



Leaf extract of *Rhizophora apiculata* as a potential bio-inducer of early blight disease resistance in tomato plant

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Received: 23 March, 2020; Accepted: 18 April, 2020; Published online: 19 April, 2020

Abstract

Tomato (*Lycopersicon esculentum* Mill.) is one of the most remunerative and widely grown vegetables in the world. Tomato has ranks second next to potato in world acreage, but it has rank first among processing crops. In the present study, tomato plants treated with *Rhizophora apiculata* as seed treatment at 15 % and as foliar spray at 30 and 45 days after treatment (DAT), co-inoculated with the *Alternaria solani* pathogen, recorded maximum activity of the defense related enzymes and PR proteins including; peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), catalase, chitinase and β - 1,3 glucanses. Induction of these defense enzymes was studied *in vivo* in treated tomato plants grown in the greenhouse. Moreover, the healthy control tomato plants also showed slight increase in activities of all these defense enzymes up to four days, and thereafter showed gradual decline. The aim of the present investigation was to access the induction of the defense enzymes and PR-proteins in tomato plants infested with *A. solani*, through the application of *R. apiculata* extract. Accordingly, we can reduce the incidence of early blight disease in these tomato plants. Current findings recorded the earlier and higher accumulation of the defense enzymes and PR-proteins mainly; PO, PPO, PAL, β - 1, 3 glucanase and chitinase in tomato plants, resulting in significant reduction in early blight infection.

Keywords: Tomato, *Rhizophora apiculata*, Early blight, ISR, Defense enzymes

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most remunerative and widely grown vegetables in the world. Tomato ranks the second next to potato in world acreage, but it ranks the first among the processing crops. The total world production of tomato is 161.7 million metric tons with a value of ~\$59

billion. According to [FAOSTAT. \(2017\)](#), India ranks the second in the total world tomato production by about 163.96 million tons and an average yield of 34.66 tons/ ha after China. In Tamil Nadu, tomato plant is cultivated throughout the year during rainy, winter and summer seasons and occupies an area of

38.73 lakh ha, with the production of 840.32 million tons in 2016-2017 ([Indian Horticulture Database, 2017](#)). The previous study of [Patil *et al.*, \(2002\)](#) highlighted that among the several fungal diseases affecting tomato, early blight caused by *Alternaria solani* is one of the most devastating disease that was managed by the use of several fungicides. However, due to the development of resistance among races of the pathogen, the factor of exposure risks, fungicide residues, and human health, a search for alternative methods of plant disease management is on increase. Earlier study conducted by [Baker *et al.*, \(1997\)](#) documented that plants have latent defense mechanism against pathogens, which can be systemically activated upon exposure of these plants to stress or infection by pathogens. [Tuzun and Kuc, \(1991\)](#) previously named this phenomenon as induced systemic resistance (ISR). According to [Dean and Kuc, \(1985\)](#), this mechanism operates through the activation of multiple defense compounds in the plant at sites distant from the point of pathogen attack. Moreover, [Arthur, \(1996\)](#) reported that there is an increased public demand for sustainable and chemical residue-free food production. In response to these chemical disadvantages, biofungicides derived either from microbes or from plants, emerged as promising alternative strategies. The induction of defense-related enzymes in plants is an indicator of the presence of induced systemic resistance in these plants. Plants are endowed with defense genes which are quiescent in healthy plants. When these genes are activated by various factors they induce systemic resistance against disease. Previous study of [Ramamoorthy and Samiyappan, \(2001\)](#) documented those biologically active compounds which are present in plants as elicitors that induce resistance in host plants, resulting in a reduction of disease development. The antibacterial activity of mangroves has been well documented against various human pathogenic bacteria including; *Excoecaria agallocha* against *Staphylococcus aureus*, *Avicennia marina* against *Pseudomonas aeruginosa*, and *Lumnitzera littorea* against *Escherichia coli* ([Ravikumar *et al.*, 2011](#); [Saad *et al.*, 2011](#); [Prakash and Sivakumar, 2013](#)). There is no significant works

