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~~ 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, three different rootstocks were used for tomato (*Solanum lycopersicum* L. cv Rio Grande): [*S. lycopersicum* L. cv. Cherry (Ch)]; eggplant [(*Solanum melanogena* L. (Ber)] and cucumber [(*Cucumis sativus* L.) (Pep)] ~~in order~~ to establish a compatible graft (RGCh), a semicompatible graft (RGBer) and an incompatible graft (RGPep). 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 ~~a period of~~ 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*; in the semicompatible graft RGBer, PPO and PAL decreased their activity after inoculation and for RGPep, inoculation with *B. subtilis* influenced CAT, POD~~,~~ and PAL by decreasing their activities. For all combinations, the quantity of total phenols decreased. These findings, together with the *in vitro* assays performed on *B. subtilis* regarding its scavenging properties, give

indications that *B. subtilis* induce antioxidant mechanisms in grafted plants. Thus, inoculation with this ~~growth~~ 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. It is very important for woody plants but in the last century, grafting has become important in the 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). Among the factors that may influence the success of grafting the time of grafting, the hormonal application, the compatibility of the species (Gainza *et al*., 2015) as well as the mechanical damage, can be determinant. ~~In fact, t~~The latter factor 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 cells to death (Baxter *et al*., 2014). Therefore, the control of tissue damage and, consequently, the success of the grafting may be related to a variation of the activity of enzymes or other non-enzymatic molecules related to the antioxidant metabolism. The enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) can be biochemical markers of ~~the~~ oxidative damage and their higher activity could be a sign of resistance to stress (Gill & Tuteja, 2010; Maksimovic *et al*., 2013). However, there are also other non-proteic substances, such as polyphenols, that are also involved in the scavenging of ROS (Foyer & Noctor, 2013). Phenolic compounds are products of the secondary metabolism of plants. Some enzymes such as polyphenoloxidase (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). Phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO) activities 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~~ growth-promoting rhizobacteria (PGPR)" and include the strains of the genus *Bacillus* (Rodríguez & Fraga, 1999; Sturz and Nowak, 2000; Sudhakar *et al*., 2000).

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; Korpela *et al*., 1997; Amanatidou *et al*., 2000, Lin and Chang, 2000).

It has also been found that *Bacillus* spp. induce antioxidant enzymes, such as SOD, CAT, POD, PPO, PAL, and ~~of~~ phenolic acids (Radhakrishnan *et al*., 2017; Rais *et al.,* 2017). Among these enzymes, *Bacillus subtilis* produces two CATs (Lowen & Switala, 1987) and SOD (Murphy *et al*.,1987) as well as other metabolites (Kaspar *et al*., 2019). ~~Positive~~ The positive impact of *B.*

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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).

This study was focused to define the effects of the PGPR *B. subtilis* on grafting of tomato plants by assessing the enzymatic activity of superoxide SOD, CAT, PAL, POD, PPO, and phenol content. To the aim, a preliminary *in vitro* antioxidant activity of *B. subtilis* was performed and then the scions of a tomato variety~~,~~ were immersed into the bacterial solution of *B. subtilis* and grafted on different rootstocks: tomato var. Cherry (compatible rootstock), eggplant (semicompatible rootstock), and cucumber (incompatible rootstock).

Commented [TA2]: What was your hypothesis of this study?

Materials & Methods

Bacterial growth

Eight strains of *B. subtilis* provided by Biotecnología Microbiana S.A. de C.V. were used. Each strain of *B. subtilis* was cultured in PD broth (Potato Dextrose) at 28 °C for 24 h on an orbital shaker at 150 rpm (preinoculum).

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. *Bacillus* cells of the eight strains were grown in 500 mL Erlenmeyer flasks containing 250 mL PD broth for 24 hours at 28 °C on an orbital shaker at 150 rpm. 1 mL of 10^6 CFU/mL ≈ 0.1 OD $_{535nm}$ of *B. subtilis* supplemented with 0.2, 0.4, 0.6, 0.8, and 1 mM hydrogen peroxide at 28 °C on an orbital shaker at 150 rpm for 24 h. The control treatment consisted ~~on~~ of the growing medium inoculated with *B. subtilis* hydrogen ~~peroxide~~ peroxide-free. Cell growth was measured spectrophotometrically by measuring optical density (OD) at 535 nm every hour.

