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ORIGINAL RESEARCH

Greenhouse and field experiments revealed that clove oil can effectively reduce bacterial blight and increase yield in pomegranate

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Abstract

Bacterial blight in pomegranate is a devastating disease caused by bacterial pathogen *Xanthomonas axonopodis* pv. *punicae* (XAP), recording huge damage to pomegranate crop worldwide. Antibiotics and copper-based chemicals are being used for the management of this blight, while in this present work, we investigated the effect of eugenol and clove oil either singly or in combination with copper oxychloride (COC) on the induction of plant defense responses and concomitant prevention of bacterial blight. Our results provided evidence that clove oil (0.2%–1%) and eugenol (0.1% and 0.2%) successfully inhibit the growth of XAP in paper disk diffusion assay. Strikingly under the greenhouse condition, clove oil (0.2%) as foliar application 24 h before XAP inoculation recorded the lowest disease severity of 7.34%, whereas eugenol (0.2%) recorded maximum disease severity of 14.56%. However, the combination of clove oil (0.2%) and copper oxychloride (0.3%) recorded the least disease severity of 2.38%. A similar trend was observed in field conditions. Prophylactic application of clove oil leads to enhanced nitrate reductase activity and nitric oxide production which was further enhanced in clove oil pre-treated plants challenged with XAP. Strikingly, the total ROS and H_2O_2 levels were reduced in response to clove oil application. Clove oil also induced the systemic response by inducing expression levels of defense genes. The reduction of disease severity by clove oil and COC combination also reflected on total yield recording via large-scale field experiments where maximum yield of 14.04 tonnes/acre was observed, whereas streptocycline application recorded 11.12 tonnes/acre. Application of COC and clove oil resulted in a high remunerative value of ₹ 1:5.6, compared to streptocycline (1:4.85) and control (1:1.85). The present study revealed that clove oil as a plant derivative and eugenol as a synthetic option can be effectively used for the successful management of bacterial blight in pomegranate.

KEYWORDS

antibiotics, copper oxy-chloride, disease severity, nitrate reductase, nitric oxide, pathogenesis-related proteins, pomegranate, streptocycline, *Xanthomonas axonopodis* pv. *punicae*

1 | INTRODUCTION

Pomegranate (*Punica granatum*) is one of the major commercial fruit crops in India and various semi-arid regions worldwide. At the global level, India and Iran are the leading producers of this fruit and collectively contributing around 70% to the global pomegranate production. According to 2019 reports, India produces 3034 thousand tonnes of pomegranate annually in an area of 262 thousand hectares (http://nhb.gov.in). Pomegranate is touted as a superfood due to its functional and nutraceutical properties (Sreekumar et al., 2014). Globally, pomegranate market is estimated worth USD 8.2 billion in 2018 and the demand is likely to increase up to 23.14 billion by the end of 2026 (https://agriexchange.apeda.gov.in).

The successful cultivation of pomegranate in recent years has also met with various challenges that emerged from pests and diseases. Among all bacterial blight (BB) disease caused by *Xanthomonas axonopodis* pv. *punicae* has become one of the major threats for pomegranate production in India, causing 60%–80% yield loss (Sharma et al., 2015). When XAP pathogen invades the pomegranate plant, it infects almost all aerial parts of the plant such as leaves, twigs, and fruits. The losses caused by XAP are in the range between 10% and 100% in various parts of India (Anonymous, 2007, 2008; Benagi & Ravi Kumar, 2009). Bacterial blight disease in pomegranate has also been reported from several countries such as Pakistan (Akhtar & Bhatti, 1992), South Africa (Petersen et al., 2010), and Turkey (Icoz et al., 2014). Superior and commercially cultivated "Bhagwa" is widely grown but this superior and high-yielding variety is highly susceptible due to the absence of resistance genes against BB (Sharma et al., 2015). Antibiotics and synthetics chemical have been a practice for disease management (Benagi & Ravi Kumar, 2009; Sharma & Sharma, 2011). However, excessive use of synthetic antibiotics leads to the development of resistance in pathogens and increased accumulation of toxic residue in fruits and has a negative impact on the environment (McManus & Stockwell, 2000). In pomegranate cultivation, it is a general practice to use streptocycline at 500 ppm and copper oxychloride at 2.5%, multiple times for management of bacterial blight in pomegranate (Sharma & Sharma, 2011). Hence, there is an increasing demand for the identification of alternative sources to treat and prevent bacterial blight in pomegranate.

Among the various alternatives, the search for plant-derived antimicrobial compounds over the years has gained considerable attention for the identification of potential agents against many bacterial diseases (Chandra et al., 2017). Plant oils are rich in phenolic compounds with broad-spectrum antimicrobial properties. Many natural compounds and essential oils have been reported as resistance inducers for restricting the pathogen invasion into the host system (Lucas et al., 2012). In the induction of disease resistance, the gaseous signal molecule nitric oxide (NO) plays an important role (Bellin et al., 2013). NO is produced by various oxidative and reductive pathways (Kolbert et al., 2019). Cytosolic nitrate reductase (cNR) pathway catalyzes the reduction of nitrate to nitrite and it can further reduce nitrite to NO (Rockel et al., 2002). NR is known to play role in plant defense in response to bacterial and fungal pathogens and their elicitors (Shi & Li, 2008; Yamamoto-Katou et al., 2006). NO is a component of salicylic acid-mediated plant defense (Klessig et al., 2000). NO activates *PR-1* expression via an NO-dependent, cADPR-independent pathway (Klessig et al., 2000) and also via S-nitrosylation process (Lindermayr et al., 2010) and so far NO production, and its role in response to XAP infection in pomegranate is not elucidated.