reported against plant bacterial pathogens. However, higher induction of the defense enzymes in rice crop treated with leaf extract of *R. apiculata* was observed during the management of bacterial leaf blight disease ([Vengadeshkumar, 2017](#)). In addition, the antibacterial potential of *R. apiculata* was recorded against *Xanthomonas oryzae* pv. *oryzae* during the recent study of [Vengadeshkumar *et al.*, \(2019\)](#). The objectives of the present study were to check the efficacy of application of mangrove leaf extracts on suppression of early blight disease, and their effects on induction of defense enzymes and PR-proteins including; peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, catalase and PR proteins such as chitinase and β -1,3glucanase.

2. Material and methods

2.1. Source of the pathogen and the antagonist

The test pathogen *A. solani* and the antagonistic mangrove species *R. apiculata* that have being used in this study, were selected based on their previous results in the study of [Mahalakshmi, \(2019\)](#), where they demonstrated potent pathogenicity and *in vitro* antifungal potential, respectively.

2.2. Pot culture assay

The efficacy of *R. apiculata* extract at a concentration of 15 % against early blight disease was carried out in pot culture assay. Tomato plant variety (PKM 1) susceptible to early blight pathogen was used. Within pots of 30 cm in diameter, the tomato plants were artificially inoculated with the test pathogen using the standard spray technique according to [Sorensen *et al.*, \(2016\)](#), and then sprayed with leaf extract of *R. apiculata* at a concentration of 15 %, as per the following treatments;

- | | |
|------------------|---|
| T ₁ - | Tomato seed treatment with <i>R. apiculata</i> (15 %) |
| T ₂ - | Foliar spray of tomato plants with <i>R. apiculata</i> (15 %) at 30 DAT |
| T ₃ - | Foliar spray of tomato plants with <i>R. apiculata</i> (15 %) at 45 DAT |

T ₄ -	Foliar spray with <i>R. apiculata</i> (15 %) at 30 and 45 DAT
T ₅ -	T ₁ + T ₂
T ₆ -	T ₁ + T ₃
T ₇ -	T ₁ + T ₄
T ₈ -	Mancozeb as seed treatment (2 g/ kg) + foliar spraying (0.25 %) at 30 and 45 DAT
T ₉ -	Healthy control plant (treated only with <i>R. apiculata</i>)
T ₁₀ -	Non treated tomato plants (Control)

The pots were maintained in the greenhouse with frequent spraying of water to provide adequate moisture and relative humidity, thus enable successful infection by the test pathogen. The assays were conducted in a randomized block design with three replicates for each treatment and a suitable control. Treatment with the fungicide Mancozeb as seed treatment at 0.2 %, and as foliar spray at 0.25 %, was used for comparison as positive control. The percentage of disease index, plant height and fruit yield were assessed on harvest of the treated tomato plants. The assay was repeated three times and the data were recorded.

2.3. Detection of the presence of defense-related enzymes

2.3.1. Collection of samples

Tomato leaf samples were collected from individual treatments to study the induction of the defense enzymes, in response to pathogen attack under pot-trials. Tomato leaves treated with *R. apiculata* and inoculated with or without *A. solani* but maintained under the same greenhouse conditions, were collected from 1-5 days at 1 day interval.

2.3.2. Preparation of leaf extract for enzyme assay

Leaf samples of treated and non-treated control tomato plants were immediately homogenized with liquid nitrogen. One gram of powdered sample was

extracted with 2 ml of 0.1 M sodium phosphate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min. at 10,000 rpm. Protein extracts prepared from tomato tissues were used for estimation of the defense enzymes. Sodium phosphate buffer 0.1 M (pH 7.0) was used for the extraction of peroxidase, polyphenol oxidase, catalase and phenylalanine ammonia lyase enzymes.