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 ~~by means of~~using the Fenton reaction, according to Kadaikunnan *et al*. (2015). Briefly, for this purpose, 1 mL of bright green reagent (0.435 mM), 2 mL of FeSO4 (0.5 mM), 1.5 mL of H2O2 (3% w/v) were mixed with different volumes of each strain (0.5, 1.0, 1.5, 2 and 2.5 mL). They were incubated at room temperature for 15 min, and 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, As is the absorbance of the simple, A0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and 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) 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 16218 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 Asample is the absorbance of the sample, Ablank is the absorbance of methanol with bacterial cells and Acontrol is the absorbance of deionized water and DPPH reagent (Brand-Williams *et al*.,1995).

Plant material and grafting

Solanum lycopersicum L. (tomato, var. Rio Grande and Cherry), *Solanum melongena* L. (eggplant), and *Cucumis sativus* L. (cucumber) 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). These plants were germinated in trays containing a mixture of lime, vermiculite, perlite, and Sunshine® Mixture no. 3. After 30, 40, and 25 days of growth, respectively (late spring), plants were grafted as follows: tomato, var. Rio Grande, was grafted on tomato var. cherry (RGCh), eggplant (RGBer), or cucumber (RGPep). 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 and both parts of the grafted plants were held with a silicone grafting clip. 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.

Commented [TA3]: Why were these tomatoes and cumber selected for grafting? Provide any reasons or preliminary resuskts, if any.

~~Inocolum~~ Inoculum

The grafted plants were divided into 2 treatment groups. In the first group, the scion parts were immersed into the bacterial solution of *B. subtilis* strain BMB 44 (10^6 CFU/mL) in 250 mL containers and incubated at room temperature for 15 min. The adopted strain was chosen among those preliminary tested and giving the highest antioxidant response. The second group was immersed in tap water and was used as a control. After treatments, grafting was performed immediately.

Post-graft plant healing

The post-grafting healing was held in containers with a plastic dome, 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, 117umol 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 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.

A randomized 2 (treatments) ~~x~~ × 6 (analyses times) ~~x~~ × 3 (grafting combinations) factorial design with 3 replications in triplicate, was used. Each experiment unit was composed of 30 seedlings. The plant material for analyses ~~were~~ was collected ~~at~~ three times: 1, 15, 28 days after grafting. The samples were represented by 1- 2 mm stem sections of seedlings at the grafting point. After each collection time, samples were frozen in liquid nitrogen and stored at –80 °C for subsequent determination of enzyme activity and total phenol content.

Enzymatic plant analyses

Superoxide dismutase (SOD)

This activity was determined according to Giannopolitis & Ries (1977) with modifications. The activity was determined by the ability of the enzyme to inhibit the reduction of Nitroblue tetrazolium (NBT) in a reaction 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 absorption at 560 nm (xMark ™ BIO-RAD). A unit 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) using the following reaction: The reaction mixture was composed of a solution of 25 mM hydrogen peroxide, 50 mM potassium phosphate buffer 10 µL of the enzyme extract. Readings were made 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)

It 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 hydrogen peroxide 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 ~~were~~ was added. The absorbance was read at 436 nm. The reading of the reaction 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 kinetics of tetraguaiacol. The results were expressed in U/mg protein.

Polyphenol oxidase (PPO)

Commented [TA4]: What experimental design was used?

Commented [TA5]: How many samples?

Commented [TA6]:

The activity of this enzyme was determined according to the protocol described in Mayer, Harel, & Ben-Shaul (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 a buffer, 20 µL of catechol, and 20 µL of the 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 prepared, a buffer Tris-HCl 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. The absorbance was measured in a 300 µL 96-well microplate (Microtiter ™) at 290. The mixture was then incubated at 37 °C in a thermostatic bath for one hour, after this time 100 µL of HCl was added ~~in order~~ 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 transcinnamic acid production and expressed in U/mg protein.

