

## Efficacy of Silicon in Strengthening Antioxidant Defenses Against Fungal Pathogens in Medicinal and Aromatic Plants

K. Chaithanya Shanthi<sup>1</sup>

1\* Telangana Social Welfare Residential Degree College for Women, Department of Botany, Armoor, Telangana University, Dichpally, Nizamabad, Telangana, India – 503322.

[chaithukamtam55@gmail.com](mailto:chaithukamtam55@gmail.com)

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### ABSTRACT

The objective of this work was to investigate the impact of silicon on the regulation of antioxidant defense mechanisms, which in turn provide resistance against different plant infections that attack medicinal plants including *Alloe vera*, *Ocimum tenuiflorum*, *Tinospora cordifolia*, as well as aromatic plants such as *Allium cepa* and *Curcuma*. Initially, we analyzed the overall levels of phenols, flavonoids, tannins, protein, MDA, and H<sub>2</sub>O<sub>2</sub> in all 5 plants that were infected with their specific pathogenic fungus. Subsequently, we assessed the effects of treating the plants with an external chemical spray of Silicon. Moreover, we assessed the activity of antioxidant enzymes in both sick and silicon treated circumstances. The findings of our study showed notable differences in all parameters among plants affected by fungal diseases, healthy plants treated with silicon, and infected plants treated with silicon, in comparison to the control group. The results of our study indicate that the addition of silicon (Si) before to treat fungal-infected medicinal and aromatic plants led to higher levels of antioxidant enzyme activity, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD), compared to control, Si-treated, and pathogenic fungus-inoculated plants. Taken together, this work emphasizes the protective function of silicon in our experimental medicinal and aromatic plants against harmful fungus by enhancing the activity of antioxidant enzymes.

**Keywords:** Antioxidant defenses, aromatic plants, medicinal plants, pathogenic fungi and silicon

### Introduction

Throughout history, India has been acknowledged as a repository of very valuable medicinal and aromatic plant species, which significantly contribute to our nation's natural resources by playing a vital role in providing essential healthcare services to the population. Medicinal and aromatic plants have extensive historical importance in the field of medicine, spanning from the ancient Ayurvedic, Siddha, and Unani medical systems of India to the modern homoeopathic and allopathic systems (Gurib-Fakim, 2006). The increasing need for medicinal and aromatic plants by many processing industries, including nutraceuticals, pharmaceutical, cosmetic, food, etc., has strained natural resources since most species in use are still obtained from their natural habitat (Gupta et al., 2013). The primary pathogens that can infect medicinal and aromatic plants are fungi, bacteria, viruses, insects, phytoplasma, nematodes, and diverse other species. Several of these are notorious for propagating epidemics under advantageous environments. Thus far, the causes and their management have received inadequate attention (Sinha et al., 2002). The medicinal value of many plant parts is decreased as a result of infections (Akhtar et al. 2017; Avan et al. 2021). The human body might be harmed if these diseased plant parts are used as medicine (Chavan and Korekar, 2011). Previous studies indicate that saprophytic and pathogenic fungus are the most probable to inhabit aromatic and therapeutic plants. (Raut et al., 2016). The predominant saprophytic fungi that invade seeds include *Alternaria*, *Cladosporium*, *Rhizopus*, *Mucor*, *Aspergillus*, and *Penicillium*. The pathogenic fungus *Fusarium spp.*, *Lasiodiplodia theobromae*, and *Ustilago coicis* require special attention due to their production of toxins and enzymes that play a critical role in pathogenesis. (Raut et al., 2016; Richardson, 1990; Titatarn et al., 1983). Apart from being continuously produced during normal growth, reactive oxygen species

(ROS) such as superoxide anion and hydrogen peroxide ( $H_2O_2$ ) also have a vital function in plants' responses to pathogen detection. (Heller and Tudzynski, 2011). According to several studies (Thordal-Christensen et al., 1997; Torres et al., 2006; Smirnoff and Arnaud, 2018), Furthermore, the oxidative burst may modify gene expression in addition to functioning as direct lethal agents or growth suppressors of infections. Additionally, it can trigger a hypersensitive reaction, alter cell membranes, synthesise antibacterial substances, and alter overall gene expression. Both suppression of the initial defensive response and scavenging of reactive oxygen species (ROS) accumulate during the early stages of infection can be employed to counteract host-derived ROS, which is essential for fungal virulence. (Heller and Tudzynski, 2011; Komalapriya et al., 2015). In addition, the control of cellular ROS levels is essential for the differentiation of fungal development (Chang et al., 2018; Gessler et al., 2007; Halliwell, 2006; Lushchak, 2011). Enzymatic and non-enzymatic defenses make up the majority of the mechanisms used in response to ROS (Gratao et al., 2005; Mittler et al., 2004; Soares et al., 2019). The non-enzymatic defense involves the production of small soluble molecules that are oxidized by ROS, such as glutathione (GSH), as well as other antioxidant compounds, including phenolics, polysaccharides, tocopherols, flavonoids, carotenoids, glycosides, ergothioneine, and ascorbic acid, which are only found in particular fungal species (Sanchez, 2017). Superoxide dismutases (SOD) and peroxidases including glutathione peroxidase (GPx) and catalase (CAT) are components of the enzymatic system (Apel and Hirt, 2004).