In plant protection, essential oil such as clove oil has been extensively studied for its antimicrobial component, insect repellent, and as a nematicide effect (Deans & Ritchie, 1987; Huang & Lakshman, 2010; Lucas et al., 2012; Sangwan et al., 1990). Clove scientifically called *Eugenia caryophyllata* generally used as a spice and food flavoring agent has a huge medicinal property. Clove oil is a rich source of a phenolic compound called eugenol (up to 76.8%) and is known to be a potent antimicrobial agent (Kishore et al., 2007; Nurdjannah & Bermawie, 2012) and antiviral agent to control yellow leaf curl virus in tomato (Wang & Fan, 2014).

Direct application of clove oil is most effective in the management of *Xanthomonas vesicatoria* in tomato (Lucas et al., 2012). Clove oil is also known to enhance the competitive ability of plants apart from its direct effect on the pathogen, and this has greater prospects in integrated disease management as well. Though clove oil is effective against various plant pathogens, the molecular mechanism of induction of plant defenses by clove oil and its combination with COC on the induction of plant defense responses and concomitant prevention of bacterial blight is not known. Hence in this present study, we assessed the effect of clove oil and eugenol in pomegranate individually and in combination with copper molecules against XAP under green house and field conditions and we found that clove oil can successfully reduce infection and improve resistance via production of NO

and induction of plant defense responses and concomitant increase in yield.

2 | MATERIALS AND METHODS

2.1 | Plant materials

One-year-old healthy pomegranate plants (variety Bhagwa) were used to study the effect of clove oil and its combinations on bacterial blight. Experimental plants were obtained from the National Research Center on Pomegranate Solapur, Maharashtra India. All the plants were maintained in the greenhouse at 30 ± 2℃ of temperature with 12-h dark and 12-h light photoperiod and relative humidity of 65%–70%.

2.2 | Isolation of *Xanthomonas axonopodis* pv. *punicae*

A pure and single colony of XAP was isolated from naturally infected leaf and fruit samples collected from pomegranate growing orchards (Figure 1a,b). The bacterial colony was oozed out from the surface-sterilized part of the infected tissue in sterile distilled water. 10 µl of that water was streaked on NGA medium (nutrient glucose agar) and incubated at 28 ± 0.5℃. Yellowish, pinheaded single colonies that appeared after 48 h were further sub-cultured on NGA medium and maintained as pure cultures.

2.3 | Preparation of bacterial inoculum and pathogenicity test

Bacterial suspension or inoculum for pathogenicity study and challenge inoculation was prepared by inoculating the single colony of the bacterial cell into NG broth and incubating at 28 ± 0.5℃ for 48 h. The final bacterial concentration used in all the experiments was set to 0.4 OD at A_{600} nm using Bio-Spectrometer (Eppendorf AG), with approximately 4×10^8 colony-forming unit (CFU) ml^{-1}. The pathogenicity of the isolate was confirmed as per Koch's postulates.

2.4 | Molecular confirmation of *Xanthomonas axonopodis* pv. *punicae*

Molecular identification of XAP was done by isolating total genomic DNA from XAP and using effector-based *Xop*Q primer (Doddaraju et al., 2019). PCR was performed in 15 µl reaction containing 1× PCR Buffer, 200 µM of dNTPs, 1 U *Taq polymerase*, (New England Biolabs), 0.25 µM each forward and reverse primer, and 50 ng of DNA as a template.

2.5 | Preparation of clove oil and eugenol

The clove oil was procured from Fine chemicals, Bengaluru (SSC/15-16/1050), and Eugenol from Sigma (97-53-0). Both clove oil and eugenol were dissolved in 1 L of water using 1% (v/v) surfactant (Tween 20) and prepared into 0.1%, 0.2%, 0.3%, and 0.5% solution. The same has been used for testing its inhibitory effect by agar disk diffusion assay, evaluation under greenhouse, field evaluation, and defense gene expression analysis.

2.6 | In vitro inhibition studies of bacterial blight pathogen

The antibacterial activity was tested in 90-mm Petri dishes, filled with 20 ml nutrient glucose agar medium according to

FIGURE 1 Pure culture of *Xanthomonas axonopodis* pv. *punicae* (*XAP*) isolated from infected fruit and leaf samples. (a) Infected fruit and (b) infected leaf sample selected from bacterial blight-infected pomegranate orchard for isolation of pathogen. (c) Pure culture of XAP on nutrient glucose agar medium. (d) Artificial inoculation under greenhouse conditions confirming typical symptoms of the pathogen

Dean's protocol (Deans & Ritchie, 1987). 100 µl of bacterial inoculum (0.4 OD_{600} nm = 4×10^8 CFU ml^{-1}) was evenly spread onto Petri dishes using a sterile L-shaped spreader. Once the culture was evenly spread and set, sterilized filter paper disks (Whatman type I, 0.6 cm in diameter) were placed on the surface of the medium, onto which 20 µl of clove oil of different concentrations 0.1, 0.2, 0.3, and 0.5 was added and a similar protocol was followed for testing eugenol at 0.1% and 0.2% concentration. For the control plate, sterile water with the same concentration of surfactant (1%, v/v) was added to the disk.

