by the method described by Kadaikunnan *et al*. (2015). Once an OD of 0.1 (10^6 CFU/mL) of *B. subtilis* cells at 535 nm was obtained, 0.5, 1.0, 1.5, 2.0, and 2.5 mL of the bacterial cells were mixed with 1 mL of the DPPH (Diphenyl-1-picryl-~~dydrazyl~~hydroxyl????) solution (0.05 mM). The mixture was stirred and incubated in the dark for 30 min at room temperature. The controls were deionized water and DPPH solution and the blanks contained only methanol and bacterial cells. The absorbance of the solution was measured at 517 nm after centrifugation of the samples at 16,218 g for 10 min. TAC was determined by the following equation:

$$Total\ antioxidant\ activity\ (\%) = \left[1 - \frac{(A_{sample} - A_{blank})}{A_{control}}\right] \times 100$$

where A_{sample} is the absorbance of the sample, A_{blank} is the absorbance of methanol with bacterial cells and $A_{control}$ is the absorbance of deionized water and DPPH reagent (Brand-Williams *et al*.,1995).

Plant material
Solanum lycopersicum L. (tomato, var. Rio Grande and var. C~~c~~erasiforme) and *Solanum melongena* L. (eggplant) seedlings were grown in the experimental greenhouse of the Ecological Biochemistry Laboratory at CINVESTAV (Advanced Research Center of the National Polytechnic Institute, Irapuato, Guanajuato, Mexico).
The commercial variety Rio Grande® was used as a scion. This is one of the industrial varieties mostly cultivated in Mexico. The tomato “C~~c~~herry” and the eggplant were used as rootstocks.
The choice of the rootstocks was made ~~on the basis of~~based on~~ a similar~~ spectrum of resistance/tolerance to biotic add~~di~~versities as well as on the degree of compatibility.

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The plants were germinated in trays containing a mixture of lime, vermiculite, perlite, leaf mold, and Sunshine® Mixture no. 3 (1:1:1:1:2:3). After 30 and 40 days of growth, respectively (late spring) when the seedlings had developed four or five true leaves in the case of tomato and two or three true leaves in the case of eggplant, plants were used for grafting~~ed as follows~~: tomato, var. Rio Grande, was grafted on tomato var. cherry (RGCh) and eggplant (RGBer).

Plant inoculation and grafting

Bacillus subtilis strain BMB 44 was prepared for inoculation by harvesting cells from ~~potato dextrose (~~PD~~)~~ broth cultures grown at 28 °C for 24 h on an orbital shaker at 150 rpm. The concentration of the inoculum was adjusted using a spectrophotometer to 10^6 CFU/mL ≈ 0.1 OD_{535} nm (Thompson *et al*., 1996). The adopted strain was chosen among those preliminary tested and giving the highest antioxidant response.

The seedlings chosen for grafting had all the same diameter (1.5-2.0 mm). The graft cut was made with a half-size double-edge razor blade. The splice grafting technique was used: the rootstock was cut at a 45 °C angle above the cotyledons and the scion was cut at the same angle as the rootstock. The height of the rootstock and the scion were 2.0-2.5 cm and 4-4.5 cm, respectively.

~~The plants were divided into two treatment groups. One group was used for inoculation and the second group for non-inoculated control plants.~~ A~~In the first group, a~~fter cutting the scion parts, inoculation was performed by immediately immersing 1 cm of the basal part of the scion~~s by 1 cm~~ in bacteria suspension (or tap water for the control) and incubated at room temperature for 15 min. After treatments, grafting was ~~performed~~ immediately performed and both parts of the plants were held with a silicone grafting clip.

~~For this study, completely randomized design was used: 2 (treatments) x 3 (analyses times) x 2 (grafting combinations). Each treatment was repeated three times (replicates). Each replicate consisted of 30 grafted plants.~~

Post-graft plant healing and grafting success rate

The post-grafting healing was held in containers with a plastic dome 23×15×14.5 cm (L×W×H), in a growing chamber of the Department of Biotechnology and Biochemistry of CINVESTAV. The conditions of the growing chamber were 25 ± 1 °C, ~~with a~~ photoperiod of 16 h, 117µmol s^{-1} m^{-2}, and relative humidity ~~was~~ between 85 % - 95 %, according to the humidity data logger. Seven days after grafting the seedlings were irrigated again but the dome was ~~opened~~ partially opened to gradually reduce humidity up to 70 %. Plantlets were kept under these conditions for 28 days. Lateral rootstock suckers were removed by hand when necessary. The observations for evaluation of grafting success were~~as~~ performed ~~at~~ 15 and 28 days after grafting (DAG). ~~At 15 days observations were focused on whether the scion would separate from the rootstock when removing the clip. At 28 days graft success rate was evaluated.~~

To determine~~prove~~ the presence of *B. subtilis* in grafted plants, a preliminary experiment was conducted based on the protocol proposed by Falcao *et al*., (2014). Fragments of leaves, stems (scion and rootstocks), and shoot apexes from 1, 15, and 28-day-old inoculated grafted plants were used. ~~Non inoculated~~Non-inoculated grafted plants were used as control.