Silicon is a versatile and advantageous element that enhances adaptability to stress and resistance to diseases caused by microorganisms and other living and non-living factors. There exist two distinct categories of plants: silicon accumulators and non-accumulators. Silicic acid is a silicon source that plants metabolize, and when accumulated in higher quantities, it is considered biocompatible. (Tubaa, 2015; Tubana et al. 2016). Numerous studies have shown that Si enhances disease resistance in plants by altering protective signaling pathways (Ghareeb et al. 2011; Reynolds et al. 2016). Plants with various pathogen infections have shoots with higher Si concentrations (Debona et al. (2014), Pereira Domiciano et al. (2015)), plants with diverse pathogen infections have branches that have greater Si contents (Mohaghegh et al. (2011) and Sun et al. (2010).

The present study focuses on five medicinal and aromatic plants that have acquired a diverse array of therapeutic qualities. The medicinal plants *Alloe vera*, *Ocimum tenuiflorum*, and *Tinospora cordifolia*, together with the aromatic plants *Allium cepa* and *Curcuma longa*, are related to fungal illnesses. These diseases can be alleviated by supplementing silicon, which modulates antioxidant defense systems.

### **Material and methods**

#### **Sample collection and study of symptoms**

Diseased samples of medicinal (*Alloe vera*, *Ocimum tenuiflorum*, *Tinospora cordifolia*) and aromatic plants (*Allium cepa* and *Curcuma longa*) leaves were collected randomly from each nursery during the survey of various nurseries, placed into labelled zip lock bags and brought into the laboratory. Each diseased sample was studied firstly with hand lens and then with dissecting microscope to assess the morphological characteristics of the disease.

#### **Isolation and purification of pathogen from diseased leaves**

Diseased samples were washed thoroughly with running tap water to remove the surface contaminants and cut into small pieces using sterile scalpel blades. These small pieces were then surface sterilized with 2% sodium hypochlorite solution ( $NaOCl$ ) for 2 mins and then washed three-four times in sterile distilled water. These surface sterilized pieces were then placed between blotting papers and aseptically inoculated onto petridishes containing Potato Dextrose Agar (PDA) media. The plates were incubated at  $25 \pm 2$  °C for 5 to 6 days and the growth of fungal colonies was recorded regularly every day.

#### **Biochemical analysis**

The infested and control leaves harvested were first cleaned with sterilized distilled water and then were dried in folds of sterilized blotting paper. Leaves were cut into small pieces and hot air oven dried at  $48-50$  °C. Three set of replicates for each treatment and control was harvested and each set having three plants. The dried leaves were grinded to fine powder and were stored at room temperature in airtight containers. The powdered leaf samples were subjected to various sample preparation for biochemical estimations. All the experiments were carried out in triplicates.

#### **Quantification of $H_2O_2$**

$H_2O_2$  quantification was done to see the effect of the pathogen and metabolites on a tomato plant. For this 0.1g of leaf sample was taken from each of the treatment was homogenized in an ice bath with 2.0 ml of 0.1% (w/v) of

TCA. The homogenate was centrifuged at 12,000g for 15 min and 0.5 ml of the supernatant was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide solution and incubated for 5 min. The oxidation product formed was measured at 390nm (Velikova, 2000). The amount of  $H_2O_2$  formed was determined from the standard curve made with known concentrations of  $H_2O_2$  and expressed as nmol  $H_2O_2$  g<sup>-1</sup> fresh weight (FW).