After inoculation, Petri dishes were incubated at 28 ± 0.5℃ until the complete growth of the pathogen was observed in the control plate (~72 h). For each treatment, three replicates were performed. The zone of bacterial growth inhibition was measured using a Vernier caliper. The same concentration (as described above) was spray-inoculated on plants and observed for any phytotoxic symptom/effect. All treatment details and observations are recorded in Table 1.

2.7 | Bacterial blight infection

Pure and single colony strain of XAP (Accession No. KX702398.1) was inoculated in NGA medium and incubated at 28 ± 0.5℃ for 48 h at 120 rpm until the OD_{600} reached between 0.35–0.4. Inoculation of the pathogen was made using an airbrush (Model Badger-200.3, Deluxe set™, Air-Brush Co.).

2.8 | Effect of clove oil and eugenol on bacterial blight under greenhouse condition

To evaluate the effect of clove oil, foliar sprays were applied using hand sprays 24 h before the pathogen inoculation. Clove oil was applied at a concentration of 1 ml L^{-1}, 2 ml L^{-1}, and in combination with copper oxychloride (53.8% WP) 2.5 g L^{-1} and compared with commercially used antibiotic compound streptocycline (0.5 g L^{-1}), and challenge inoculated with pathogen was made 24 h after carrying out each spray. Eugenol was also tested as a foliar application at 0.2%, and challenge inoculation was made using pathogen after 24 h of spray. Control plants were sprayed sterile water before pathogen inoculation, and the experiment was repeated twice with three replicates of each treatment. Plants were routinely observed for the development of the symptom.

2.9 | Disease severity analysis

Disease severity was measured 15 days post-inoculation when the control plant displayed a minimum of 50% disease severity. Scoring for disease severity was made by counting the total number of healthy and infected leaves and scaling them on disease grades of 0–5 according to Singh et al. (2015). Disease severity and percent disease protection were calculated based on the following formulae.

TABLE 1 Antibacterial activity of clove oil against *Xanthomonas axonopodis* pv. *punicae* at different concentrations

Sl. no.	Concentration (%)	Inhibition (mm) in in vitro assay		Phytotoxicity observed in plants when sprayed on pomegranate plans	
		Clove oil	Eugenol	Clove oil	Eugenol
1	0.1	16.3 (0.61)	24.46 (0.38)	–	–
2	0.2	23.43 (0.34)	27.38 (0.23)	–	+
3	0.3	32.16 (0.42)	–	+	+
4	0.5	35.33 (0.31)	–	+	+
5	Control	00.00	00.00	–	–
C.D @ 0.05		1.60	1.51		
CV		3.68	2.47		
SE(m)±		0.49	0.32		
SE(d)		0.7	0.46		

Notes: All the figure numbers in parentheses represent the standard error, and the letters indicate the significant difference between mean values at $p < 0.05$ according to Duncan's new multiple range tests. After the different treatments, plates were incubated at 28 ± 0.5℃ and the bacterial growth was measured after complete growth of the pathogen in the control plate, the data expressed in mm. Phytotoxic effect of clove oil was also evaluated for similar concentration. Results are indicated as average mean data. "+" indicates exhibition of phytotoxicity, "–" indicates no phytotoxicity on plants.

Abbreviations: CD, critical difference; CV, critical variance; SE(d), standard deviation; SE(m), standard error mean.

$$\text{Percent disease severity} = \frac{\text{Number of infected leaves} \times \text{Grade obtained}}{\text{Total number of leaves} \times \text{Maximumgrade}} \times 100$$

$$\text{Percent disease protection was calculated using} = \frac{\text{Disease severity in control} - \text{Disease severity in treatment}}{\text{Disease severity in the control}} \times 100$$

3 | MEASUREMENT OF NITRATE REDUCTASE ACTIVITY

100 mg pomegranate leaf sample of control or clove oil alone or XAP or XAP + clove oil-treated was homogenized in liquid nitrogen. Samples were extracted in 1 ml of extraction buffer (100 mM HEPES pH 7.6, 3.5 mM β-mercaptoethanol, 15 mM $MgCl_2$, 0.5% PVP, 0.5% BSA, and 0.3% Triton X-100). The homogenate was centrifuged at 14,000 *g* for 10 min at 4℃, and reaction was stopped by Zn acetate. The supernatant was collected followed by adding 1 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene dihydrochloride) and incubated for 10 min at 25℃. Absorbance was recorded at 546 nm.

3.1 | Determination of nitric oxide by DAF fluorescence and gas-phase chemiluminescence

For diaminofluorescein (DAF-FM) fluorescence, section of leaves was cut manually by sharp razor blade and incubated in 10 μM of DAF-FM DA (Molecular Probes, Life Technologies) for 15 min in darkness and then washed 2–3 times with 100 mM of HEPES buffer pH 7.2 to remove excess of dye and visualized using a fluorescence microscope (EVOS M 5000 Invitrogen by Thermo Scientific) with excitation at 488 nm and emission at 515 nm. For chemiluminescence nitric oxide measurement, approximately branch weighing 10 g of leaves (treated with clove oil, clove oil + XAP or XAP or control) was placed in customized polyacrylic boxes containing inlets and outlet and inlet was connected to air pump and the produced NO was passed to chemiluminescence analyzer nCLD AL (Ecophysics) and NO levels were recorded for several hours.