Total phenols

Total phenols were determined according to Mng'omba, du Toit & Akinnifesi (2008) with some modifications. The extraction of phenols was done by grinding in a mortar and with a pestle 0.05 g of tissue with liquid nitrogen and 1 mL of a methanol-acetone-water solution was added (7: 7: 1). The mixtures were centrifuged at 62 G for 4 min.

50 µL of the supernatant ~~were~~ was used and 200 µL of distilled water and 250 µL of Folin-Ciocalteau reagent were added, then they were shaken at 62 g for 3 min. Then 500 µL of a 7.5 % w/v NaCO3 solution ~~were~~ was added, the mixture was vigorously homogenized for 1 min and put to rest for 15 min at 45 °C in a thermostatic shaker. Absorbance was measured at 760 nm. The concentration of phenols was expressed as meq gallic acid/mg protein.

Statistical analysis

The study design was a randomized block with three biological replicates. Each replicate consisted of 30 grafted plants. The data for each combination was evaluated separately by analysis of variance (ANOVA) and significance within treatments was analyzed by Least Significant Differences (LSD) test at $P<0.05$. Data were analyzed using R statistical software.

Results

The bacterial antioxidant activity was studied ~~by means of~~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.

Commented [TA7]: Poor data analysis performed - just uing mean comparisons. It would be better to analyze interactions between enzymes and grafted tomatoes or at different times (days after gratfting), and regression and correlation analyses to explain your results.

In vitro* antioxidant activity of *B. subtilis

Resistance to hydrogen peroxide (H_2O_2)

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

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

The scavenging activity for hydroxyl radicals 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 % ~~of~~ scavenging rate while the lowest percentage was 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 ~~a~~ 100 % ~~of~~ inhibition but even at a lower concentration (0.5 mL), *B. subtilis* showed about 30 % of scavenging activity.

Effect of Bacillus subtilis on grafted plants

The *in vitro* methods used in this study for measuring the antioxidant scavenging activity ~~are~~ is based on the measurement of the variation of enzymatic antioxidant activities of the sample. In detail, the variations of antioxidant enzyme activities were assessed ~~in relation~~ 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~~ Table 1 are reported the activities of SOD and CAT 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 and in the incompatible graft combination RGPep the inoculated plants have also higher activity and the difference in respect to the control plants is of 70 units.

On day 15, the RGCh and RGPep activity in the controls was significantly higher in respect to the inoculated plants (difference of 238 and 29 units, respectively), while in the case of RGBer the bacterized plants showed ~~a~~ higher activity in respect to the non-inoculated plants. It was also observed that in the case of the control grafts, the compatible graft, RGCh, has a difference of 189.4 units in respect to the incompatible one, but this difference in activity was significantly reduced when the same combinations were inoculated presenting a difference of only 17.9 units.

~~At~~ On day 28, an increase of SOD activity was observed in RGCh, while in the semi-compatible graft (RGBer) and ~~in~~ the incompatible graft combination (RGPep) the increase in activity in the inoculated plants in respect to the control was not observed.

The variation in activity of enzyme CAT with the different graft combination ~~are~~ is reported in Table 1. ~~At~~ On day 1 after grafting in the RGCh combination, it can be observed a higher enzyme activity in the non-inoculated plants presenting ~~these~~ 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, and in the case of the non-compatible combination (RGPep), it can be seen, however, that on day 1 the control has a higher activity.

Fifteen days after grafting, in contrast with RGCh and RGPep, the RGBer graft showed ~~a~~ higher activity when inoculated. However, it was observed that within the non-inoculated grafts when comparing the RGCh and RGPep combination the difference in activity was ~~of~~ 20 units but when inoculated the difference for these two grafts was ~~of~~ 3.46 units only.

~~At~~ On day 28 after grafting, ~~a~~ 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. It can be seen, however, that on day 28, the inoculated plants of RGPep present a higher activity (difference of 133 units) 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 ~~on~~ in the other cases there is only a slight tendency to increase ~~of~~ activity of 0.1 and 0.3 units, respectively, on the control plants of RGBer and RGPep.