#### **Lipid Peroxidation**

MDA content reveals the amount of total lipid peroxidation and was determined by the thio barbituric acid (TBA) reaction. The assay was carried out by the method described by Ohkawa et al. (1979). A leaf sample (0.1g) from each of the treatment was homogenized and incubated with 2.0ml of 20%TCA (w/v) containing 1% TBA (w/v) for 30 min at 95°C. The reaction was stopped by placing the samples on ice for 10min and then centrifuged the samples at 10,000 rpm for 15 min. Reaction product absorbance was measured at 532nm and the amount of MDA was expressed as mmol MDA g<sup>-1</sup> fresh weight.

#### **Determination of Total Protein Content**

Total protein content was determined spectrophotometrically using bovine serum albumin, BSA (Sigma Aldrich co, USA) as standard at 640nm according to the Lowry's method (1951). Data were expressed as  $\mu$ mol per g FW.

#### **Determination of Antioxidant Enzyme Assays**

For enzyme assays, 100mg of leaf sample from control and treated plants were weighed and thoroughly ground to a paste in liquid nitrogen in a mortar and pestle and then transferred to 1ml of ice cold extraction buffer (100mM potassium phosphate buffer pH 7.0, 1mM EDTA). The homogenate was filtered using muslin cloth and centrifuged at 5,000 rpm for 15 min and the collected supernatant was used to analyze activities of SOD, CAT, APX, GR, POD and POX.

In all the enzyme assays, the quantity of total soluble protein concentration was determined using bovine serum albumin, BSA (Sigma Aldrich co, USA) as standard at 640nm according to the Lowry's method (1951). Tissue samples (100 mg each) were ground in ice cold potassium phosphate buffer (10 mM, pH 6.8), then centrifuged at 15 000  $\times$  g for 20 min. The supernatant was used to determine soluble protein content. Obtained values were expressed as mg per g fresh weight (FW).

**Superoxide dismutase (SOD)** (EC 1.15.1.1) activity was monitored as per the method of Beyer and Fridovich (1987). The leaf samples weighing 100mg were homogenized in 1 ml of ice-cold 100 mM Na-phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone, and 0.5% (v/v) Triton X-100. The reaction was initiated with the photochemical reduction of nitro blue tetrazolium (NBT) and a unit of SOD activity was expressed as the amount of enzyme consumed resulting in 50% inhibition of NBT as monitored at 560 nm.

**Catalase activity (CAT)** (1.11.1.6) was measured as per Aebi et al. 1984, wherein, decrease in  $H_2O_2$  was monitored at 240nm absorbance and quantified by its molar extinction coefficient ( $36\text{ M}^{-1}\text{cm}^{-1}$ ). The activity of enzyme was expressed as micro moles of  $H_2O_2$  decreased  $\text{min}^{-1}\text{mg}^{-1}$  protein.

**Ascorbate Peroxidase (APX)**, (1.11.1.1) activity was determined according to Nakano and Asada (1981) at an absorbance of 290 nm and quantified by its molar extinction coefficient ( $2.8\text{ mM}^{-1}\text{cm}^{-1}$ ). The activity of enzyme was expressed as micro moles of AsA decreased  $\text{min}^{-1}\text{mg}^{-1}$  protein.

**Glutathione Reductase (GR)**, (1.6.4.2) activity was measured according to Foyer and Halliwell (1976) at an absorbance of 340 nm and was calculated using the extinction coefficient of NADPH ( $6.2\text{ mM}^{-1}\text{cm}^{-1}$ ). One unit of GR was defined as 1 m mol  $\text{ml}^{-1}$  GSSG reduced  $\text{min}^{-1}$ .

**Polyphenol oxidase (PPO)** activity was determined by following the method of Zauberman et al. 1991.

**Peroxidase (POD)**, activity was assayed by using 3 ml of 0.05 M guaiacol, 20  $\mu$ l of enzyme extract and 0.1 ml of 0.8 M  $H_2O_2$ . The reaction was initiated by adding  $H_2O_2$  and rate of change in absorbance was recorded at 470 nm (Shannon et al., 1966).

#### **PAL Activity:**

It was assayed spectrophotometrically by measuring the amount of transcinnamic acid at 290 nm for PAL using double beam UV-Vis spectrophotometer. The 3 ml reaction mixture consisted of either 6  $\mu$ mol of L-phenylalanine (for PAL), 500  $\mu$ mol of Tris-HCl buffer (pH 8.0), and 100  $\mu$ l of enzyme. Reaction mixtures were incubated for 90 min at 37 °C and reaction was stopped by the addition of 0.05 ml 5N HCl (Beaudoin-Eagan and Thorpe 1985). Protein content of each enzyme extract was estimated by the method of Lowry et al. (1951). Standard curve of cinnamic acid was prepared to express enzyme activity as  $\mu$ M cinnamic acid h<sup>-1</sup> g<sup>-1</sup> protein

for PAL activity, respectively. A one-way ANOVA analysis of the gene expression level of the samples at different days after inoculation was performed using the software SigmaPlot 12. The individual treatment means were compared using the LSD (least significance difference) test.