3.2 | Total ROS measurements by DCF fluorescence

For 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence, section of leaves was cut and incubated in 10 μM of DCF (Molecular Probes, Life Technologies) for 15 min in darkness and then washed with 100 mM HEPES buffer and visualized under fluorescence microscope (EVOS M 5000 Invitrogen by Thermo Scientific) upon excitation at 492 nm and emission at 515 nm.

3.3 | Measurement of hydrogen peroxide levels by DAB staining

H_2O_2 accumulation in leaves was visualized by DAB (3,3′-Diaminobenzidin) staining. Pomegranate leaves were excised placed in 50-ml falcon tubes containing 1 mg/ml DAB and incubated on shaker for a period of 8 h. The stain was removed, and chlorophyll was bleached by boiling in bleaching solution (ethanol: acetic acid: glycerol = 3:1:1) for 10–12 min. DAB is rapidly absorbed by the plant and is polymerized locally in the presence of H_2O_2 and peroxidase giving a visible brown stain which was photographed using stereo zoom microscope (Nikon AZ100).

3.4 | Relative DNA quantification using qPCR

Subjected treatments were evaluated for relative DNA abundance of XAP from the leaves collected after 15 days of pathogen inoculation. Random leaf samples were collected from each treatment, and 100 mg of the grounded tissue was used for isolation of total genomic DNA. Pathogen colonization was calculated by the total biomass of its genomic DNA isolated from XAP-inoculated leaves using a modified CTAB method (Mondal et al., 2013), and qPCR was performed using SYBR Green Supermix in StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Pathogen-specific XopQ primers F-GCGAGGAACTTGGAATGCTC XopQR-AGGTCGAAGGCTTTTTGCG were used to amplify and detect XAP DNA, and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for normalizing the cycle threshold values. The pathogen was quantified based on relative pathogen load ($2^{-\Delta C_T}$) and copy number which was correlated with the disease severity based on visual symptoms.

3.5 | Defense responses associated with clove oil treatment

3.5.1 | Sampling

Leaf sampling was done from control and clove oil-treated plants at 0, 6, 12, 24, 72, 120, and 168 h post-inoculation

(hpi). The collected leaves were frozen in liquid nitrogen and stored at −80℃ for gene expression analysis.

3.5.2 | RNA isolation and cDNA synthesis

Total RNA was isolated from leaves of clove oil-treated and controls. Leaves were ground to a fine powder using liquid nitrogen, and 75 mg of the ground leaf was processed for RNA extraction using Spectrum Plant Total RNA kit (Sigma). DNA cross-contamination was eliminated using DNase I, RNase-free (Thermo Fisher Scientific) using the manufacturer's protocol. Total concentration and quality of the RNA were measured using NanoDrop (ND-1000; Thermo Scientific) at 260 nm, and the quality of the RNA was determined by the absorbance ratio of A_{260}/A_{280} and A_{260}/A_{230}. A total of 1000 ng of RNA was converted to cDNA using RevertAid H-minus M-MuLV Reverse Transcriptase following the manufacturer's instructions.

3.5.3 | qPCR analysis of gene expression

A total of seven genes were selected for the analysis (Table 2). A one-step qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Gene-specific primers were designed using Oligo Explorer software (version 1.1.0), and all the primers used in the present study are listed in Table 2. GAPDH was used as a reference gene for normalizing gene expression data (Doddaraju et al., 2021). The qPCR was performed in a 10 µl reaction composed of 1X SYBR Green Master Mix (Applied Biosystems) and 0.25 µM for primer (forward and reverse each) and 2 µl of 10-fold diluted cDNA and a non-template control. The qPCR program was used as follows: 2 min of initial activation at 50℃, 2 min at 95℃ followed by 40 cycles of 15 s denaturation at 95℃ and 1 min annealing and extension at 60℃. Post-PCR amplification, melt curve analysis of the amplicons was performed between 60 and 95℃ and data were collected at every 0.3℃ intervals to determine the specificity of the primers. Relative gene expression of each gene was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Relative fold change in each of the target genes was compared with control (XAP inoculated) in the respective time intervals.

3.5.4 | Field evaluation for effective treatment

Field evaluation of clove oil was conducted in Ambe bahar (flowering regulation during January–February) and Hasta bahar (flowering regulation in September–October) of 2017–2018 in farmer field (16.1725° N × 75.6557° E) Bagalkot taluk and district. Treatment of clove oil was evaluated individually and in combination with COC for disease severity on leaves and fruits (Table 3). A total of 5 sprays of each treatment were given in 25 days intervals. These treatments' bio-efficacy was compared with streptocycline and untreated control. Scoring of disease severity on leaves and disease incidence on fruits was carried out 180 days after the first spray as described earlier.

After successful completion of sprays, observations were recorded for the number of fruits per plant and total yield per acre area, at the time of harvest. The total number of fruits per plant was calculated by manually counting the number of fruits from 10 plants, from each treatment. Fruit yield per acre was calculated by multiplying average fruit yield per plant into the total number of plants per acre area (350 plants per acre area), and total yield is expressed in terms of tonnes/acre area.