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Sample collection and~~,~~ enzyme extraction ~~and assays~~

To evaluate e~~Alterations of defense e~~nzyme~~s~~ activities and total phenol content ~~was assessed in~~ the stem sections of the treated and control plants were used. For each treatment, the samples were represented by 2 mm stem sections (1mm above and 1 mm below the grafting point) per replication. These were collected ~~after~~ 1, 15, and 28 DAG. ~~After each collection time,~~ Collected s~~s~~amples were frozen in liquid nitrogen and stored at –80 °C for subsequent determination of enzyme activity and total phenol content. The method described by Giannipolits & Ries (1977) was adopted for the extraction of antioxidant~~tive~~ enzymes SOD, CAT, POD, and PPO. The stem samples (0.1 g) were homogenized in pre-chilled pestle and mortar. 450 µL of ice-cold 50 mM phosphate buffer, pH 7.0, and 50 µL of 10 mM EDTA
solution (1:5 w/v) were added to the homogenate and centrifuged at 18000 g at 4 °C for 15 min. The supernatants were immediately used for the determination of the activities of the enzymes. All steps in the enzyme extraction were carried out at 0-4 °C. Enzyme activities were expressed as U/mg protein.
The method described by Beaudoin-Eagan & Thorpe (1985) was used for the extraction of enzyme PAL. The stem samples (0.1 g) were homogenized in pre-chilled pestle and mortar. 200 µL of ice-cold and 0.5M Tris-HCl, ~~0.5M~~ pH 8, (1:2 w/v) were added to the ~~h~~homogenized sample~~omogenate~~ and centrifuged at 15000 g at 4 °C for 10 min. Enzyme activity was expressed as U/mg protein.

Enzyme activity assays

Superoxide dismutase (SOD)
This activity was determined according to Giannopolitis & Ries (1977) with modifications. The activity eassay was based on ~~was determined by~~ the ability of the enzyme to inhibit the reduction of Nitroblue tetrazolium (NBT) in a reaction mixture composed of 13 mM L-methionine, 100 µmol NBT, 0.1 mM EDTA, 16.7 µmol ~~of~~ riboflavin, and 50 mM potassium phosphate buffer (pH 7.8). The production of blue formazan, resulting from the photo-reduction of NBT, was determined by monitoring the sample absorption at 560 nm with a spectrophotometer (xMark ™ BIO-RAD). An unit (U) of SOD was defined as the amount of enzyme required to inhibit 50 % of NBT photo-reduction. The enzymatic activity was expressed in U/mg protein.

Catalase (CAT)
The CAT activity evaluation was based on Beers & Sizer (1952) method using the following reaction: t~~T~~he reaction mixture was composed of a solution of 25 mM H_2O_2~~hydrogen peroxide~~, 50 mM potassium phosphate buffer, and 10 µL of the enzyme extract. Readings were made spectrophotometrically (xMark ™ BIO-RAD) at 240 nm ~~(xMark ™ BIO-RAD)~~. The enzyme activity was determined by the kinetics of H_2O_2 degradation and expressed in U/mg protein.

Peroxidase (POD)

Peroxidase was determined by the procedure described by Sadasivam & Manickam (1996) with modifications. Guaiacol was used as a substrate~~ for the peroxidase~~. The assay was performed using 50 mM phosphate buffer, a 20 mM guaiacol solution, and a 25 mM H_2O_2 solution. In a 96-well microplate (Microtiter ™), 300, 5, and 10 µL of the above solutions were placed, respectively, and, finally, 10 µL of the enzyme extract was added. The absorbance was read spectrophotometrically (xMark ™ BIO-RAD)at 436 nm. The reading of the reaction started~~began~~ when the reaction absorbance was 0.05 and stopped when it reached an absorbance of 0.1. The enzymatic activity was determined by the production level ~~kinetics ~~of tetraguaiacol. The results were expressed in U/mg protein.

Polyphenol oxidase (PPO)

The activity of this enzyme was determined according to the protocol described in Mayer~~, Harel, & Ben-Shaul~~ et al, (1995) with some modifications. In this case~~,~~ catechol was the substrate of the enzyme. 50 mM phosphate buffer, ~~solutions~~ pH 7, and 0.1 M catechol were used. In a 96-well microplate (Microtiter ™) 150 µL of the ~~a ~~buffer, 20 µL ~~of ~~catechol, and 20 µL of the sample~~enzyme source~~ were placed. Absorbance was read at 495 nm at intervals for 3 min. The specific enzymatic activity was determined by the kinetics of quinone production. The activity was expressed in U/mg protein.

Phenylalanine ammonia lyase (PAL)

The activity of this enzyme was determined by the protocol described in Beaudoin-Eagan & Thorpe (1985)~~,~~ with some modifications. Three solutions were used~~prepared~~:~~,~~ a ~~buffer ~~0.5 M Tris-HCl buffer~~ 0.5 M~~, pH 8, one of 10 mM L-phenylalanine, and one of 5 M HCl. For the reaction, 250 µL of phenylalanine solution, 125 µL of distilled water, 500 µL of the buffer, and 125 µL of the enzyme extract were added. Absorbance was spectrophotometrically measured in a 300 µL 96-well microplate (Microtiter ™) at 290 nm. The mixture was then incubated at 37 °C in a thermostatic bath for one hour, after this time 100 µL ~~of ~~HCl were~~as~~ added to stop the reaction and the absorbance was again measured at the same wavelength. The specific activity of the enzyme was determined by the kinetics of the trans-cinnamic acid production and expressed in U/mg protein.