2.3.2.1. Assay of phenylalanine ammonia-lyase (PAL)

One gram of leaf sample was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer (pH 7.0), containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinyl pyrrolidone (PVP). The PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer (pH 8.8) and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min. at 30°C. The amount of trans-cinnamic acid synthesized was calculated using an extinction coefficient of 9630 M⁻¹cm⁻¹ ([Dickerson *et al.*, 1984](#)). The enzyme activity was expressed in fresh weight basis as nmol of trans-cinnamic acid/ min/ mg of sample.

2.3.2.2. Assay of peroxidase (PO)

Assay of PO activity was carried out according to [Hammerschmidt and Kuc, \(1982\)](#). The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 % (v/v) of guaiacol in 0.01 M sodium phosphate buffer (pH 6.0), and 0.1 M hydrogen peroxide. About 0.1 ml of the enzyme extract was added to the mixture to initiate the reaction. The crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/ min. The boiled enzyme was used as blank. The PO enzyme activity was expressed as the increase in absorbance at 470 nm/ min./ mg of protein.

2.3.2.3. Assay of polyphenoloxidase (PPO)

About one gram of leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C, and then the homogenate was centrifuged at 20,000 rpm for 15 min. at 4°C. The supernatant served as the enzyme source, and then the PPO activity was determined in reference to the procedure of [Mayer *et al.*, \(1965\)](#). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added, and the activity was expressed as change in absorbance / min./ mg of the protein.

2.3.2.4. Assay of catalase (CAT)

The CAT activity was assayed spectrophotometrically as described by [Chaparro-Giraldo *et al.*, \(2000\)](#). About 3 ml of the assay mixture was used, containing 100 mM potassium phosphate buffer (pH 7.5), 2.5 mM H₂O₂ prepared immediately before use, and 100 µl of the enzyme extract. The activity was measured by monitoring the degradation of H₂O₂ using UV-visible Spectrophotometer (Varian Cary 50) at 240 nm over 1 min., against a plant extract-free blank. The decrease in H₂O₂ was followed as the decline in optical density at 240 nm, whereas the CAT activity was calculated using the extinction coefficient ($\epsilon_{240\text{nm}} = 40 / \text{mM} / \text{cm}$) for H₂O₂, and was expressed in µmol / min./ mg of sample.

2.3.2.5. Assay of chitinase

One gram of leaf sample was ground using a chilled pestle and mortar with 0.1 M Sodium citrate buffer (pH 5.0) at 41°C. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was used as a crude enzyme extract for assaying the chitinase activity. The changes in the chitinase activity were determined through colorimetric assay described by [Boller and Mauch, \(1988\)](#).

2.3.2.6. Assay of β -1,3 glucanase

The β -1,3 glucanase activity was detected using the Laminarin-dinitro-salicylic acid method. An aliquot of 62.5 µl of the crude enzyme extract was

added to 62.5 µl of laminarin, incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitro-salicylic acid, heated for 5 min. in boiling water bath, vortexed, and then absorbance of the mixture was measured at 500 nm. The crude extract preparation with laminarin at zero time incubation served as the blank. The enzyme activity was expressed as µg equivalent of glucose/ min./ mg of protein, in reference to [Kavitha *et al.*, \(2005\)](#).

2.4. Statistical analyses

The statistical analysis of the obtained results was performed using the computer software package 'SPSS' by Duncan Multiple Range Test (DMRT), and the values are expressed as mean according to [Gomez and Gomez, \(1976\)](#).

3. Results

3.1. Induction of the *in vivo* production of defense enzymes in the treated tomato plants

Among the various treatments, treatment (T₇) involving seed treatment at 15 % and foliar spray at 15%, with *R. apiculata* at 30 and 45 DAT recorded higher peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase activities (2.55, 2.12, 12.86 and 2.31 / min./ g of unit) on the 4th day, when compared with other treatments. This was followed by (T₆) (1.99, 1.96, 11.96 and 2.02 / min./ g of unit), (T₅) (1.98, 1.89, 11.43 and 1.88 / min./ g of unit) and (T₄) (1.83, 1.85, 10.36 and 1.75 / min./ g of unit) treatments, in the decreasing order of merit.