TABLE 2 Oligonucleotide primers used for qPCR analysis of different defense gene induced after clove oil treatment

Target gene	Primers (5′−3′)	Accession no.	Amplicon size (bp)
Pathogenesis-related protein 1 (*PR-1*)	F-ACTACGCCAACAAGCACATTG R-GTCCACCCACATTTTCACTG	KU977458	122
Pathogenesis-related protein 10 (*PR-10*)	F-GCCAGTACAAATCAGTGAAG R-TACTTGCTCGTGTTCTTGC	KY635883.1	175
Pathogenesis-related protein 4 (*PR-4*)	F-GCACAACTGGGACCTGAATG R-TGTCACCCTGAGGCATCTTC	KU977460	154
Phenylalanine ammonia lyase (*PAL*)	F-TCGGGAAGCTGATGTTTGC R-CCCCTTGAAGCCATAGTCC	KX450397	116
Chitinase	F-AAGGGACGAGAGGAAGACTG R-CTGAGCGCCGAAATAAGGAG	KU977459.1	164
Callose synthase 3 (*CS-3*)	F-AGCCTATGGAGGTGAAGAC R-CTGGGAATGCTTTGACTTTC	KU977465	112
Peroxidase (POD)	F-CTTCCGACTCTTCTTTCACG R-GGGCACACCTTCTCAACAG	KY635883.1	168

TABLE 3 Effect of clove oil on bacterial blight disease severity on leaves and disease incidence on fruit under field conditions, evaluated for two seasons

	Leaf blight (%)		Fruit blight (%)		Pooled data (%)	
Treatment	**Season 1**	**Season 2**	**Season 1**	**Season 2**	**Leaf**	**Fruit**
T1	7.33^{d} (0.34)	6.85^{d} (0.40)	5.36^{b} (0.55)	4.08^{c} (0.35)	7.09^{d} (0.39)	4.72^{c} (12.39)
T2	13.82^{b} (0.69)	12.42^{b} (0.44)	5.68^{b} (0.37)	6.60^{b} (0.41)	13.14^{b} (0.41)	6.14^{b} (14.26)
T3	11.05^{c} (0.6)	12.06^{c} (0.35)	7.04^{b} (0.57)	7.56^{b} (0.71)	11.56^{c} (0.36)	7.3^{b} (15.52)
T4	2.41^{e} (0.28)	4.08^{e} (0.31)	1.72^{c} (0.19)	1.96^{c} (0.202)	3.25^{e} (0.28)	1.84^{c} (7.66)
T5	60.13^{a} (0.99)	62.32^{a} (1.24)	57.96^{a} (1.39)	62.8^{a} (1.63)	61.23^{a} (1.83)	60.38^{a} (51.02)
C.D @ 0.05	1.77	1.93	2.18	2.23	1.86	1.66
CV	10.25	10.88	15.39	14.78	10.79	16.37
SE(m) ±	0.61	0.67	0.75	0.77	0.46	0.58
SE(d)	0.87	0.952	1.07	1.09	0.66	0.83

Notes: All the figure numbers in parentheses represent the standard error and the letters indicate the significant difference between mean values at $p < 0.05$ according to Duncan's new multiple range tests. Pooled data represent the average value of two seasons. T1—streptocycline 0.5 g L^{-1}, T2—clove oil 2 ml L^{-1}, T3—COC 2.5 g L^{-1}, T4—clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}, and T5—water control. Results are indicated as average mean data.

Abbreviations: CD, critical difference; CV, critical variance; SE(d), standard deviation; SE(m), standard error mean.

3.5.5 | Statistical analysis

All greenhouse and field experiments were designed as a completely randomized block design (RCBD). The statistical analysis was carried out using R software and Microsoft Excel (2007). Critical differences were calculated at 5% level of significance to each treatment. All the statistical analyses were applied at the level of $p < 0.05$ to the significant data, and Duncan's multiple range test was applied to differentiate the mean values.

3.5.6 | Cost-to-benefit ratio

The cost-to-benefit ratio was calculated after the successful harvest of each treatment and represented as per hectare area. The total cost for cultivation was calculated by considering all the components required for the cultivation of one hectare of land as described by the national horticulture board government of India (Anonymous, 2017). To the total cost of cultivation, the additional cost of each treatment (clove oil, streptocycline, and COC) was added. After the complete harvest, net gross returns were calculated by multiplying the cost per kilogram by the total yield. Then, net returns were calculated by subtracting the total cost of cultivation from gross returns. Finally, the cost-to-benefit ratio was calculated by dividing the total cost of cultivation from the net gross returns.

