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Antioxidative capacity and electrolyte leakage in healthy versus phytoplasma infected tissues of Euphorbia coerulescens and Orbea gigantean

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Antioxidative Capacity and Electrolyte Leakage in Healthy Versus Phytoplasma Infected Tissues of *Euphorbia coerulescens* and *Orbea gigantea*

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Abstract

The present study reports on reactive oxygen species (ROS), antioxidant enzyme activities, electrolyte leakage and levels of abscisic acid (ABA) in healthy and phytoplasma infected (PI) tissues of *Euphorbia coerulescens* and *Orbea gigantea*. Histochemical staining for ROS indicated that PI tissues possess higher levels of hydrogen peroxide (H_2O_2) rather than superoxide ($O_2^{\cdot-}$) as compared with healthy tissues in both plant species. The results indicated that superoxide dismutase (SOD) is not playing an important role in eliminating O_2- in PI tissues. This was confirmed by a significantly decreased activity of SOD and a non-significant difference in $O_2^{\cdot-}$ content in PI tissues as compared to healthy tissues in both plant species. Peroxidase (POX) activity was significantly decreased while polyphenol oxidase (PPO) significantly increased in PI tissues compared with healthy tissues in both plant species. PI tissues was associated with a significant increase in catalase (CAT) and ascorbate peroxidase (APX) activity in *E. coerulescens* and a significant increase in glutathione reductase (GR) activity in *O. gigantea*. However, other antioxidant enzymes were lower compared with the healthy tissues. In both plant species, electrolyte leakage was significantly increased in PI tissues compared to healthy tissues.ABA level was decreased in PI tissues as compared to healthy tissues.

Keywords

Antioxidant enzymes; Fasciation; *Euphorbia; Orbea;* Phytoplasma

Abbreviations

ABA: Abscisic acid; APX: Ascorbate peroxidase; CAT: Catalase; DAB: 3,3-diaminobenzidine; GR: Glutathione reductase; NBT: Nitro blue tetrazolium; PI: Phytoplasma infected; POX: Peroxidase; PPO: Polyphenol oxidase; ROS: Reactive oxygen species; SOD: Superoxide dismutase

Introduction

Phytoplasmas are plant pathogenic bacteria that belong to the class Mollicutes and are associated with several hundred plant species worldwide [1]. Succulent plants are sensitive to phytoplasma infection and develop characterized symptoms such as irregular growth and fasciation [2]. On account of the artistic appearance and economic value of these plants, they have been cultivated and introduced to other parts of the world as ornamentals. Moreover, phytoplasma infected succulent species are also highly evaluated for their ornamental value as pot plants [2] but, on the other hand, infected plants can be phytoplasma-host and cause infection to many food crops. Phytoplasma are intracellular parasites that have obligate symbiotic relationships with their hosts [3]. It has been reported that phytoplasmas are able to directly interact with host cells via their secreted proteins [4] and, like other plant pathogens, induce host defence responses [5,6].

In our previous study, we identified fasciation phytoplasmas in *Euphorbia coerulescens* (*Euphorbiaceae*; phytoplasma accession number GenBank HG421070) and *Orbea gigantea* (*Asclepiadaceae*; phytoplasma accession number GenBank HG421073). The phylogenetic analysis placed the fasciation phytoplasmas in 16SrII group [2]. The morphological symptoms of phytoplasma infection in *E. coerulescens and O. gigantean* have been described as cristation or fasciation which is characterized by abnormal cohesion of organs, broadening of the shoot apical meristem and flattening of the stem [2]. They also reported that these phytoplasma induced fasciation characteristics were associated with perturbation in the plant hormonal balance. The phytohormone abscisic acid (ABA) plays a regulatory role in many physiological processes in plants. Early study on apical dominance in the tomato by Tucker (1978) suggested that the role of auxin may be one of inducing or maintaining a high level of ABA in the region of bud initiation and that it is this ABA which inhibits full bud development. Like other plant pathogens, phytoplasmas induce host defense responses [5,6]. One of the major host defenses against bacteria is the production of reactive oxygen species (ROS) such as superoxide (O_2-) and its reduced products, such as hydrogen peroxide (H_2O_2). In eukaryotic cells, O_2- is constantly released from aerobic processes such as respiration and photosynthesis. In addition, when host cells are infected with pathogenic bacteria, high levels of ROS production are triggered [7]. O_2- and H_2O_2 can damage DNA, proteins, and lipids, resulting in a toxic effect on the pathogenic bacteria [7]. Various antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) participate in ROS metabolism during the pathogen attack. The development of an antioxidant defense system in plants can protect them against oxidative stress damage by the scavenging of ROS [8,9].