The maximum peroxidase activity was observed on the 4th day in all the treatments, and thereafter a gradual decrease was observed. Plants treated with the synthetic fungicide and the healthy control plants also showed slight increase in all the enzymes activities up to the 4th day, thereafter presented a gradual decline. On the other hand, the non-treated control plant did not showed any increase in the activity of enzymes, as clear in Fig. (1, 2, 3 and 4).

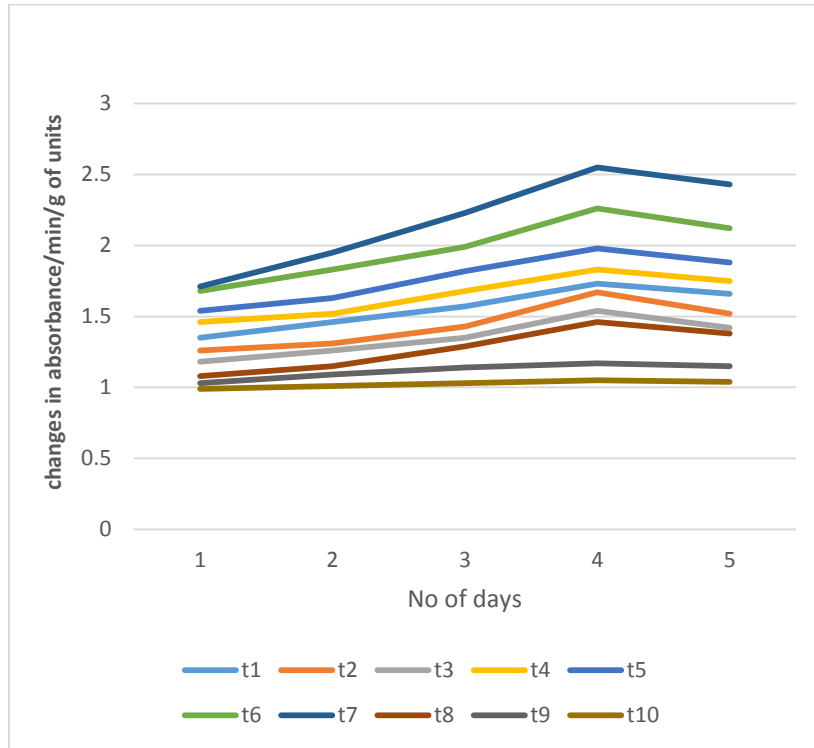


Fig. 1: Induction of Peroxidase (PO) activity in treated tomato plants

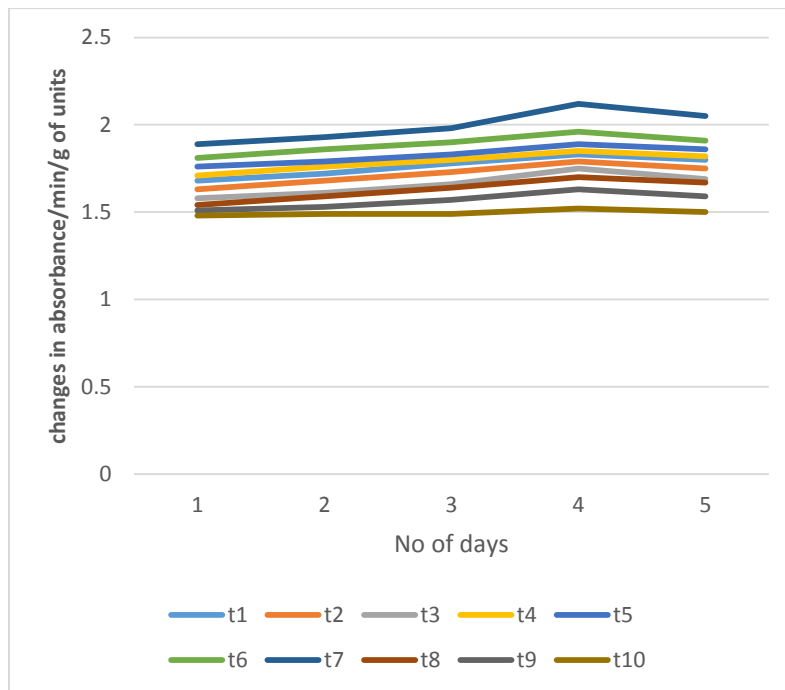


Fig. 2: Induction of Polyphenoloxidase (PPO) activity in treated tomato plants

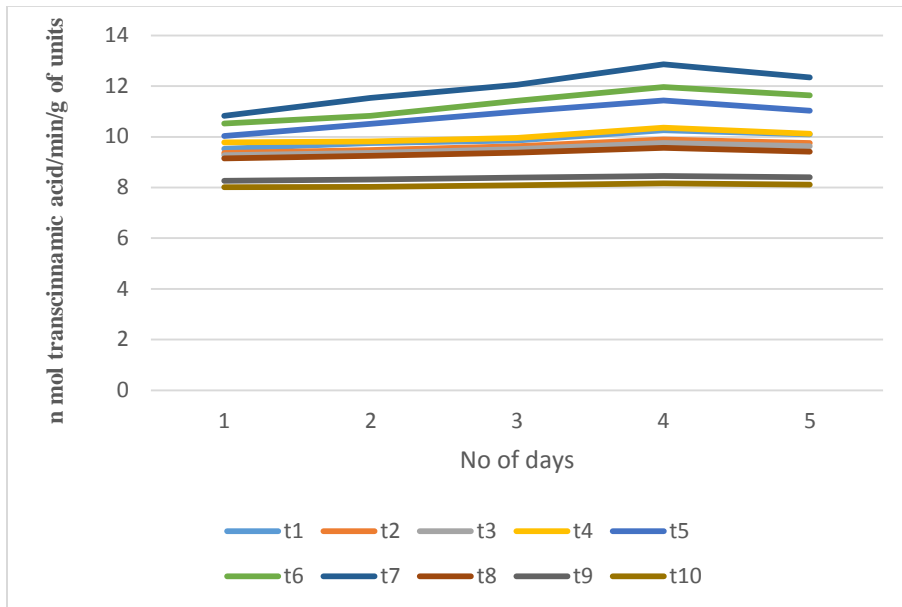


Fig. 3: Induction of Phenylalanine ammonia lyase (PAL) activity in treated tomato plants