Total phenols

Total phenols were determined according to Mng'omba, du Toit & Akinnifesi (2008). The stem samples (0.05 g) were homogenized in pre-chilled pestle and mortar. 1 mL of ice-cold methanol-acetone-water solution was added (7: 7: 1) to the homogenate and centrifuged at 10,000 g at 4 °C for 4 min. The supernatant was used for the quantification of total phenols~~ was~~ performed by using 50 µL of the supernatant and adding 200 µL of distilled water and 250 µL of Folin-Ciocalteau reagent. The mixture was shaken at 800 rpm for 3 min. Then, 500 µL of a 7.5 % (w/v) NaCO3 solution were added. The mixture was homogenized for 1 min at 800 rpm and incubated for 15 min at 45 °C in a thermostatic shaker. Absorbance was measured in a 300 µL 96-well microplate (Microtiter ™) at 760 nm. The concentration of phenols was expressed as mE~~e~~q gallic acid/mg protein.

Statistical analysis

Principal component analysis (PCA) was performed. R software (3.5.1) was used to plot the PCA map of 15 days after grafting samples.

For this study, a completely randomized design was used.~~:~~ ~~2 (treatments) x 3 (analyses times) x 2 (grafting combinations).~~ Each treatment was repeated three times (replicates). Each replicate consisted of 30 grafted plants.

~~To determine whether the observed differences in enzyme activity and total phenols were significant, t~~The data ~~for each graft combination~~ were~~as~~ evaluated ~~separately~~ by analysis of variance (ANOVA) and significance among ~~within~~ treatments was analyzed by Least Significant Differences (LSD) test at the 5% level ($P<0.05$). Data were analyzed using R statistical software (3.5.1).

Results

The bacterial antioxidant activity was studied using free radical scavenging and a ferric reducing power assay. The tests were performed on eight strains (data not shown). The following results were obtained for strain BMB 44 which was the best performing strain.

In vitro antioxidant activity of B. subtilis

Resistance to hydrogen peroxide ~~(H_2O_2)~~

In Fig. 1, the effect of H_2O_2 ~~hydrogen peroxide~~ on the growth of the *B. subtilis* strain BMB 44 is shown. The results showed that all concentrations reached their maximum OD after 18 hours. The highest OD, 1.6, corresponded to the control. However, despite the ~~increasing~~ increase of H_2O_2 concentrations, the lowest OD registered was 1.2. Surprisingly, at the highest concentration of H_2O_2, an OD of 1.4 was measured.

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Hydroxyl radical scavenging activity ($OH^{\cdot}$)

The scavenging activity for hydroxyl radicals of ~~by~~ the strain BMB 44 of *B. subtilis* is shown in Fig. 2A. It was observed that the increase in the scavenging activity was directly proportional to the concentration of the cells. At 2.5 mL of cells at 10^6 CFU/mL, there was a 37 % scavenging rate while the lowest percentage was found in the control with a 5 % scavenging ability.

Total antioxidant activity (DPPH free radical scavenging activity)

The *B. subtilis*, strain BMB 44, was also checked for its DPPH reducing capability. The DPPH free radical scavenging activity was measured by the reduction of stable DPPH radical to non-radical DPPH-H. The scavenging activity was highly dependent and directly proportional to the concentration of cells (Fig. 2B). The highest inhibition activity was found at 2.5 mL (10^6 CFU/mL) with 100 % inhibition but even at a lower concentration (0.5 mL), *B. subtilis* showed about 30 % of scavenging activity.

Effect of Bacillus subtilis BMB 44 *on tomato grafting*

~~In order t~~To confirm~~prove~~ the ability of *B. subtilis* to colonize the tomato grafted plants, a preliminary experiment was conducted. In all examined tissues of the inoculated plants, *B. subtilis* was observed (data not shown). ~~Thus, the subsequent results are based on these findings~~.

Grafting success rate

To evaluate the effects of *B. subtilis* on the tomato-grafting success rate in the two combinations, the strain was used in the grafting procedure and compared to the use of water (Table 1). The data of the treatments were analyzed by (…) Fifteen days after grafting, the success rate of plants treated with BMB 44 was evaluated based on whether the graft union was secured even after removing the silicone clip. In the case of inoculated RGCh plants, the grafting success rate was 6% higher than the rate of plants treated with water, while for RGBer, it was 10% higher.

On day 28, the grafting success rate was evaluated as the result of the overall grafting procedure. For the bacterized and control RGCh plants, there were no significant differences, while for RGBer the inoculated plants showed 5% higher grafting success rate

~~Alteration in~~ ~~E~~Eenzyme activity and total phenols

~~The *in vitro* methods used in this investigation for measuring the antioxidant scavenging activity are based on the measurement of the variation of enzymatic antioxidant activities of the sample. In detail, the variations of antioxidant enzyme activities were assessed to oxidative stress produced in the tissues when a plant is grafted and the variation in the activity of the enzymes SOD, CAT, POD, PAL, and PPO were measured in the plants grafted with the different scion/rootstock combinations.~~

In this study the variations of antioxidant enzyme activities were assessed in relation to oxidative stress produced in the tissues when a plant is grafted: the variation in activity of the enzymes SOD, CAT, POD, PAL, and PPO were measured in the plants grafted with the different scion /rootstock combinations in relationship to the *B. subtilis* inoculation.

