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Quantitative PCR (qPCR) Data Analysis of SOD and its Activity and Expression in *Elettaria cardamomum* under Biotic Stress

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The aim of the study was to compare the relative gene expression of Superoxide dismutase (SOD) in tolerant and susceptible cardamom plants under shoot borer attack. The difference in the SOD gene expression was supported by measuring the activity of the enzyme. Relative quantification of SOD gene was done in tolerant and susceptible plants of cardamom by qPCR. The variation of SOD activity was determined in methyl jasmonate (MeJa) treated and untreated plants by spectrophotometric and in gel assays. The free radical scavenging ability of tolerant plants was measured by DPPH assay. The quantitative gene expression of SOD was higher in tolerant plants than the susceptible ones indicating its resistant nature. The MeJa treated plants showed two fold increase in SOD gene expression, compared to untreated plants revealing the effect of MeJa in inducing resistance. The variation noticed in the qPCR analysis was reflected in the assay data of SOD in susceptible and tolerant plants as well s MeJa treated and untreated plants. The higher rate of free radical scavenging quality of tolerant plants than susceptible obviously supports the active phase of defense mechanism in tolerant plants. qPCR analysis and the assay data of SOD clearly supports the defense mechanism of SOD against biotic attack.

Key words: Real-time PCR; Methyl jasmonate; Defense mechanism; Relative expression.

Superoxide dismutase (SOD) is the key enzyme in defense mechanism of plants because of its antioxidant properties during infestation[1-2]. Recent reports on the physiological correlation of superoxide dismutase (SOD) and stress tolerance have shown that the up-regulation of SOD levels enhance the stress defense potential in plants[3-4]. Earlier research studies in the pathophysiology of cardamom have been confined to the field management practices for controlling them. Cardamom (*Elettaria cardamomum;* Family: *Zingeberaceae*) is a perennial herbaceous spice of tropical country that has been identified as a plantation crop for more than two centuries. It is a crop adapted under forest cover and the possibilities of infestation by various pests has become more pronounced. The tissue injuries and wounds caused by these pests will lead to other microbial infections as a consequence. The serious pest problems of cardamom growth are the borers (*Conogethes punctiferalis*) and thrips (*Sciothrips cardamomi*), that affects the leaves, stem and capsules. Even though integrated pest management (IPM) programs have put more effort in controlling these infestations, it remains as a consistent threat for cardamom growth and development in the country. Despite the wide popularity of cardamom as the queen of spices, investigations evaluating the antioxidant capacity and the associated enzymatic pathways in the plant during pest attacks have not been done satisfactorily for designing an appropriate control measure. Hence, in the present

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study, an attempt was made to quantify the activity and expression of SOD in tolerant and susceptible plants of cardamom by biochemical and qPCR analysis. The effect of MeJa on SOD was measured on treated and untreated cardamom plants.

MATERIALS AND METHODS

Plant Material

Cardamom (*Elettaria cardamomum* L.) plants susceptible to thrips and shoot borer attack were identified from the experimental plantations of Indian Cardamom Research Institute (ICRI), Idukki, Kerala, India. Phenotypically healthy plants with better yield were selected from the infected area and they are marked as tolerant. Susceptible plants were selected based on their poor growth, low yield and other pathophysiological symptoms. A random sampling was performed for collecting leaf samples based on morphological features. The juvenile leaf samples were kept in chilled condition for enzyme assay and the analytical studies.

Free Radical Scavenging Potency

Free radical scavenging potency was measured by DPPH (di-phenyl-2,4,6-trinitrophenyl-iminoazanium) assay. Methanolic extract was prepared from the leaf samples by refluxing it for 10 min and 50 µL of methanolic extract was added to 3 mL of DPPH dye. Initial reading was taken and final absorbance was measured after 30 min at 517 nm. Rate of DPPH scavenging was measured by percentage inhibition[5-6].