In continuation of our previous study, the aim of this research was to improve the understanding of the physiological mechanisms associated with phytoplasma infection in *Euphorbiaceae* and *Asclepiadaceae*. Therefore, activities of antioxidant enzymes (SOD, POX, PPO, CAT, APX and GR), ROS (H_2O_2 and $O_2^{\cdot-}$), electrolyte leakage and ABA level were determined in healthy versus phytoplasma infected (PI) tissues of *Euphorbia coerulescens* and *Orbea gigantea*.

Materials and Methods

Plant materials

Healthy and PI stem segments of *Euphorbia coerulescens* and

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Orbea gigantea were used in this study. The plants were showing symptoms of phytoplasma infection and the infection was confirmed using nested PCR assay in our previous report by Omar et al. [2]. The plants were purchased from a local nursery and were grown in 20 cm diameter clay pots containing a mixture of clay:sand (1:1, *v/v*). Plants were irrigated once a week during the summer season and once a month during the winter season. The plants were grown under 50% shade net and a compound fertilizer (19:19:19 N:P_2O_5:K_2O) at 1 g L^{-1} was applied once a month.

Histochemical analysis of ROS

Detection of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were visualised as a blue coloration of nitro blue tetrazolium (NBT) and a reddish-brown coloration of 3, 3-diaminobenzidine (DAB), respectively. Cross and longitudinal stem discs were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1% (w/v) NBT (Sigma - Aldrich, Steinheim, Germany) according to Ádám et al. [10] or 0.1% (w/v) DAB (Fluka, Buchs, Switzerland). NBT and DAB treated samples were incubated under daylight for 20 min and 2 hours, respectively and subsequently cleared in 0.15% (w/v) trichloroacetic acid in ethanol: chloroform 4:1 (v/v) for 1 day [11]. Cleared samples were washed with water and placed in 50% glycerol prior to evaluation. Discoloration of stem discs resulted by NBT or DAB staining was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

Preparation of protein extracts

Extracts were prepared as described by Ratkevicius et al. [12]. Between 5 and 30 g of frozen plant biomass was ground to powder in liquid nitrogen using a mortar. A solution containing 100mM potassium phosphate buffer (pH 7) with 5mM 2-mercaptoethanol was added in a ratio of 1g:3 mL. The mixture was filtered through Miracloth paper (Calbiochem) and centrifuged at 13,000 rpm for 10 min at 4°C. To precipitate proteins, the supernatant was transferred to a new tube and 0.5 g per millilitre of ammonium sulphate added; the mixture was vortexed at 400 rpm for 2h at 4°C. The mixture was centrifuged at 13,000 rpm for 30 min at 4°C, and the pellet was re-suspended in 100 mM potassium phosphate buffer (pH 7), containing 2 mM 2-mercaptoethanol and 10% glycerol. Protein extracts were adjusted to a final concentration of 1mg mL^{-1} using the Bradford method and bovine serum albumin as standard [13]. Extracts were stored at -80°C for subsequent enzymatic activity analyses.