#### **Silicon supplementation**

Prior to *fungal* infection, plants were supplemented with Si in the form of potassium silicate (2 mM) as described by Ouellette et al. (2017) and control plants were treated with potassium chloride (2 mM) solution.

#### **Statistical Analysis**

The data are mean values ( $\pm$  SE) of experiments performed in 3 replicates at different time points which were subjected to further statistical analysis by one way ANOVA (Holm-Sidak method) using SigmaPlot Version 14.0.

### **Results**

#### **Effect of Si ROS and Lipid peroxidation levels**

In the present work, we initially investigated the levels of reactive oxygen species (ROS) as indicated by the presence of hydrogen peroxide ( $H_2O_2$ ) in five distinct plants after infection with their respective pathogenic fungus. The findings of our study indicate a significant increase in  $H_2O_2$  levels following infection with fungal diseases in all assessed plants. This rise in the  $H_2O_2$  concentration was seen to be greatest in *O. tenuiflorum* and lowest in *C. longa* after 48 hours of infection by each respective pathogen. Application of Si (2 mM) treatment to all the samples examined led to a significant reduction in the  $H_2O_2$  level. The reduction in  $H_2O_2$  levels following Si treatment was particularly significant in *O. tenuiflorum* and least significant in *C. longa* after 48 hours of infection (Fig. 1A).

Analogous to  $H_2O_2$ , the oxidative damage of the cell membranes was assessed indirectly by quantifying the malondialdehyde (MDA) concentration. In this work, we quantified the lipid peroxidation in the leaves of all 5 plant species and found notably increased levels of MDA after fungal infection, as compared to the control plants (Fig. 3B). Maximum increase in MDA concentration was documented in *A. cepa*, whereas the lowest accumulation of MDA content was seen in *A. vera*. Additional silicon (2 mM) supplementation to all examined samples led to a significant reduction in the MDA concentration. The greatest decrease in MDA content was seen in *C. longa* and the smallest in *O. tenuiflorum* after treatment with silicon.

#### **Antioxidant enzyme activities**

The objective of this work was to investigate the activity of PAL in infected, Si treated, infected plant treated with Si, and control plants after 45 hours. Infection with the pathogen caused a significant decrease in PAL activity in *C. longa*, while no reduction was seen in *T. cordifolia* (Fig. 3A). The use of silicon treatment significantly increased the levels of PAL activity in *A. vera* after 48 hours of infection, but no significant increase was seen in *A. cepa*.

Furthermore, we examined the antioxidant activities of infected, Si-treated, infected plant treated with Si, and control plants after 48 hours. Our observations revealed a significant increase in the activity of PPO and POD in *C. longa*, but it was minimal in *O. tenuiflorum* after 48 hours (Fig 3B & 3C). Applying silicon treatment resulted in a significant increase in PPO activity in *C. longa* and showed the lowest level of activity in *A. vera*.

The highest SOD activity was seen in *O. tenuiflorum* and the lowest in *C. longa* 72 hours after infection (Fig 4A). Silicon supplementation greatly increased superoxide dismutase (SOD) activity in *Aloe vera* and somewhat increased it in *Triticum cordifolia*. The activity of catalase was found to be highest in *A. cepa* and lowest in *T. cordifolia* after 48 hours of infection (Fig 4B). Induction of catalase activity was found to be maximal in *A. vera* following the Si treatment, but minimal in *T. cordifolia*.

Following 48 hours of infection, the activity of APX was quantified and found to be high in *A. vera* and lowest in *O. tenuiflorum* (Fig 4C). In *A. cepa*, the APX activity was most boosted by Si treatment, whereas in *A. vera*, the increase was relatively minimal. After 48 hours of pathogen infection, the genetic resistance activity was found to be significant in *C. longa* and minimal in *A. vera* (Fig 4D). Stimulation of GR activity was shown to be maximal in *A. cepa* following Si treatment, while *O. tenuiflorum* showed a very minimal increase.