4 | RESULTS

4.1 | In vitro antimicrobial effect by clove oil revealed that it can retard XAP effectively

To check the in vitro effects of clove oil on XAP first, the pathogen was isolated from the infected fruit samples (IA&B). Yellow, pin headed colonies appeared after 48 h of incubation were sub-cultured on NGA medium (Figure 1c). Pathogenicity was seen as extended blight symptoms on the leaf confirmed the pathogen (Figure 1d). Further, pathogen identity was established using the XAP effector protein *XAPQ*. Amplification of 190 bp of the partial gene sequence of the XAP effector primer confirmed the molecular identity of *X. axonopodis* pv. *punicae* (Figure 2). In vitro antimicrobial property of clove oil and eugenol was tested against XAP. 0.1%, 0.2%, 0.3%, and 0.5% of clove oil and 0.1 and 0.2% eugenol were used to test its effect on inhibitions against the pathogen (Table 1). All concentrations displayed clear inhibition of bacterial growth. The maximum zone of inhibition was observed at 0.5% with 35.33 mm, followed by 0.2% of clove oil with a concentration of 32.16 mm zone inhibition. Similarly, eugenol also displayed a maximum zone

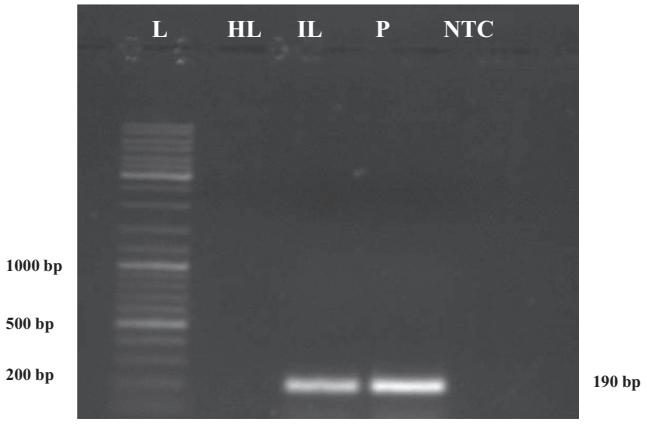

FIGURE 2 Molecular identification of *Xanthomonas axonopodis* pv. *punicae* (*XAP*) using specific primer *XopQ*. 1. Healthy leaf, 2. XAP isolated from leaf, 3. *XAP* DNA, 4. non-template control. L—1 Kb bp ladder

of inhibition at 0.2% with 27.38 and 24.46 mm at 0.1%, respectively (Figures S1 and S2).

4.2 | Greenhouse evaluation of clove oil and eugenol on bacterial blight revealed it is very effective against blight

After establishing the in vitro, we sought to check the effect of eugenol and clove oil on the prevention of pathogen infection in intact plants grown in the greenhouse. For this purpose, different concentrations of clove oil and eugenol were tested to evaluate the phytotoxicity effect on plants. Individual 0.1% of eugenol or clove oil up to 0.2% did not display any phytotoxic symptoms on plants. Thus, combination of eugenol (0.1%, v/v) and clove oil (0.1% and 0.2%, v/v) was evaluated for its efficiency in controlling pathogens in combination with COC (2.5 g L^{-1}). Eugenol recorded 14.56% disease severity and 71.15% of disease protection at 15 days post-challenge inoculation, whereas clove oil treatment recorded the disease severity of 7.34% at 0.2% and 8.83% at 0.1% with disease protection of 85.46% and 82.51%, respectively, over control (Figure 3). Clove oil (0.2%) + COC 0.25% (or clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}) were found compatible and recorded least disease severity of 2.38% with highest disease protection of 95.36%. Streptocycline 0.5 g L^{-1} recorded 3.65% disease severity and COC 2.5 g ml^{-1} recorded disease severity 5.24%, with disease protection of 92.78% and 89.61%, respectively. The corresponding calculations of percent disease protection are also represented in Figure 3. Based on the preliminary analysis, it was observed that 0.2% or 2 ml of clove oil per liter (2 ml L^{-1}) of the water is the best concentration, hence the same was used for further experiments.

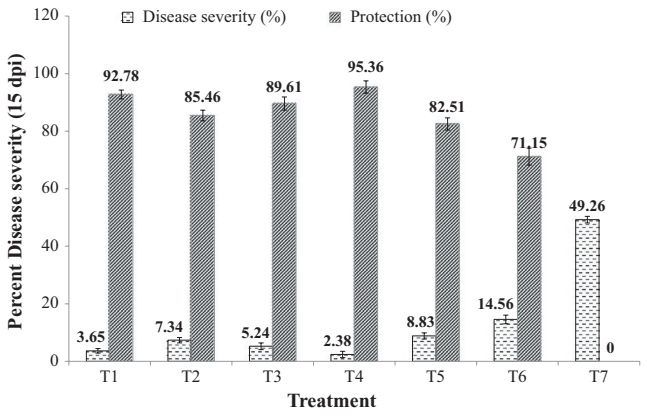

FIGURE 3 Effect of clove oil, eugenol, streptocycline, and COC on bacterial blight severity, under greenhouse conditions. T1—streptocycline 0.5 g L^{-1}, T2—clove oil 2 ml L^{-1}, T3—COC 2.5 g L^{-1}, T4—clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}, T5—clove oil 1 ml L^{-1}, T6—eugenol 2 ml L^{-1}, and T7—water control. The bar represents the standard error of mean value

4.3 | In vivo pathogen quantification in response to treatments

To corroborate disease severity, pathogen quantification was carried out by measuring the DNA concentration of the pathogen in all the treatments using XAP primers. The relative abundance of the pathogen DNA was found high in pathogen-treated control plants with 214.22 folds. Clove oil (0.2%) in combination with COC showed very minimal pathogen DNA abundance of 9.85 folds. Standard control streptocycline and COC recorded 23.20 and 24.03 folds, respectively. These results were found correlating with the visual scoring of the disease, thereby indicating efficiency and accuracy in quantifying pathogen using qPCR (Figure 4).