Superoxide dismutase assay

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured in a plate reader with modifications to Mishra et al. [14]. 290 µL of a mixture containing 100mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 11 µM cytochrome-c, 11 µM xanthine, and 0.002 Units of xanthine oxidaseto 20 µg of protein extracts. Xanthine oxidase controls produce an increase in the absorbance due to the reduction of cytochrome-c in the range of 0.025 ± 0.005 min^{-1}. Activity of SOD was expressed in units as described by McCord and Fridovich [15].

Peroxidase assay

Peroxidase (POX; EC 1.11.1.7) activity was determined according to procedure proposed by Hammerschmidt et al. [16]. The reaction mixture consisted of 2.9 ml of a 100 mM sodium phosphate buffer (pH 6.0) containing 0.25 % (v/v) guaiacol (2- methoxy phenol) and 100 mM H_2O_2. The reaction was started by adding 100 µl of crude enzyme extract. Changes in absorbance at 470 nm were recorded every 30 sec intervals for 3 min. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh weight.

Polyphenol oxidase assay

Polyphenol oxidase (PPO; EC 1.10.3.1) activity was determined according to the method described by Malik and Singh [17]. The reaction mixture contained 3.0 ml buffered catechol solution (0.01 M), freshly prepared in 0.1 M phosphate buffer (pH 6.0). The reaction was started by adding 100 µl of crude enzyme extract. Changes in the absorbance at 495 nm were recorded every 30 sec intervals for 3 min. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh weight.

Catalase assay

Catalase (CAT; EC 1.11.1.6) activity was determined according to method adjusted from, the activity of CAT was measured by adding 15 µg of protein extracts to 1 mL of 100 mM potassium phosphate buffer (pH 7) containing 16 mM H_2O_2. The decrease of absorbance due to H_2O_2 consumption was followed at 240 nm for 30s, and the activity was calculated using an extinction coefficient of 43.1 M cm^{-1}.

Ascorbate peroxidase assay

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured with modifications to Nakano and Asada [18]. The reaction was started by adding 16 mM H_2O_2 to a 1 mL mixture containing 15 µg of protein extracts and 100 mM potassium phosphate buffer (pH 7) containing 0.5mM ascorbate. The decrease of absorbance at 290 nm due to ascorbate consumption was monitored for 30s, and the activity was calculated with an extinction coefficient of 2.8 mM cm^{-1}.

Glutathione reductase assay

Glutathione reductase (GR; EC 1.6.4.2) activity was adapted to a plate reader from Sen Gupta et al. [19]. 290 µL of 100 mM potassium phosphate buffer (pH 7) containing 0.5 mM oxidized glutathione and 0.15 mM NADPH was added to 10 µg of protein extracts. The decrease in absorbance due to NADPH consumption was measured at 340 nm for 5 min, and the activity was calculated using an extinction coefficient of 6.22 mM cm^{-1}.

Electrolyte leakage

Measurements were carried out as described by Szalai et al. [20] and Whitlow et al. [21] with some modification. Stem discs of healthy and PI tissues were placed individually into 25 mL deionized water (Milli-Q 50, Millipore, Bedford, Mass., USA). Flasks were shaken for 20 hr at ambient temperature to facilitate electrolyte leakage from injured tissues. Initial electrical conductivity measurements were recorded for each vial using an Acromet AR20 electrical conductivity meter (Fisher Scientific, Chicago, Il., USA). Flasks were then immersed in a hot water bath (Fisher Isotemp, Indiana, PA) at 80°C for 1 hr to induce cell rupture. The vials were again placed on the Innova 2100 platform shaker for 20 hr at 21°C, final conductivity was measured for each flask. Electrolyte leakage percentage was calculated as: (initial conductivity/final conductivity) × 100.