### **Discussion**

Recent evidences have clearly illustrated the multifaceted role of Si in plants by modulating several biochemical, physiological and molecular adaptation processes for their survival under stress conditions. Several evidences demonstrated that the supplementation of Si to fungal pathogen infected plants led to decreased severity to various fungal diseases (Fortunato et al. 2015; Rodrigues et al. 2015). Interestingly, in susceptible crop plants, Si supplementation enhanced disease resistance to the level of cultivars with race-specific resistance (Resende et al.

2012b; Seebold et al. 2004). Hence, present study was carried out to further investigate the positive effect of Si on improving fungal disease resistance in selected medicinal and aromatic plants.

Reactive oxygen species are byproducts of the typical cellular metabolic activities that are produced by plants (Tripathy and Oelmüller 2012). Although plants generate higher levels of ROS under stressful situations, such as superoxide anion, H<sub>2</sub>O<sub>2</sub>, and hydroxyl radical, these ROS severely impede plant growth, development, and productivity, mostly through oxidative stress (Thannickal and Fanburg 2000; Sies et al. 2017). It's interesting to note that plants have a well-developed defense system for neutralizing ROS, which includes enzymatic and non-enzymatic antioxidants to maintain ROS-homeostasis, guard against cellular toxicity, and fine-tune ROS-mediated signal transduction (Gong et al. 2005; Gechev et al. 2006; Shi et al. 2014). Si is known to regulate the activity of antioxidant enzymes under stress, which makes it a crucial element for mitigating the detrimental effects of ROS in crop plants during stress conditions.

The effect of Si on antioxidant enzymes in medicinal and aromatic plants following fungal pathogen infection is the topic of the present investigation. The antioxidant enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), GR (EC 1.6.4.2), and POD (EC 1.11.1.7) are significantly more active in fungal-infected medicinal and aromatic plants when Si is supplemented, according to our research. Contrarily, medicinal and aromatic plants that have not received Si treatment and are infected by fungi have lower levels of antioxidant enzymes, which may exacerbate the cell damage brought on by oxidative stress. Our findings show that Si is essential for reducing the adverse effects of oxidative stress induced by fungus infection in medicinal and aromatic plants. Similar observations of elevated antioxidant enzymes were made during the rust, sheath blight, and powdery mildew diseases in several crops (Zhang et al., 2006), as well as other diseases such powdery mildew (Guoqiang wei, 2004). Al-Huqail et al. 2019, Al-aghabary et al. 2005, Shi et al. 2014, and Shi et al. 2016 showed that si treatment boosts SOD, CAT, APX, GR, and POD activities, inhibiting the generation of hydroxyl radicals. Another study found that the application of Si boosted the activity of SOD, GPX, and APX in cucumber (Zhu et al. 2004), SOD, CAT, and POD in turfgrass (He et al. 2010), and SOD, GR, and CAT in peas (Tripathi et al. 2015).

Our findings and earlier research suggesting Si enhances the levels of antioxidant enzymes in plants indicate towards a potential function of Si in helping to mitigate the adverse consequences of stress (Liang et al. 2003; Shekari et al. 2017). When given to the plants during stressful conditions, si supplementation lowers the levels of oxidative stress indicators such malondialdehyde (MDA) and hydrogen peroxides (H<sub>2</sub>O<sub>2</sub>). According to reports, supplementing plants with Si alters the activity of antioxidant enzymes, which is mostly dependent on the type of stress (Cooke and Leishman 2016). Comparably, in this research, the addition of Si considerably reduces the negative effects of various reactive oxygen species (ROS) during fungal infection in aromatic and medicinal plants by enhancing the activity of an array of antioxidant enzymes.