~~To explore the relationship between enzyme activity, total phenols and survival rate with the graft combination and the effect of *B. subtilis*, a PCA analysis was performed using the enzyme activity, total phenol content and the survival rate as descriptors. As shown in Figure 3, the two principal components (PC1 and PC2) represented 83.98% of the data variance. The first component accounted for 53.1% of variance and the second one to 30.88% of variance.~~

~~PC1 is strongly correlated with five of the original variables. The first principal component increases with decreasing CAT, SOD, POD, PPO and ~~tTotal phenols scores but PC1 correlates most strongly with CAT and POD (Table S1). Dominant variables for the second component (PC2) were PAL, PPO, SOD and survivors. PC2 increases with increasing SOD and PAL and with decreasing PPO and survivors (Table S1).~~

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~~As shown in the PCA plot (Figure 3), the grafted plants were divided in four clusters. Based on PC1, clusters RGCh and RGBer (control) are clearly separated from inoculated RGCh and RGBer clusters. Such differences are likely to be due to the enzymes that have heavy influences on PC1. Based on PC2, there are also four clusters: RGCh (control) and RGCh (inoculated) and, RGBer (control) and RGBer (inoculated). Such differences are likely to be due to the enzymes that have heavy influences on PC2. Given that PC1 reveals the most variation, differences among clusters along PC1 are larger than PC2 differences.~~

In ~~In order to understand whether the measured enzyme activity and total phenol quantity between treatments and graft combinations is significant, in T~~table 2 are reported the activities of SOD and CAT in~~of~~ grafted plants treated with *B. subtilis*. In the case of SOD, 1 day after grafting, in the RGCh combination, the compatible one, there is a lower activity (difference of 51 units) *in B. subtilis* treated plants in respect to the control. On the other hand, in the case of the semi-compatible graft (RGBer) there is a significant increase of 40 units at day 1.

On day 15, the RGCh activity in the control was significantly higher in respect to the inoculated plants (difference of 238 units), while in the case of RGBer the bacterized plants showed higher activity in respect to the non-inoculated plants.

On day 28, an increase of SOD activity was observed in RGCh, while in ~~the semicompatible graft (~~RGBer~~)~~ the increase in activity in the inoculated plants in respect to the control was not observed.

The variation in the activity of enzyme CAT with the different graft combinations is reported in Table 2. On ~~day~~ 1 day after grafting, in the RGCh combination, ~~a~~ higher enzyme activity can be observed in the non-inoculated plants presenting a difference of 671 units in respect to the grafted plants treated with *B. subtilis*. On the other hand, in the case of RGBer, the inoculated plants present higher enzyme activity (573 units) in respect to the control.

Fifteen days after grafting, in contrast with RGCh, the RGBer graft showed higher activity when inoculated. On day 28 after grafting, higher activity was observed in compatible grafted plants treated with the bacterium in respect to the non-inoculated plants, while, a reduction of activity was observed in grafted plants of RGBer treated with the bacterium in respect to the control.

Considering PPO, on day 1 after grafting (Table 2) the enzyme activity is higher in the case of the inoculated plants of RGCh, presenting a difference of 3.8 units, while in the other cases there is only a slight tendency to increase the activity of 0.1 and 0.3 units, respectively, on the control plants of RGBer. On day 15, the activity of the non-inoculated grafted plants was higher for the RGBer combination, while the bacterized plants presented the highest activity for the compatible graft and the lowest activity for the RGBer combination. On day 28, the activity is slightly higher in the control grafts.

The variation in the activity of POD with the different graft combinations is reported in Table 3. On day 1 after grafting, the greatest difference in activity was found in the RGBer combination where the bacterized plants present a higher activity of more than 47 units ~~with respect to~~for the control. The POD activity for RGCh is similar in the inoculated and non-inoculated plants. On day 15, RGCh and RGBer grafted plants had the highest activity when non-inoculated, while the

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inoculated plants showed higher activity ~~with respect to~~for the control only when comparing the compatible graft. On day 28 the RGCh and RGBer inoculated plants had higher activity.

Concerning the enzyme PAL (Table 4), one ~~1~~ day after grafting, regardless of the graft combination, lower activity in bacterized grafted plants in respect to the controls was observed. The control in the RGCh graft showed the highest difference (7.2 units) in respect to the inoculated graft, while the semi-compatible combinations have a difference of 3.8 units.

On day 15, the non-inoculated grafted plants presented the highest activity, regardless of the combination. On day 28, the control grafted plants of RGBer combinations present a slightly higher activity than the inoculated plants but with a difference of only 0.5, while no difference was found in RGCh.

In the case of total phenols (Table 4), one ~~1~~ day after grafting, the non-inoculated plants presented higher content of phenols for both RGCh and RGBer; on day 15, a greater content of phenols was measured in the controls for the semi-compatible (6.15 units) grafts ~~with respect to~~for the bacterized grafts presenting a tendency to increase as incompatibility also increased. On the other hand, in the case of the inoculated grafts, the phenol content for RGBer presented a lower content (4.05 units). The opposite pattern was observed on day 28 where the non-inoculated RGCh and RGBer grafted plants have a lower content of total phenols, even if the differences are limited.