Extraction procedure and gas liquid chromatography (GLC) quantification of ABA

Healthy and PI tissues were extracted and the levels of ABA were

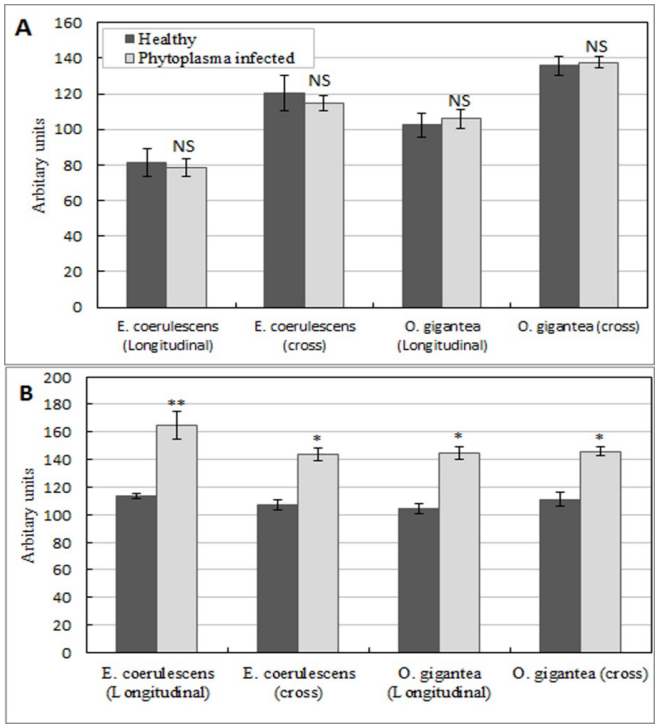

Figure 1: Quantification of reactive oxygen species (ROS) in longitudinal and cross stem discs of *Euphorbia coerulescens* and *Orbea gigantea* A) superoxide ($O_2^{\cdot-}$); B) hydrogen peroxide (H_2O_2).NS, * and ** = non-significantly, significantly different at $P \leq 0.05$and $P \leq 0.01$, respectively, according to Student's unpaired *t*-test. Error bars are ± 1 SD, n = 3).

quantified according to Shindy and Smith (1975) with modifications. For each different sample, 5 g fresh weight of plant tissue was ground and soaked in 80% (v/v) aqueous methanol and allowed to extract for 72 h and then filtered through Whatman no 42 paper. The filter paper and the residue were returned to the flask with a fresh volume of methanol and filtered again. The procedure was repeated once more and the combined extracts were evaporated to the aqueous phase using a rotary evaporator. The aqueous phase was adjusted to pH 2.8 with 1% HCl and extracted three times with ethyl acetate and evaporated to dryness then dissolved in 1 mL of high pressure liquid chromatography (HPLC) grade methanol and used for ABA quantification.The extract was filtered through a membrane filter (0.45 m) before injecting 1 µL into GLC (trace GC ultra, thermo TR-FAME -70% cyanopropylpolysilphenylenesiloxane; FID detector) equipped with capillary column (30 m × 0.25 mm ID × 1 um film). Gases flow rate of N_2, H_2 and air was adjusted to 1.5 mL min^{-1}, 35 mL min^{-1} and 350 mL min^{-1}, respectively. The temperature of injector and detector was adjusted to 350°C and 250°C, respectively.The concentrations of hormones in the samples were calculated from the response ratio of target compound and the appropriate internal ABA Sigma standard – 48880.

Statistical analysis

There were three replicates of both healthy and PI tissues used for each assay. The data were analyzed by Student's unpaired *t*-test, and the mean values were compared at $P \leq 0.05–0.001$.