Isolation and assay of superoxide dismutase (SOD, EC 1.15.1.1)

The superoxide dismutase (SOD) was extracted and assayed following the method of Smitha *et.al.,* [7]. Fresh leaf tissues of *Elettaria cardamomum* L. plants were homogenized in cold extraction buffer containing 2.6 mM potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was taken as the source of enzyme. SOD activity was measured as the inhibition of the rate of reduction of cytochrome C by the superoxide radical observed at 550 nm. An aliquot of the sample was assayed in a reaction cocktail containing 2.6 mM extraction buffer, 10.7 mM EDTA, 1.1 mM cytochrome C solution, 0.108 mM xanthine solution (total 3 mL volume) at pH 7.8 at 25 °C. Two tests were carried out during the assay. The uninhibited test was done without adding the enzyme source (without SOD), the assay mixture contained 2.8 mL of reaction cocktail, 0.1 mL of XOD (Xanthine oxidase) and 0.1 mL of deionized water. This was followed by the inhibited test where the assay mixture containing 2.8 mL of reaction cocktail, 0.1 mL of XOD and 0.1 mL of the sample and recorded the increase in A550 nm for 5 min. The $A550nm.min^{-1}$ was obtained by using the maximum linear rate for both the uninhibited and inhibited tests. A blank was maintained without adding XOD and sample. SOD activity was measured as per the standard calculation.

In Gel Assay of Superoxide Dismutase (SOD) and Preparation of Zymogram

The freshly prepared SOD extract was analyzed by native PAGE using 12.5% gel. After the electrophoretic run at 4 ºC, the gel was soaked in a solution of 0.2% nitro blue tetrazolium, 0.028 M N,N,N,N tetra methyl ethylene diamine (TEMED) and 25 µM riboflavin in 50 mM potassium phosphate buffer (pH 7.8) for 30 min in dark at 37 ºC . The gel was then illuminated until achromatic zone was visible and documented in gel documentation system[8].

Application of Methyl Jasmonate (MeJa) on Cardamom Plants

Methyl Jasmonate was applied on susceptible cardamom plants at a concentration of 50 mM with 0.1% (v/v) tween 20 [9]. The fresh leaf tissues from cardamom plants before and after treatment were assayed for SOD enzyme activity at a time interval of 24 h, 48 h, 72 h, 96 h and 120 h respectively.

Real-time PCR

RNA was isolated from the juvenile leaf samples of tolerant and susceptible plants using GTC method. A pool of 25 plants was used for the study from each category. In the case of MeJa treatment, RNA was isolated from cardamom plants before and after MeJa treatment. The primers for the qPCR study were designed for a size of 150 bp by eprime software (eppendorf) – FP -5'aacaatggtgaaggctgttgctgt 3'; RP- 5'agtgcatgca catggaacccatg 3', from the partial gene sequence of CuZn SOD (GQ925541). 4 µg of total RNA was used for RT reaction using AMVRT enzyme. Real time quantitative PCR was performed in a 20 µl PCR mixture containing 150 nM of each primer, 10 µl of 2X SYBR green master mix containing

dATPs, dTTPs, dGTPs and dCTPs and 1.5 mM $MgCl_2$. For the generation of standard curve for SOD gene and 18S rRNA, the cDNA was serially diluted to a concentration ranging from 200 ng to 0.02 ng. The reactions were run on realplex using the following programme: 10 minutes of 95ºC, followed by 40 cycles of 15 seconds at 95ºC, 15 seconds at 60ºC and 45 seconds at 72ºC. The PCR efficiency curve was calculated according to the equation $E=10^{-1/slope}-1$. Quantification was performed by interpolating a standard regression curve of Ct values generated from the samples of known concentrations. The curve analysis was performed using realplex software. The expressions of SOD in 25 samples were determined from the efficiency curve of the normalized PCR assay by using calibrator. The normalized relative quantity for each gene was calculated using the equation $2^{-\Delta\Delta Ct}$ method [10].

Statistical analyses

The significance of variation in tolerant and susceptible means was assessed using paired student's t-test at $P \leq 0.05$. Data were also analyzed by one-way analysis of variance (ANOVA). All statistical analyses were done with graphpad software (Graphpad software, inc. USA).