On day 15 the activity of the non-inoculated grafted plants was higher for the RGBer and RGPep combination, however, the tendency in these plants was to have a high activity for the incompatible graft and ~~a~~ low activity for the semicompatible one, while the bacterized plants presented the highest activity for the compatible graft and the lowest activity for the RGBer combination. On day 28, except for the incompatible combination where no significant difference was observed, 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 2. 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 ~~in~~ with respect to the control. In the RGPep combination, the activity is also significantly higher in the inoculated plants but with a difference of 4 units. 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 inoculated plants showed higher activity ~~in~~ with respect to the control only when comparing the compatible graft. On day 28 the RGCh and RGBer inoculated plants had higher activity while ~~on~~ in the case of RGPep the higher activity was presented in the control plants.

Concerning the enzyme PAL (Table 3), on 1 day after grafting, regardless of the graft combination, ~~a~~ 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 and non-compatible combinations have a difference of 3.8 units and 1.6 units, respectively.

~~At~~ On day 15, the non-inoculated grafted plants presented the highest activity, regardless of the combination, however, the RGPep combination showed the highest activity: 1.97 units with respect to the compatible RGCh graft. 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 incompatible graft, however, the bacterized plants have a difference of 6.4 units more in respect to the control.

In the case of total phenols (Table 3), at 1 day after grafting the non-inoculated plants presented higher content of phenols in the case of the RGCh and RGBer; on the other hand, RGPep has a lower endogenous total level of these compound in non-inoculated plants. On day 15, a greater content of phenols was measured in the controls for the semicompatible (6.15 units) and incompatible (8.50 units) grafts with respect to 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 the RGCh and RGPep grafts were very similar (7.6 units) while the 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. For the RGPep a higher total phenols content (10.1 units/ug of gallic acid) was found in the control than in inoculated plants (8.8 units/ug of gallic acid).

Discussion

In vitro* antioxidant activity of *B. subtilis

Microbial cells have ~~a number of~~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 radical 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). ~~There are several growth promoting bacteria that~~Several growth 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 shown to possess an adaptation mechanism against H_2O_2 and 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 against higher concentrations (10 mM) of this oxidant and ~~a number of~~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_20_2 treatment and it is only after 18 hours that it reaches its plateau. In the same way, our results confirm previous results of Yan *et al*., 2006) showing that *B. subtilis* has the capacity of scavenging H_20_2 radicals presenting a scavenging activity of more than 35 %. In a biological system, ~~there is no enzyme that~~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 directly breakdown H_2O_2 such as CAT or sequestration of transition metal and repression of iron uptake (Hameed & Lee, 2009). In our study, ~~a~~ 37 % ~~of~~ scavenging activity was obtained. In this sense, in the present study when measuring the DPPH antioxidant capacity, the results obtained were similar to other studies (Kadaikunnan *et al*., 2015) Similarly, in our study, even at low concentrations, *B. subtilis*, already scavenges 30 % and at a greater concentration, it is capable of neutralizing 100 % of the radicals. This suggested that the antioxidant properties may help ~~reducing~~ to reduce the level of oxidative stress associated with mechanical injuries created during grafting and ~~during~~ different physiological stages.

Effect of Bacillus 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. Thus, during the different stages, enzymes are differently regulated and the effect that *B. subtilis* may have on this regulation was also studied. ~~A number of~~Many developmental stages can be recognized in the formation of a graft union. The early stage in herbaceous plants begins within 4 d 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.

Several studies have demonstrated the benefits of inoculating plants with PGPR (Bonaterra *et al*., 2003; Vardharajula *et al*., 2011). In plants having an infection of bacterial disease inoculation with *B. subtilis* induced improved conditions (Gajbhiye et al., 2010; Singh et al., 2012) In that study authors found that the plants that had been inoculated with *B. subtilis* presented an increase ~~of~~ in the antioxidant enzymes such as CAT, POD, PPO, PAL, and phenolic acids. In another study, *Bacillus* spp was also assessed to induce an increase in activity of antioxidant enzymes against *Pyricularia orizae* (Rais *et al*., 2017). The application of *Bacillus* enhanced PPO and PAL activity but also changes in SOD and POD were observed. 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 increasing salinity stress but plants treated with PGPR, such as *B. subtilis* and *Arthrobacter*, showed reduced activity of the 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 within incompatible rootstock/scion interfaces either the level of reactive oxygen species is increased or a less efficient detoxification system is initiated here (Aloni *et al*., 2008; Nocito *et al*., 2010).