A summary of the effect of *B. subtilis* is shown on the bar graph (Fig. 4). The bars are the result of the difference between the enzyme concentration of inoculated grafted plants and control grafted plants. Positive values mean that the difference in concentration is given by the inoculated plants. Negative values mean that the control plants have a greater concentration. The same applies ~~for~~ to total phenols.

For RGCh day 1 and 15, negative values can be observed for CAT and SOD while on day 28, ~~for~~ the same enzymes have a positive value including POD. In the case of RGBer, on day 1, remarkable positive values can be observed for CAT, SOD, and POD while on day 15, this happens only in the case of POD. On day 28, CAT and POD have positive values while SOD has a negative value. A positive value for t~~T~~otal phenols is registered on day 1 in the RGCh combination and on day 28 of the RGBer combination.

To better describe and quantify the association within the enzymes and between the enzymes and the total phenols, the Pearson's correlation coefficient was used (Figure S1). Considering RGCh day 1, CAT and POD showed a strong positive correlation while on day 15, SOD and PAL have a strong negative correlation. On day 28, PPO and Total phenols are strongly negatively correlated.

In the case of RGBer, such as for RGCh, CAT and POD presented a positive correlation on day 1. On the contrary, PPO and PAL presented a negative correlation. On day 15, POD and Total phenols, are negatively correlated as well as POD and PPO, on day 28.

To explore the relationship between enzyme activity, total phenols, and survival rate with the graft combination and the effect of *B. subtilis*, a PCA analysis was also performed using the enzyme activity, total phenol content and the survival rate as descriptors. As shown in Figure 3, the two

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principal components (PC1 and PC2) represented 83.98% of the data variance. The first component accounted for 53.1% of the variance and the second one to 30.88% of the variance.

PC1 is strongly correlated with five of the original variables. The first principal component increases with decreasing CAT, SOD, POD, PPO, and total phenols scores but PC1 correlates most strongly with CAT and POD (Table S1). Dominant variables for the second component (PC2) were PAL, PPO, SOD, and survivors. PC2 increases with increasing SOD and PAL and with decreasing PPO and survivors (Table S1).

As shown in the PCA plot (Figure 3), the grafted plants were divided into four clusters. Based on PC1, clusters RGCh and RGBer (control) are ~~clearly~~ separated from inoculated RGCh and RGBer clusters. Such differences are likely to be due to the enzymes that have heavy influences on PC1. Based on PC2, there are also four clusters: RGCh (control) and RGCh (inoculated) and, RGBer (control) and RGBer (inoculated). Such differences are likely to be due to the enzymes that have heavy influences on PC2. Given that PC1 reveals the most variation, differences among clusters along PC1 are larger than PC2 differences.

Discussion

In vitro antioxidant activity of B. subtilis

Microbial cells have several defense mechanisms. To prevent damage by ROS, organisms have evolved multiple detoxification mechanisms including various enzymatic or non-enzymatic systems (Asada, 1994; Ahmad *et al*., 2010). Among these enzymes, the combined action of SOD and CAT is critical in mitigating the effects of oxidative stress. They maintain the free radicals at levels that are not toxic to the cells. However, the ability of bacteria to overcome oxidative stress is related to the levels and types of antioxidant enzymes that they possess (Amantidou *et al*., 2001; Poole, 2012). Several growth-promoting bacteria have been reported to possess antioxidant activity (Han & Lee, 2005; Upadhyay *et al.,* 2012; Kang *et al*., 2014). *B. subtilis* has been extensively studied (Hecker & Völker, 2001) and ~~is~~ shown to possess an adaptation mechanism against H_2O_2. This bacterium undergoes a typical bacterial stress response when exposed to low concentrations (0.1 mM) of hydrogen peroxide but protection was also shown to be induced at higher concentrations (10 mM) and many proteins are induced including the scavenging enzymes, CAT (Loewen & Switala, 1987; Dowds, 1994), SOD and POD (Mols & Abee, 2011). Our results ~~are encouraging and~~ confirm the capacity of *B. subtilis* to react to stress conditions. At very high concentrations (1.0 mM) of hydrogen peroxide, the bacteria are unaffected by the H_2O_2 treatment and it is only after 18 hours that it reaches a plateau. In the same way, our results confirm previous results of Yan *et al*. (2006) showing that *B. subtilis* has the capacity of scavenging radicals presenting a scavenging activity of more than 35 %. In a biological system, no enzyme specifically destroys OH^-. The most effective defense against OH^- induced damage is to reduce the intracellular concentration of components in the Fenton reaction such as H_2O_2 and iron. This can be achieved by enzymes ~~which~~ that directly breakdown H_2O_2 such as CAT or sequestration of transition metal

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and repression of iron uptake (Hameed & Lee, 2009). In our study, 37 % scavenging activity was obtained.

In this sense, the results of DPPH antioxidant capacity measured in our study~~,~~ were similar to other studies (Kadaikunnan *et al*., 2015). In our study, *B. subtilis* already scavenges 30 % even at low concentrations and is capable of neutralizing 100 % of the radicals at greater concentrations. This suggested that its ~~the~~ antioxidant properties may help to reduce the level of oxidative stress associated with mechanical injuries created during grafting and different physiological stages.