RESULTS AND DISCUSSION

Free radicals scavenging potency

Figure 1 demonstrates the free radical scavenging potency of tolerant and susceptible cardamom plants. The scavenging potency was found in a higher level in tolerant cardamom plants than the susceptible ones. A sound reduction of scavenging potency noticed in the susceptible plants indicates the lack of innate ability of the plant in removing the free radicals accumulated in leaf tissues during infection as well as the suppression of resistance. The variation observed in the scavenging potency of free radicals in plants during pathological conditions indicates the intensity of infection[11].

Superoxide dismutase (SOD) activity

Figure 2 displays the comparison of SOD activity in cardamom plants collected from the infected area. The plants are grouped into tolerant and susceptible based on the phenotypic features. It could be seen that the leaf samples of susceptible plants showed a lower pace of activity than the tolerant samples. The depletion observed in the activity of SOD in susceptible cardamom plants indicates the down regulation of defense mechanism. Being an enzyme actively involved in the defense against oxidative damage in diseased plants, SOD has been considered a good candidate of pathological research for decades[11].

In-Gel SOD activity

The difference noticed in the level of activity between tolerant and susceptible plants were further checked by an in-gel assay of SOD enzyme in the presence of riboflavin and NBT. Figure 3 demonstrates the zymogram of SOD showing its in-gel activity in tolerant and susceptible cardamom plants. The intensity of white band was more in tolerant plant than the susceptible indicates the higher activity of SOD in tolerant plants.

Effect of methyl jasmonate (MeJa)

As a naturally occurring compound that has a pertinent role in plant growth and development by inducing disease resistance with respect to environmental stresses, the importance of methyl jasmonate is unquestionable[12-13]. Treated plants showed an increase in SOD activity at the initial phase with a gradual decline from the third day onwards (Figure 4). But the activity at the fifth day was found higher than the control plants. It has been reported that MeJa treatment played a positive role in the signaling pathway and induced the defense response during infection[14]. Hence the activation of SOD enzyme in MeJa treated plants further supports the previous reports.

Relative expression of SOD gene

For the quantitative gene expression, the efficiency of the PCR reaction was checked by using the reference gene 18S rRNA in different dilutions. The standard curve was drawn for 18S rRNA and SOD gene based on the concentrations of cDNA (Fig. 5). The relative expression of

Table 1. Relative expression of SOD gene in susceptible and tolerant plants

S. No	Tolerant Plants	Susceptible Plants
1.	1.05 ± 0.015	0.136 ± 0.003
2.	1.25 ± 0.056	0.115 ± 0.002
3.	1.49 ± 0.035	0.125 ± 0.004
4.	1.10 ± 0.015	0.335 ± 0.007
5.	1.22 ± 0.025	0.848 ± 0.022

SOD gene in tolerant and susceptible cardamom plants was analyzed. The values are the mean with standard deviation. Tolerant plants showed higher level of expression compared to susceptible plants, which inturn supports the biochemical data (Table 1). The activity of SOD in MeJa treated plants was

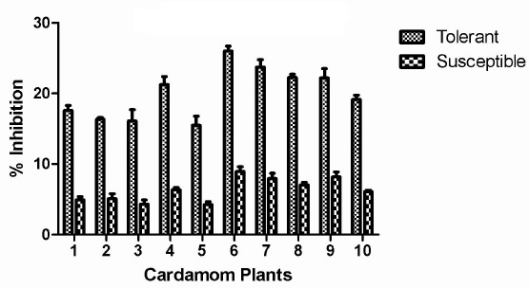

Fig. 1. Free radical scavenging potency in tolerant and susceptible plants measured by DPPH assay. Methanolic extracts of both tolerant and susceptible plants were taken and percentage inhibition of DPPH was calculated

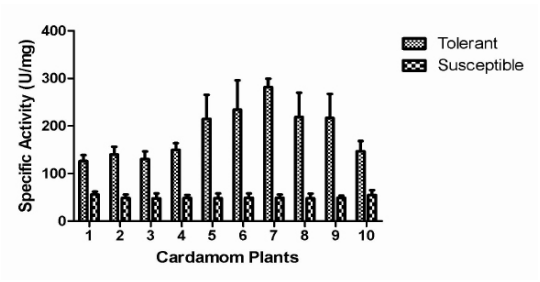

Fig. 2. SOD activity was measured by xanthine method. A significant increase in SOD activity was noticed in tolerant plants when compared to the susceptible ones