Results and Discussion

Antioxidative capacity in healthy versus PI tissues of *E. coerulescens and O. gigantea*

Histochemical staining for ROS including $O_2^{\cdot-}$ and H_2O_2were visualised in both longitudinal and cross stem discs of healthy and PI tissues as a blue coloration and brown coloration, respectively. Quantification of discoloration of stem discs resulted by NBT or DAB staining indicated that PI tissues possess high levels of H_2O_2 rather than $O_2^{\cdot-}$ compared to that of healthy tissues in both plant species (Figures 1A and 1B).It has been reported that ROS are produced within the plant host in response to phytoplasma infection [22,23]. Enhanced ROS production in plants is termed 'oxidative burst' [24]. ROS derived from the oxidative burst can directly damage bacteria [25] and can also function as signaling molecules. In plant cells, for example, ROS derived from the stress-induced oxidative burst activate a variety of defense responses including synthesis of phytoalexins, synthesis of pathogenesis-related proteins, and suppression of pathogen growth by programmed cell death [26]. Therefore, it is important for pathogenic bacteria to inactivate ROS by employing antioxidant enzymes for their survival.

Based on our ROS results, we expected up-down regulation of antioxidant enzymes activities especially SOD could be down-regulated. Therefore, we investigated the activities of antioxidant enzymes in *E. coerulescens* and *O. gigantean*. SOD plays a major role in the dismutation and degradation processes of O_2- in ROS detoxification pathways [27]. SOD activity was significantly decreased in PI tissues of both plant species *E.coerulescens* and *O. gigantea* as compared to healthy tissues (Figure 2A). Although SOD is usually considered the first line of defence against oxidative stress [28], the results suggest that SOD is not playing an important role in eliminating O_2- in PI tissues. This was confirmed by a significantly decreased activity of SOD (Figure 2A) and a non-significant difference of superoxide content in PI tissues as compared to healthy tissues (Figure 1A).

H_2O_2 plays central role in the oxidative burst, acting as a signal for the localized death of challenged cells [29] and as a diffusible signal for the induction of cellular protective genes in adjacent healthy cells and tissues [30]. H_2O_2 generated by SOD is further degraded and detoxified by other antioxidant enzymes such as POX, APX or CAT. In *E. coerulescens,* CAT activity was significantly higher in PI tissue as compared to healthy tissue. For *O. gigantea,* CAT in PI tissues was below detection limit (Figure 2B). A significant decreased POX activity while a significant increased PPO activity was observed in PI tissues of *E. coerulescens* and *O. gigantea* as compared with healthy tissues (Figures 2C and 2D). Previous reports demonstrated that POX and PPO may participate in the responding defense reaction by inducing plant resistance against pathogenic agents [31]. An increase in PPO activity in phytoplasma infected *Citrus auarntifolia* has been reported [9].

A significant increase in GR activity was observed in PI tissues of *O. gigantea* (Figure 2E) but no significant difference in APX activity in PI tissue of *O. gigantea* compared to healthy tissue. In contrast for *E. coerulescens*, there were no significant differences in GR activity whilst the activity of APX increased significantly in PI tissue as compared to healthy tissue (Figure 2F). APX is primarily located in both the chloroplasts and cytosol, and as the key enzyme of the glutathione ascorbate pathway, it eliminates peroxides by converting ascorbic acid