Effect of B. subtilis on grafted plants

Graft compatibility may influence the antioxidant response when subjected to certain conditions such as the initial wound response and the subsequent physiological stages that the grafted plant goes through to reconnect the vascular tissue. Therefore, ~~in order~~ to find out the average grafting success rate in the presence or absence of *B. subtilis* a visual observation of the graft survival and the graft success rate was performed. In the case of RGCh, 15 DAG, the survival success percentage reached up to 86% when the plants were inoculated, that is, 6% higher compared to the control plants. The compatibility of this graft combination as well as the beneficial characteristics of *B. subtilis* (Sabir, 2013; Falcao *et al*., 2014) may be promoting a greater graft survival. In the case of the semi-compatible combination, RGBer, the survival rate is higher (10%) even though the non-inoculated plants have an initial lower survival rate with respect to the compatible combination. This result ~~gives an indication~~indicates that despite the lower compatibility, *B. subtilis* ha~~i~~s a positive influence.

On day 28, there were no significant differences between the inoculated and the control RGCh plants as there was 100 and 99% graft success, respectively. However, for the inoculated RGBer plants, there was an overall 95% graft success rate with respect to the 90% success rate reached by the control plants.

Many developmental stages can be recognized in the formation of a graft union. The early stage in herbaceous plants begins within 4 days and is characterized by the death of cell layers at the graft interface as a wound reaction (Moore, 1984; Tiedemann, 1989). The differentiation of callus parenchyma to form new cambial initials and the subsequent union of the newly formed vascular strand with the original vascular bundle in both rootstock and scion begins between days 4 and 8 and is fully developed after 15 d (Fernandez-Garcia *et al*., 2004). After that, the graft assemblage between the cells of the rootstock and scion was developed, differentiation of the new vascular system begins. Thus, enzymes are differently regulated during the different stages and the effect that *B. subtilis* may have on this regulation was also studied.

Several studies demonstrated the benefits of inoculating bacteria in plants (Bonaterra *et al*., 2003; Vardharajula *et al*., 2011). Inoculation of plants with *B. subtilis* growth and mitigation of abiotic and biotic stress effects (Gajbhiye et al., 2010; Singh et al., 2012).

~~In this study a PCA analyses was also performed in order to analyze which of the measured enzymes was more important and the influence of the total phenols on the inoculated e non-~~

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~~inoculated compatible and semicompatible grafted plants. In Figure 3, it is possible to observe that all measured variables had a strong effect.~~

~~It should be noted that there exists an inverse relationship between CAT, SOD, POD, PPO, and Total phenols and the first component (PC1) (Table S1). This indicates that *B. subtilis* might be affecting the enzyme and total phenols at this stage (15 DAG) which could be important for the graft survival. The dominant variables for PC2 were PAL PPO, SOD and survivors. However, SOD and PAL are directly related to PC2 while PPO and survivors are inversely related to the second principal component.~~

~~The inoculated plants are clearly influenced by *B. subtilis*. As it is shown in Figure 3, if the PC1 is considered, grafted plants form separated clusters, two regarding the inoculated plants and two regarding the control plants. On the contrary, respect to PC2 four clusters can also be observed but in respect to the graft combination: RGCh and RGBer. This confirms that also compatibility confers specific characteristics based on the graft combination.~~

~~In order to analyze whether the enzyme activity measured and the total phenols are significantly different,~~I~~ we report i~~n this study, that plants inoculated with *B. subtilis* presented an increase in the antioxidant enzymes ~~such as~~ CAT, POD, PPO, PAL, and in total phenols levels. In another previous study, *Bacillus* spp. were also assessed to induce an increase in activity of antioxidant enzymes against *Pyricularia ory~~i~~zae* (Rais *et al*., 2017). The application of *Bacillus* enhanced PPO and PAL activity but also changes in SOD and POD were observed in that study as response to the fungal infection. It has also been demonstrated that in the case of abiotic stresses, such as salinity stress, the activity of antioxidant enzymes in wheat increase with the increasing of salinity stress but plants treated with PGPR, such as *B. subtilis* and *Arthrobacter*, showed a reduction of activity of antioxidant measured enzymes as compared to uninoculated plants and among all antioxidants activities studied, the maximum reduction was recorded in CAT activity (Upadhyay *et al*., 2012). Initially, when the mechanical damage is induced in the grafted plants, there is a burst of free radicals (Savatin *et al.,* 2014) and the antioxidant machinery activates. Later on, when the graft union has been reestablished, the lignification processes may intervene (Aloni *et al*., 2008).

Superoxide dismutase is an important antioxidant enzyme and constitutes the first level of defense against superoxide radicals in plants. SOD catalyzes the dismutation of $O_2^{\,-}$ to H_2O_2 and O_2. Although exposing plants to stress situations, such as grafting, would trigger the antioxidant defense systems, there are indications that in incompatible rootstock/scion combination either the level of reactive oxygen species can be increased or decreased if a less efficient detoxification system is initiated (Aloni *et al*., 2008; Nocito *et al*., 2010).