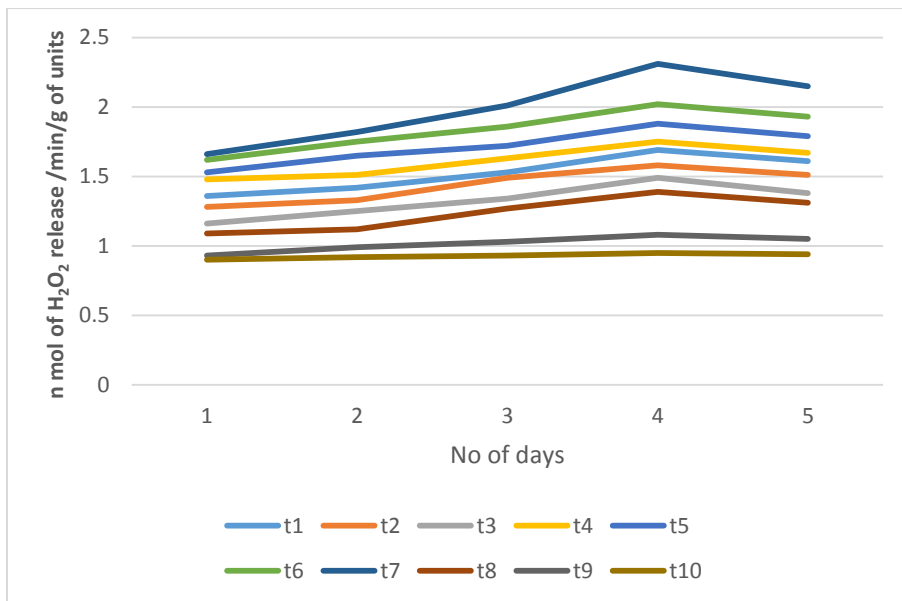


Fig. 4: Induction of Catalase activity in treated tomato plants

where; T1: - Seed treatment with *R. apiculata* (15 %); T2: - Foliar spray with *R. apiculata* (15 %) at 30 DAT ; T3: - Foliar spray with *R. apiculata* (15 %) at 45 DAT; T4: - Foliar spray with *R. apiculata* (15 %) at 30 and 45 DAT; T5: - T1 + T2; T6: - T1 + T3; T7: - T1 + T4; T8: - Mancozeb as ST (2 g/ kg) + FS (0.25 %) at 30 and 45 DAT; T9: - Healthy control plants (treated with *R. apiculata* only); T10: -Non-treated control plants

3.2. Induction of the *in vivo* production of PR-proteins in the treated tomato plants

Results revealed the increased activities of the chitinase and β -1,3 glucanase enzymes, upon treatment with leaf extract of *R. apiculata* (15 %), and challenge inoculation with the tested pathogen. Among the treatments, seed treatment as well as foliar application with *R. apiculata* (15 %) at 30 and 45 DAT (T₇) recorded higher chitinase and β -1,3 glucanase activities, compared to the other treatments. This was followed by (T₆) (2.13 and 25.68 / min./ g of unit) (T₅) (1.93 and 22.62 / min./ g of unit) and (T₄) (1.89 and 20.57 / min./ g of unit) treatments, in decreasing order of merit. The maximum chitinase and β -1,3 glucanase activities (2.76 and 30.45 / min./ g of unit) were observed on the 4th day, and thereafter a gradual decrease was observed. The plants treated with the synthetic fungicide and the healthy controls also demonstrated slight increase in the catalase activity up to the 4th day, and then showed a gradual decline. Whereas, the non-treated control plants did not exhibit any increase in the enzymes activities, as shown in Fig. (5 and 6).

4. Discussion

The induced resistance as a technique of phyto-immunity has received great attention. Moreover, inducing the plants own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy ([Ramamoorthy and Samiyappan, 2001](#)). Plants are bestowed with various defense related genes. Previous studies conducted by [Patricia *et al.*, \(2009\)](#); [Karthiba *et al.*, \(2010\)](#); [Meera *et al.*, \(2013\)](#) highlighted that these defense genes are sleeping genes, and appropriate stimuli or signals are needed to activate them. They added that various types of biological agents and plant extracts are used as inducers for the induction of resistance in the various crops.

In the present study, tomato plants treated with *R. apiculata* (15 %) as seed treatment and foliar spray at

30 and 45 DAT (T₇), and artificially inoculated with the pathogen recorded the maximum activity of the defense related enzymes and the PR proteins including; peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL), catalase, chitinase and β -1,3 glucanase enzymes. Similar to the current results, [Bhuvaneshwari *et al.*, \(2015\)](#) positively correlated the treatment with botanicals on the induction of defense enzymes and PR proteins against wilt of tomato plants. Hence, the enhanced resistance against early blight in tomato plants treated with leaf extract of *R. apiculata* (15 %) + foliar spray (15 %) at 30 and 45 DAT (T₇), can be attributed to the direct inhibitory effect of these extracts, as well as their ability to induce systemic resistance against *A. solani*.