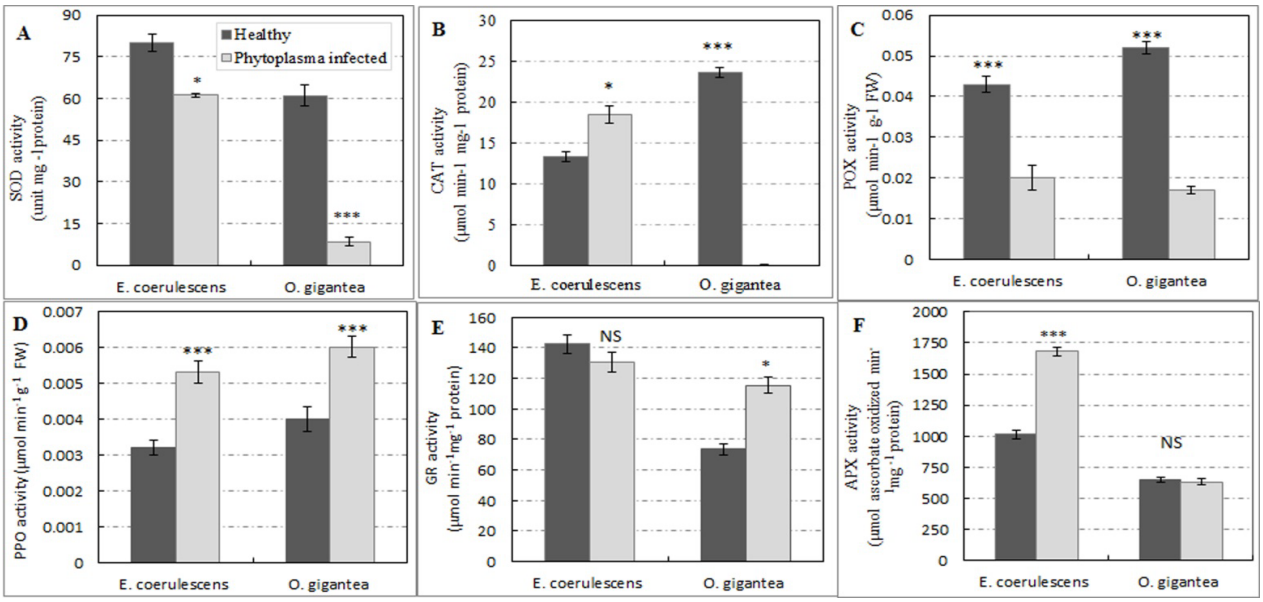

Figure 2: Level of antioxidant enzyme activities in healthy versus phytoplasma infected tissues of *Euphorbia coerulescens* and *Orbea gigantea*. A) superoxide dismutase (SOD); B) catalase (CAT); C) peroxidase (POX); D) polyphenol oxidase (PPO); E) glutathione reductase (GR); F) ascorbate peroxidase (APX).(CAT in fascinated *Orbea gigantea* was below detection limit. NS, * and *** = non-significantly, significantly different at $P \leq 0.05$and $P \leq 0.001$, respectively, according to Student's unpaired *t*-test. Error bars are ± 1 SD, n = 3).

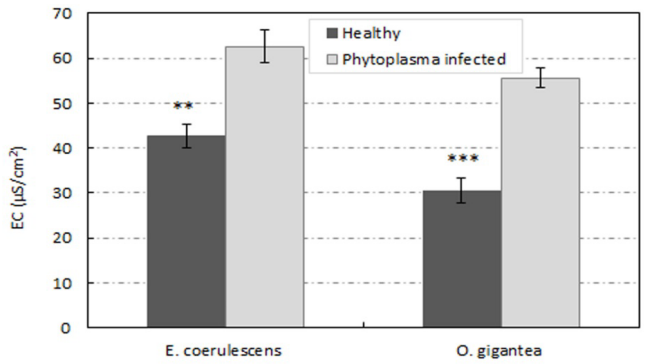

Figure 3: Electrolyte leakage in healthy versus phytoplasma infected tissues of *Euphorbia coerulescens* and *Orbea gigantea* (** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, according to Student's unpaired *t*-test).

to dehydroascorbate [32] and it is one of the most important enzymes playing a crucial role in eliminating toxic H_2O_2 from plant cells [33]. The activities of the two enzymes primarily involved in H_2O_2 scavenging, namely CAT and APX were significantly increased in *E. coerulescens* PI tissues but were below detection or non-significant, respectively, in *O. gigantea.* Therefore, the increased CAT and APX activity observed in *E. coerulescens* might have been due to the increased H_2O_2 production *in planta* (Figure 1B). On the other hand, CAT and APX were significantly decreased in *O. gigantea*. Therefore, scavenging H_2O_2 were dependant on GR and PPO rather than CAT and APX. A decreased CAT activity was also reported in PI potato plants [34]. The role of GR and glutathione in the H_2O_2 scavenging in plant cells has been well established as the Halliwell–Asada pathway [35]. GR is involved in the recycling of reduced glutathione, providing a constant intracellular level of GSH [36], the main cell antioxidant [37-39]. In our opinion, the ROS production (Figure 1) and regulation of antioxidant enzymes activities (Figure 2) in *E. coerulescens* and *O. gigantea* are governed by a defence mechanism by the host plant tissues, which is a species-dependant, and a survival mechanism by the phytoplasma.