Our results ~~give an indication of~~indicate the response in tomato grafting when comparing the two grafting combinations with different compatibility. In the case of RGPep there is a higher activity at day 1 and the bacterized plants showed an even higher activity which could mean a higher protecting activity. During the following days, the tendency in both cases is to diminish but the bacterized plants keep the units of SOD even lower. ~~Our results indicate that this could be the case when comparing the two graft combinations. In the case of the RGCh and RGBer~~

~~combinations where there is an efficient antioxidant system the bacterized plants tend to decrease the enzyme activity compared to the control plants.~~

The highest level of CAT activity was observed in the compatible graft on day 1 after grafting, while the opposite response was observed in the incompatible graft. These results confirm findings of Fernandez-Garcia *et al*. (2004) who reported that ~~in~~ tomato catalase is the enzyme more involved in the cell controlling process of H_2O_2 production that takes place after grafting. In addition, the most noticeable effect of *B. subtilis* was seen on day 1 in the compatible graft where the activity is considerably reduced in respect to the control. On day 28 the CAT activity increases but mainly could be due to the degradation process of the tissues which explains the low activity and maybe less efficient antioxidant system of the control grafts respect the bacterized plants.

It is known that genes encoding for the enzymes like PAL, PPO, and POD are developmentally and tissue-specifically regulated and may be induced by environmental stresses (Pina & Errea, 2008). PAL ~~is~~ has generally recognized as a marker of environmental stress and an important step ~~a potential site for~~in the pathway regulating~~on during~~ the synthesis of flavonoid compounds, xylogenesis, and formation of lignin, one of the main cell wall polymers (Rogers & Campbell, 2004). Pina & Errea (2008) demonstrated for the first time that the enhancement of the level of PAL transcription ~~is enhanced~~ results~~ing~~ in an accumulation of phenol and ~~. Ou~~our observations on~~, described for~~ the two graft combinations, are consistent with the above-mentioned studies. In the case of the bacterized grafted plants enzyme activity is always lower than in the controls suggesting that, in the case of the inoculated plants, the control of the stressful conditions by *B. subtilis* could have reduced the activity of PAL.

POD is also reported as an important antioxidant enzyme involved in stress response by previous studies ~~Nevertheless, in the case of the bacterized grafted plants enzyme activity is always lower. In the case of the inoculated plants, the stressful conditions could have reduced indirectly the activity of PAL.~~

~~POD is reported as a stress enzyme by previous researchers~~ (Has-Schön *et al*., 2005; Rajeswari *et al*., 2008). Assuming that grafting is a relevant stress factor for herbaceous plants, the ~~increasing~~ increase of peroxidase activity following grafting may explain this idea. In our study, it was observed that there was an increas~~inge~~ in POD ~~ed peroxidase~~ activity 15 days after grafting~~after day 15~~. Some researchers also reported that in tomato grafts, peroxidase activity increased day by day after the graft (Fernandez-Garcia *et al*., 2004). Similarly, in another study ~~compared~~ peroxidase activity increase was found in melon ~~at~~ 14 and 24 days after grafting (Aloni *et al.,* 2008). Some researchers suggested that different graft combinations give different reactions to grafting (Feucht *et al*., 1983; Hudina *et al*., 2014; Pina & Errea., 2005). The POD activity in the bacterized plants tends to be lower in the semi-compatible graft, and this response suggests that it may have a radical scavenging effect in this graft combination.

~~PThe POD activity has also been associated with the lignification process (Olson & Varner, 1993; Quiroga *et al*., 2000), the possibility thus that the higher activity in the incompatible grafts may be due to the more active lignification process which might take longer in the incompatible grafts. The POD in the bacterized plants generally tends to be lower the activity of the enzyme except for the compatible graft suggests that it may have a radical scavenging effect.~~

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POD is also ~~considered the~~a catalyzer in the ~~of~~ polyphenol biosynthesis and~~, however~~, together with ~~POD and~~ PPO, ~~enzymes ar~~is~~e~~ responsible for the production of phenolic compounds which contribute to the reinforcement of cell barriers and therefore they confer ~~a~~ resistance against diseases. In addition, they are involved in the ~~stress and~~ wounding stress response (Gainza *et al*., 2015; Saltveit *et al*., 2015). Recent data demonstrate that several biochemical pathways are affected during graft union formation (Koepke & Dhingra, 2013). One of these is the metabolism of phenolic compounds (Mng'omba *et al*., 2008). As expected in a normal wound reaction, an intense production of new phenolic compounds has been reported during the establishment of a graft union (Tiedemann, 1989; Hartmann, Kesler & Geneve, 2002). Phenolic compounds are uncommon in ~~bacteria~~bacteria, but their accumulation is a distinctive characteristic of plant response to stress. Our results show higher total phenol content for the compatible grafts on day 1 even though on the following days the content tends to decrease. This response may be due to the nature of the scion and rootstock itself. The inoculated plants, however, in both cases have a lower phenol content. This could be due to the antioxidant effect of ~~*Bacillus* which takes the plant to a lower stress condition.~~of *B. subtilis* which takes the plant to a lower stress condition.

PPO physiologically has an important role in plant defense and is also involved in the lignification of plant cells. This could explain the peaks that can be observed after 15 days in the RGCh control combination. T~~n while t~~he inoculated plants have higher activity than in the control in the compatible grafts but ~~tend to decrease~~ the activity tend to decrease in the case of the semicompatible grafts and this response could be also related to modulation of the response by *B. subtilis*.