In accordance, similar induction of defense enzymes and PR proteins in tomato crop treated with *Zimmu* leaf extract against *A. solani* was reported by [Latha *et al.*, \(2009\)](#). Likewise, [Kahkashan *et al.*, \(2012\)](#) reported the increased induction of defense enzymes and PR proteins in tomato crop due to treatment with garlic extract, against *Fusarium* wilt. Recently, [Magesh and Ahiladevi, \(2017\)](#) study added that tomato plants treated with *Allium sativum* extract as a foliar spray (15 %) at 30 and 45 DAT, plus seed treatment with bacterial antagonists co-inoculated with *A. solani*, demonstrated earlier induction and increased levels of defense enzymes mainly; PO, PPO, PAL and catalase, as well as PR proteins such as chitinase and β -1, 3 glucanase.

[Pala *et al.*, \(2011\)](#) positively correlated the treatment with botanicals on the induction of defense enzymes against sheath blight of rice. Also, [Vengadeshkumar, \(2017\)](#) reported the induction of defense enzymes and PR-proteins in rice plant treated with extract of *R. apiculata* as foliar spray and seed treatment, co-inoculated with *P. fluorescens*.

In accordance with the present findings, several earlier workers reported the enhanced induction of defense enzymes (i.e. PO, PPO, PAL, catalase) and