Our results ~~give an indication~~indicate that this could be the case when comparing the three combinations. In the case of RGPep there is a higher activity ~~at~~ on day 1 however, 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. In the case of the RGCh and RGBer combinations where there is a more 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 which confirms what was reported by Fernandez-Garcia *et al*. (2004), catalase is considered an enzyme involved in the cell defense process against H_2O_2 production that takes place after grafting. The most noticeable effect of *B. subtilis* can be seen, in fact, on day 1 where the activity is considerably reduced. This confirms the antioxidant activity that was observed in the *in vitro* antioxidant tests. The opposite was observed in the incompatible graft. 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 may be a less efficient antioxidant system of the control grafts with respect to 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 generally recognized as a marker of environmental stress and a potential site for pathway regulation 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 level of PAL transcription is enhanced resulting in an accumulation of phenol. Our observations, described for the three graft combinations, are consistent with the abovementioned studies. Nevertheless, in the case of the bacterized grafted plants enzyme activity is always lower except for day 28 of the incompatible graft. 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 stress factor for herbaceous plants, increasing peroxidase activity following grafting may explain this idea. In our study, it was observed that there was an increased peroxidase activity in the incompatible graft after day 15. Some researchers 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 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). It is expected thus that the highest peroxidases activity will be given in a more incompatible graft such as in the case of RGPep after day 1.

The POD activity has also been associated ~~to~~ 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.

POD is considered the catalyzer of polyphenol biosynthesis, however, together POD and PPO enzymes are 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 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 but their accumulation is a distinctive characteristic of plant stress. Our results show ~~in fact~~ a higher total phenol content for the compatible and incompatible grafts on day 1 even though on the following days the content tends to decrease while in the case of the incompatible graft the initial phenol content is lower and then it increases. This response may be due to the nature of the scion and rootstock itself. The inoculated plants, however, in the three 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.

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 at 15 days in the RGCh and RGPep combinations while the inoculated plants have ~~a~~ higher activity in the compatible grafts but tend to decrease the activity in the case of the incompatible grafts.

In the present research, the activity was enhanced or reduced depending on the enzyme, the time where the activity was measured, and the graft combination. In general, *B. subtilis* decreased the activity of SOD, CAT, POD, 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. However, in the case of the incompatible grafts, the CAT and the PAL decreased its activity. 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. As for the RGPep, the SOD, POD decreased their activities as well as the total phenols. ~~In fact,~~ Krishna *et al*. in 2011 also tested *B. subtilis* for antioxidant activity by enzymatic and non-enzymatic parameters and changes of antioxidant activity ~~was~~ were observed. These results are an implication of the same positive effect and indicate that inoculated plants were subjected to less stress as compared to non-inoculated plants. Moreover, at this stage, the grafted plants are 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 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 in day 1 where for the SOD, CAT and PAL enzymes the activity was significantly decreased while it was elicited for the PPO, while there was not a significant change in POD. 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 ~~a~~ plant ~~growth~~ 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.

Conclusions

Bacillus subtilis strain BMB 44 was tested for its antioxidant properties and it was confirmed that *in vitro* it showed antioxidant capacity. Our *in vitro* results confirmed the *in vivo* effect using grafted plants ~~in order~~ to observe the different scavenging activity when there is an outburst of free radicals as well as in the other stages of the recovery period where the oxidative stress can be associated to the reconnection of the vascular tissue. In both cases, it can be inferred that the enzyme activity and the total phenols changed due to the presence of the bacteria. Moreover, the enzyme activity and the total phenols were measured in a ~~time~~ time-lapse and it was observed that the bacteria have the capacity of lowering or increasing the enzyme activity and total phenols but it also depends on whether the graft combination is compatible, semicompatible or incompatible. Further studies are needed to better understand how these enzymes and the total phenols together with the ~~growth~~ growth-promoting factors of these bacteria may influence the grafted plants at a physiological level. However, considering the results of this study suggest that inoculation with *B. subtilis* ~~it~~ may represent a positive approach for enhancing graft success and survival rate.

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