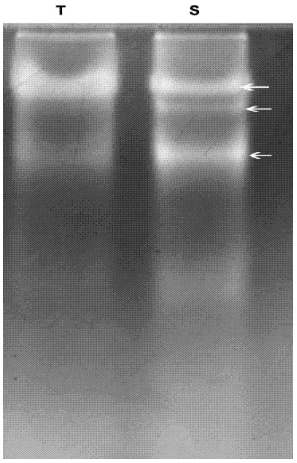

Fig. 3. Zymogram of SOD by In-Gel assay. Achromatic bands were visible in the non-reduced regions due to the action of SOD (T-tolerant, S- susceptible, the arrows (←) indicate iso-enzymes of SOD)

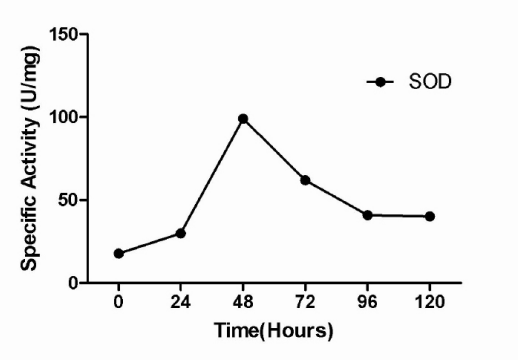

Fig. 4. Specific activity of SOD in cardamom plants showing the induced effect of MeJa elicitor. The SOD activity reached its peak level after 48hrs of MeJa treatment

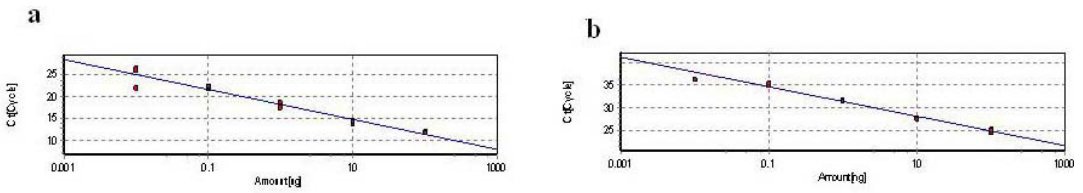

Fig. 5. Standard curve of the house keeping gene 18S rRNA (a) and CuZn-SOD gene (b) in cardamom for relative quantification by qPCR

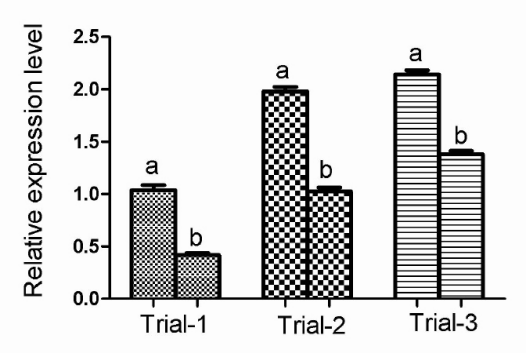

Fig. 6. Expression of SOD gene quantified by qPCR in MeJa treated cardamom plants. Treated (a), untreated (b)

further confirmed by quantifying the expression of SOD in MeJa treated plants by qPCR. As control, samples from the untreated plants were used. It is obvious from the data that the SOD gene exhibited a higher level of expression in the treated plants due to the effect of MeJa elicitor[14] and it further supports the assay data of treated plants (Fig. 6).

CONCLUSION

During pathogenesis, the response of the host plant to the pathogens or insects and the interference of the pathogen to the host metabolism have a crucial role in detecting the tolerance and susceptibility of the host plant. How far these interactions affect the effectiveness of resistance and the efficacy of defense system has become a border line to assess the intensity of infection. Despite the role of SOD in the formation of reactive oxygen species (ROS) – H_2O_2 in cell system, the role of the enzyme in inducing defense mediated pathways was ascertained. The up-regulation of SOD in enhancing the defense system in plants has become a trend of biotechnology research today. Hence the data of the present study provides a firm foundation in developing resistant cardamom plants against biotic attack.

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