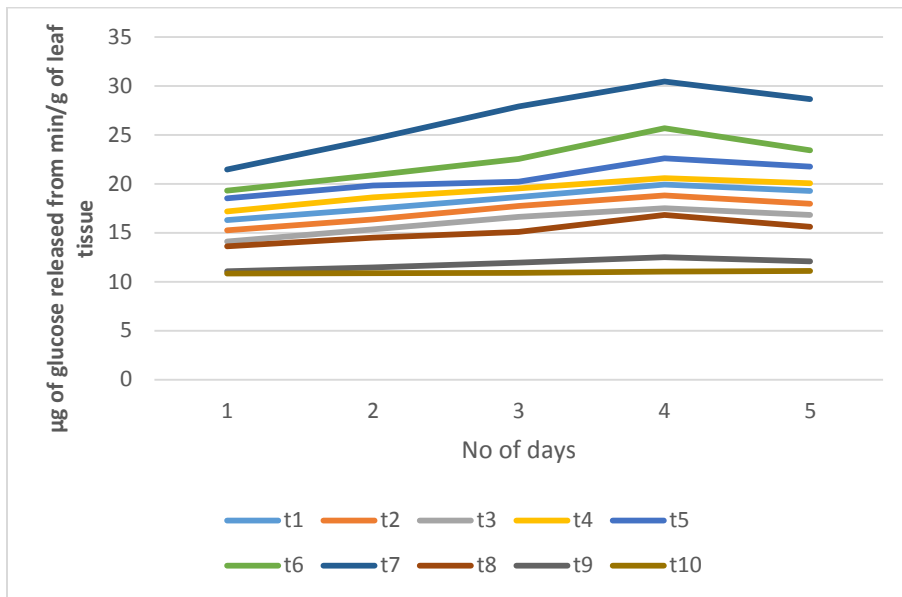


Fig. 5: Induction of β -1,3 glucanase activity in the treated tomato plants

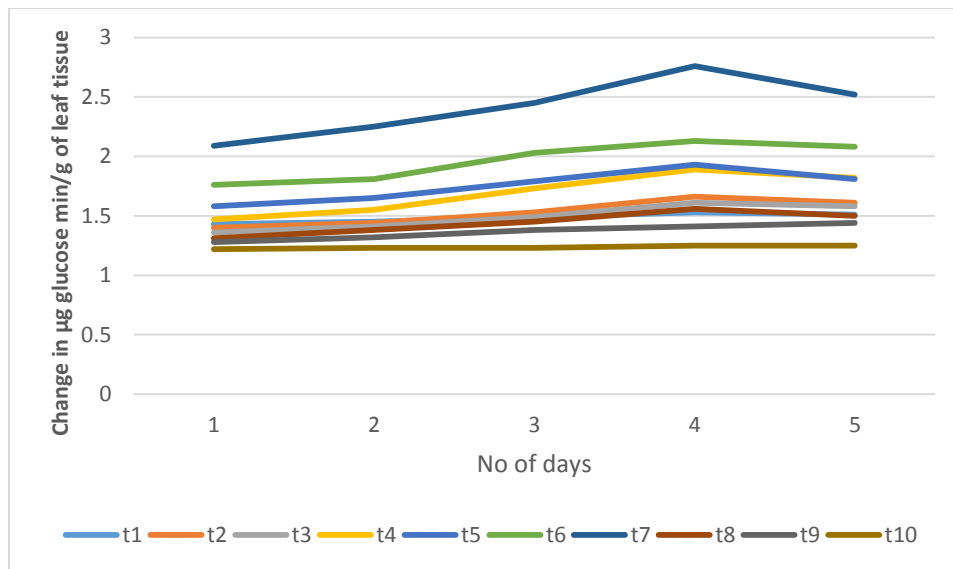


Fig. 6: Induction of Chitinase enzyme activity in the treated tomato plants

where; T1: - Seed treatment with *R. apiculata* (15 %); T2: - Foliar spray with *R. apiculata* (15 %) at 30 DAT ; T3: - Foliar spray with *R. apiculata* (15 %) at 45 DAT; T4: - Foliar spray with *R. apiculata* (15 %) at 30 and 45 DAT; T5: - T1 + T2; T6: - T1 + T5; T7: - T1 + T4; T8: - Mancozeb as ST (2 g/ kg) + FS (0.25 %) at 30 and 45 DAT; T9: - Healthy control plants (treated with *R. apiculata* only); T10: -Non-treated control plants

PR-proteins (i.e. chitinase and β - 1, 3 glucanase) in various crops, due to treatment with different plant extracts mainly; in Cucumber due to *Ocimum gratissimum* extract (Colpas *et al.*, 2009), in Cumbu due to *Viscum album* extract (Chandrashekhara *et al.*, 2010), in Rice due to both of *Datura metal* extract (Kagale *et al.*, 2004) and *Adathoda vasica* extract (Govindappa *et al.*, 2011), in Banana due to *Solanum torvum* extract (Jadesha *et al.*, 2012), and recently in Apple due to Neem extract (Gholamnezhad, 2019).

Conclusion

The susceptibility of different varieties of tomato, pathogen resistance to fungicides and possible pollutions to the environment have created concerns worldwide, and also necessitated the phytopathologists to look for eco-friendly alternatives for managing early blight disease of tomato. The use of synthetic fungicides although showed limited success, however was the common practice followed for the management of early blight of tomato. Results of the current study proved that application of leaf extract of *R. apiculata* (15 %) as seed treatment and foliar spray expressed higher induction of the defense enzymes (i.e. peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase and catalase), and PR-protein (such as; chitinase, β -1,3 glucanase), thus caused reduction of early blight incidence caused by *A. solani*. Induction of systemic resistance exerted by *R. apiculata* extract improved the consistency of biological control of tomato early blight under varied climate conditions. Moreover, treatment with plant extracts (*R. apiculata*) without the use of chemical pesticides as demonstrated in this study, will be of great interest to the growing organic crop industry.

Acknowledgement

The authors express sincere thanks to Directorate of Research, Annamalai University, Tamilnadu, India.

Conflict of interest

The authors declare that there is no any conflict of interests.

Funding source

This study was not funded by any profit or non-profit organization.

Ethical approval

This article does not contain any studies with human participant's and\ or animals performed by the authors.

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