4.4 | Clove oil treatment can enhance the NR activity and NO emissions

The observed disease resistance in response to clove oil probably also due to nitric oxide emissions. Nitrate reductase can be a major enzyme responsible for NO emission; hence, after 24 h of application of clove oil to the plants, the NR activity was measured in clove oil-treated, XAP-treated, XAP + clove oil-treated leaves along with the controls. Interestingly, clove oil application leads to 2.5-fold induction of NR activity (Figure 5a). XAP application also showed significant enhancement of NR activity which was further accelerated in response to clove oil application (clove oil + XAP; Figure 5a). Since NR activity enhanced, we further checked the NO emissions by gas-phase chemiluminescence detection, for

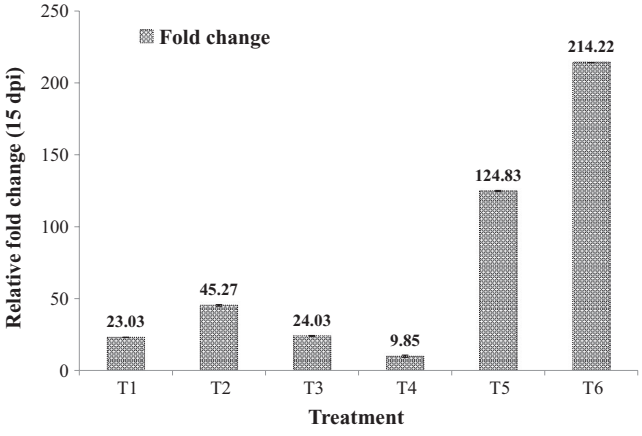

FIGURE 4 Relative quantification of *XAP* DNA in pomegranate leaves with different treatment of clove oil, streptocycline, and COC. Leaf samples are collected 15 days post-inoculation (dpi) of the pathogen, and DNA was quantified by qPCR using *XopQ* primer. T1—streptocycline 0.5 g L^{-1}, T2—clove oil 2 ml L^{-1}, T3—COC 2.5 g L^{-1}, T4—clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}, T5—clove oil 1 ml L^{-1}, and T6—water control. The bar represents the standard error of the mean value