Electrolyte leakage in healthy versus PI tissues of *E. coerulescens and O. gigantea*

Electrolyte leakage was significantly increased in PI tissues of *E. coerulescens* and *O. gigantea* as compared with healthy tissues (Figure 3). The cellular membrane dysfunction due to stress is well expressed in increased permeability and leakage of ions out, which can be readily measured by the efflux of electrolytes. One of the most common effects of pathogens on the plant cells is to increase their permeability. Electrolyte leakage has been applied to quantify damages to cell membranes in response to biotic stresses [40,41] as well as abiotic stresses [42-44]. In the present study, phytoplasma infection increased electrolyte leakage in *E. coerulescens* and *O. gigantea* (Figure 3) because it is an obligatory parasite which depends heavily on their host cells for essential metabolic compounds. It has been stated that phytoplasma imports many metabolites from host cells as possible consequences of their life in a nutrient-rich environment [45-48].

ABA level in healthy versus PI tissues of *E. coerulescens and O. gigantea*

In the present study, the phytohormone ABA was significantly decreased in PI tissues of both plant species *E. coerulescens and O. gigantea* as compared to healthy tissues (Figure 4). It was conclude that the release of axillary buds from apical dominance in *Elytrigia repens* does not require IAA content to be reduced, but is associated with reduced ABA content [49]. On the other hand, it is well established

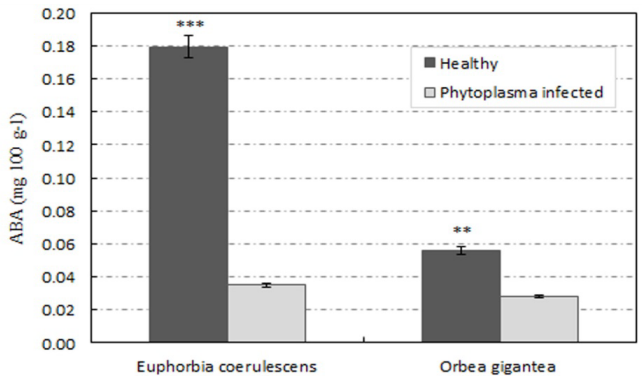

Figure 4: Abscisic acid (ABA) levelinhealthy versus phytoplasma infected tissues of *Euphorbia coerulescens* and *Orbea gigantea* (** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, according to Student's unpaired *t*-test).

that ABA level is increased in response to different biotic and abiotic stresses; therefore, it is considered the plant stress hormone. The extent of ABA production is positively related to the degree of resistance to a given stress factor [50-52]. However these observations alone do not establish that ABA is a necessary intermediary for acquisition of stress tolerance. Our results showed that ABA level was decreased in PI tissues as compared to healthy tissues in both plant species (Fig. 4). This indicating that ABA may not be associated in stress defense mechanism in PI tissues but it is involved in the release of axillary buds representing an "overgrowth" effect due to loss of apical dominance.

Conclusion

The results demonstrated a significantly high content of H_2O_2 while no significant difference of O_2– content of in PI tissues compared to that of healthy tissues in *E. coerulescens* and *O. gigantea*. In both plant species, PI tissues were associated with a significant decrease in SOD and POX as well as a significant increase in PPO. PI tissues of *E. coerulescens* were associated with a significant increase in CAT and APX activities while a significant increase in GR activity was obtained in *O. gigantea* as compared to healthy tissues. PI tissues had a significant increase in electrolyte leakage but a significant decrease in ABA level in both plant species.

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