In the present research, the activity was enhanced or reduced depending on the enzyme, the time when~~re~~ the activity was measured, and the graft combination. In general, *B. subtilis* decreased the activity of SOD, CAT, and PAL as well as the quantity of total phenols, on day 1 on the compatible grafts. In the case of the semi compatible grafts, the activity of the PAL, PPO, and the total phenols quantity was decreased. On day 28, CAT, PAL, and PPO showed reduced activity for RGCh but in the case of RGBer, the SOD, CAT, and PAL showed reduced activity as well as the total phenols. Krishna *et al*. in 2011 also tested *B. subtilis* for antioxidant activity by enzymatic and non-enzymatic parameters and changes of antioxidant activity were observed. These results can be considered ~~are~~ implications~~an implication~~ of the same positive effect and suggest~~indicate~~ that inoculated plants were subjected to less stress as compared to non-inoculated plants. Moreover, in the ~~at this~~last stage, the grafted plants are should ~~supposed to~~ have their vascular connections formed, and, therefore, the enzymatic activity could change accordingly to the graft union formation.

Taken together, the above results, showed that the mechanical damage, such as the one caused by wounding in grafted plants, generates ROS~~reactive oxygen species~~. In the present study, the activity of the measured antioxidant enzymes SOD, CAT, PAL, PPO, and POD in the graft union of different graft combinations treated with strain BMB 44 of *B. subtilis* was significantly reduced or increased as compared to control plants (non-inoculated). The most evident effect can be noticed indeed on day 1 where for the SOD, CAT and PAL enzymes the activity was significantly decreased while it was elicited for the PPO and~~, while~~ there was not a significant change in POD.

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This can be attributed to the ability of bacteria to limit producing ROS through modulation of the enzymatic defense system by increasing or decreasing antioxidant enzyme activities according to the physiological stage of the graft and the compatibility level of the graft combination. ~~This can be attributed to the ability of bacteria to limit producing types of active oxygen species through stimulating enzymatic defense system by increasing antioxidant enzyme activity or on the contrary decreasing the antioxidant enzyme activity of the plant suggesting the positive effects that plant growth-promoting bacteria may have depending on the physiological stage of the graft and the compatibility of both the variety and the rootstock. The total soluble phenols and the variation may exist and could be related to the PAL and PPO activity influenced by the presence or absence of *B. subtilis*. However, further research is needed to better clarify this mechanism.~~

In order to better understand ~~the visually~~ the effect of *B. subtilis* a comparative analysis is also proposed (Figure 4)~~. The result is given~~ by calculating the difference of the enzyme activity ~~(~~or total phenols~~)~~ in the inoculated plants minus the enzyme activity ~~(~~or total phenols~~)~~ of the control plants. For the RGCh combination, on days 1 and 15, the inoculated plants present a lower enzyme activity (CAT and SOD) but on day 28 this effect is reverted.

For the RGBer combination the inoculated plants on day, present a greater activity or concentration for CAT, SOD, POD, and total phenols. However, on day 28, only ~~the~~ POD and total phenols. The SOD is characterized by ~~a~~ lower enzyme activity.

In this study, a PCA analyses was also performed ~~in order~~ to analyze which of the measured enzymes was more important and the influence of the total phenols on the inoculated e non-inoculated compatible and semi-compatible grafted plants. In Figure 3, it is possible to observe that all measured variables had a strong effect.

It should be noted that there exists an inverse relationship between CAT, SOD, POD, PPO, and Total phenols and the first component (PC1) (Table S1). This indicates that *B. subtilis* might be affecting the enzyme and total phenols at this stage (15 DAG) which could be important for ~~the~~ graft survival. The dominant variables for PC2 were PAL PPO, SOD, and survivors. However, SOD and PAL are directly related to PC2 while PPO and survivors are inversely related to the second principal component.

The inoculated plants are ~~clearly~~ influenced by *B. subtilis.* As it is shown in Figure 3, if the PC1 is considered, grafted plants form separated clusters, two regarding the inoculated plants and two regarding the control plants. On the contrary, respect to PC2 four clusters can also be observed but ~~in~~with respect to the graft combination: RGCh and RGBer. This confirms that also compatibility confers specific characteristics based on the graft combination.

Conclusions

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Bacillus subtilis, strain BMB 44 was shown, through *in vitro* evaluation, to have high antioxidant capacity. The *in vivo* application of this strain on grafting tomato plants showed its relevant effect on the modulation of enzyme activities and total phenols level ~~*B. subtilis* strain BMB 44 was tested for its effect on tomato grafting by evaluation of its capacity of modulate antioxidant response in colonized plants. The study confirmed its *in vitro* antioxidant capacity. In addition, the studies on *in vivo* effects on grafted plants showed scavenging activity~~ immediately after grafting when there is an outburst of free radicals as well as in the other stages of the graft recovery period when~~re~~ the oxidative stress can be associated ~~to~~ with the reconnection of the vascular tissue~~. In both cases it can be inferred that the enzyme activity and the total phenols in plants changed due to the presence of the bacteria~~. Moreover, it was observed that the capacity of the bacterium~~a~~ ~~have the capacity~~ of lowering or increasing the enzyme activity and total phenols level in plants ~~and that this response also~~ depends on whether graft combination is compatible or semi-compatible.

The results of this study suggest that *B. subtilis*, acting on the modulation of the antioxidant response, may represent a useful tool for mitigation of the adverse effect of grafting enhancing graft success and survival rate.

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