### **Conclusions:**

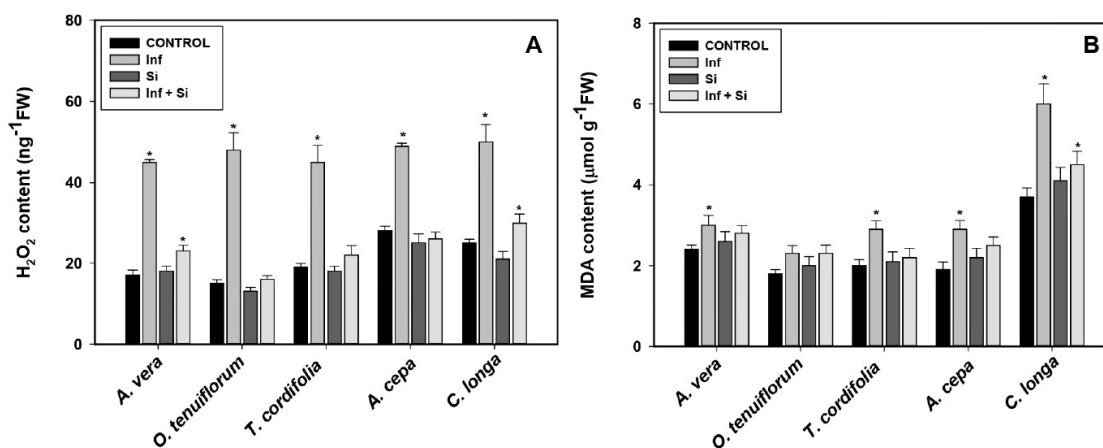
The current investigation aimed to examine the antioxidant defense status of five distinct plants against their specific pathogenic fungus and assess how this status might be improved by the addition of silicon. The results of our study indicate that the addition of silicon promoted pathogenic resistance through the activation of antioxidant defense mechanisms. According to our understanding, this is the first study of its nature that explores the involvement of silicon in regulating the antioxidant activity of several medicinal and aromatic plants against harmful fungus.

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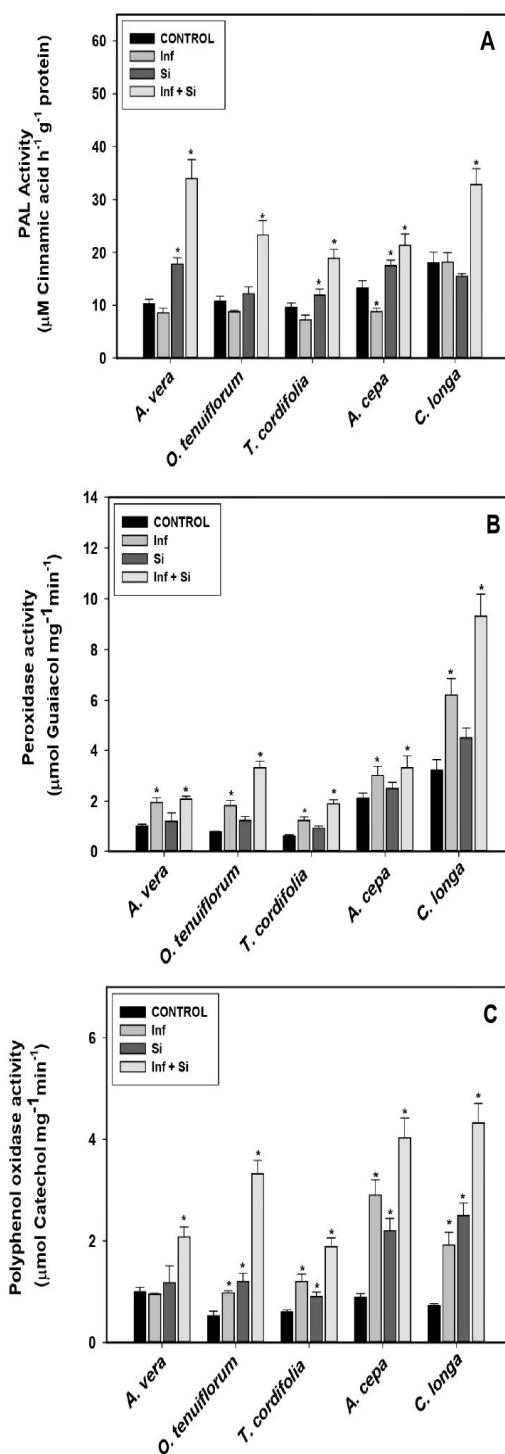
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## Figures

**Fig. 1.** Effect of Si (2 mM) on the levels of H<sub>2</sub>O<sub>2</sub> (A) and MDA (B) in different medicinal and aromatic plants from control tissue and tissue inoculated with their respective fungal pathogens after 72 hours of inoculation,

(DOI). The data represented are the mean of three replicates with standard error.



**Fig. 2.** Effect of Si (2 mM) on the activity of PAL (A), PPO (B) and POD (C) in different medicinal and aromatic plants from control tissue and tissue inoculated with their respective fungal pathogens after 72 hours of inoculation, (DOI). The data represented are the mean of three replicates with standard error.



**Fig. 3.** Effect of Si (2 mM) on the activity of SOD (A), CAT (B), APX (C) and GR (D) in different medicinal and aromatic plants from control tissue and tissue inoculated with their respective fungal pathogens after 72 hours of inoculation, (DOI). The data represented are the mean of three replicates with standard error.