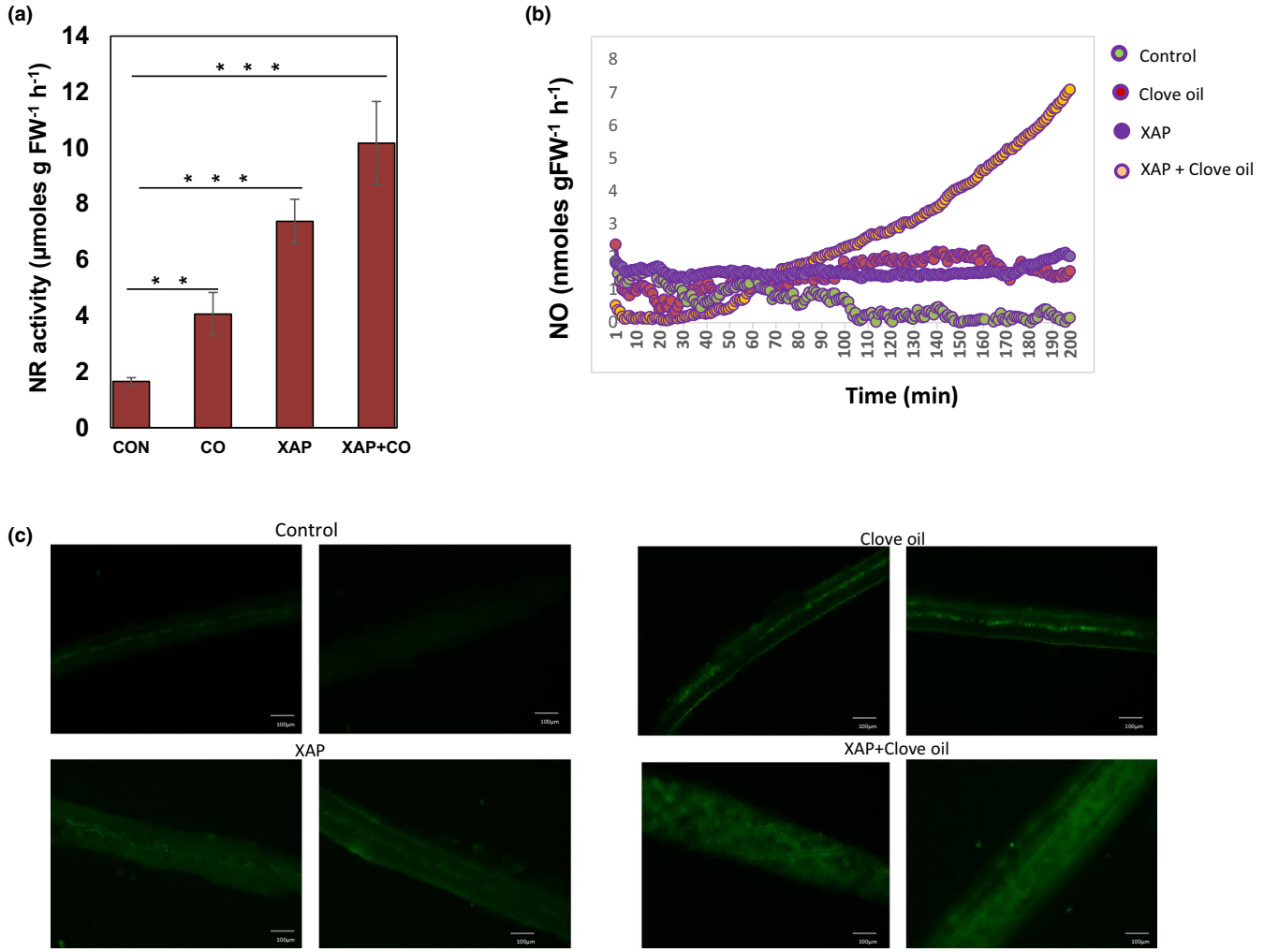

FIGURE 5 Nitrate reductase, nitric oxide emission, and levels in response to clove oil, XAP, and XAP + clove oil: (a) total NR activity of control and treated leaves. Error bars indicate SD. p values for differences are $^{**}p < 0.01$ and $^{***}p < 0.001$. (b) NO emission from leaves in response to clove oil, XAP, and XAP + clove oil and control as indicated in the figure. The NO curve is representative example of three biological replicates. (c) DAF-FM fluorescence from transverse sections of leaves in response to clove oil, XAP, and XAP + clove oil and control as indicated in the figure. Image is representative example of four independent replicates

this purpose after 24 h of application of clove oil or XAP-treated or XAP + clove oil treatment, branch of plants (approximately 10 g) was cut and measured the NO emissions into gas phase which was detected by chemiluminescence detector. The NO emission was low in control plants and clove oil alone or XAP alone treatment stimulated NO emission and strikingly the application of clove oil + XAP caused very significant increase in NO emissions (Figure 5b). To corroborate the chemiluminescence data, we measured the NO levels by using DAF-FM DA fluorescence, for this purpose in response to treatments above we made transverse sections of leaves and measured the DAF-FM fluorescence. The control tissues produced low levels of DAF-FM fluorescence and NO levels were higher in response to clove oil alone, XAP alone treatments and clove oil + XAP caused very significant increase in DAF-FM fluorescence (Figure 5c). Taken together, these results suggest that the clove oil can activate NR activity and concomitant increase in NO levels which can play role in induction of plant defenses.

4.5 | Expression analysis of defense gene in response to clove oil treatment

The effect of clove oil in inducing systemic resistance against pathogens was determined through qRT-PCR analysis. A total of seven different defense genes belonging to different pathways were tested for their expression upon clove oil treatment (Table 2). Among various defense response genes, pathogen-related (*PR*) protein gene families have been frequently used as marker genes for studying systemic acquired resistance in many plant species.

The PR1 gene upregulation was recorded from 12 hpi (15.5 folds) and continued till 168 hpi, with significant

upregulation observed at 120 and 168 hpi with 77 and 82 folds, respectively. The expression level of *PR 4* gene was upregulated from at 6 hpi (11 folds) and reached a maximum at 120 hpi (27 folds) over control. Similarly, *PR 10* gene was also upregulated from 6 hpi with 1.85 folds, and a higher fold change was observed at 24, 72, and 120 hpi with 12.2, 12, and 15.46 folds, respectively (Figure 6). It is very interesting to note that all *PR* genes expressed maximum at 120 hpi of the pathogen, which is the key time for pathogen expression phenotypically. Defense enzymes such as PAL, peroxidase, chitinase, and callose synthase (CS3) were also studied for understanding the systemic nature of clove oil in protecting against XAP.

PAL is a gateway enzyme in the phenylpropanoid pathway and was found to increase upon treatment with clove oil. Regulation of the *PAL* gene was found significant in all time points. A gradual increase of *PAL* was observed from the initial 6 (2.95 folds) to 168 hpi (61.7 folds) with slight decreases at 72 hpi with 14 folds (Figure 6). CS greatly contributes to the plant's constitutive resistance and clove oil treatment consistently found increasing *CS3* expression from 6 to 168 hpi over control and maximum upregulation was found at 120 hpi with 14.12-fold change (Figure 7).

Chitinase, an important cell wall-degrading enzyme, actively involved in plant protection during pathogen attack and also found highly expressed with clove oil. The upregulation of the *chitinase* gene was observed from 6 hpi (2.6 folds), and consistent transcript abundance was observed in 48, 72, and 120 with 2.1, 1.5, and 2.2 folds, respectively. Similarly, the antioxidant peroxidase gene was also found to increase with clove oil. The gene was found to be increased gradually from 24 h (5.72 folds) and continued till 120 hpi, and maximum upregulation was found at 120 hpi with 9.85 folds and subsequently, the activity was truncated (Figure 7).

4.6 | Clove oil treatment can reduce ROS levels

We further sought to explore other beneficial effects of clove oil in enhancing defense responses; hence, we checked the reactive oxygen species production in response to clove oil, XAP, and clove oil + XAP in 24 h of application. First, we measured hydrogen peroxide (H_2O_2) levels by using DAB (3,3′-Diaminobenzidine) staining. Control uninfected leaves produced basal levels of H_2O_2 and levels have enhanced in

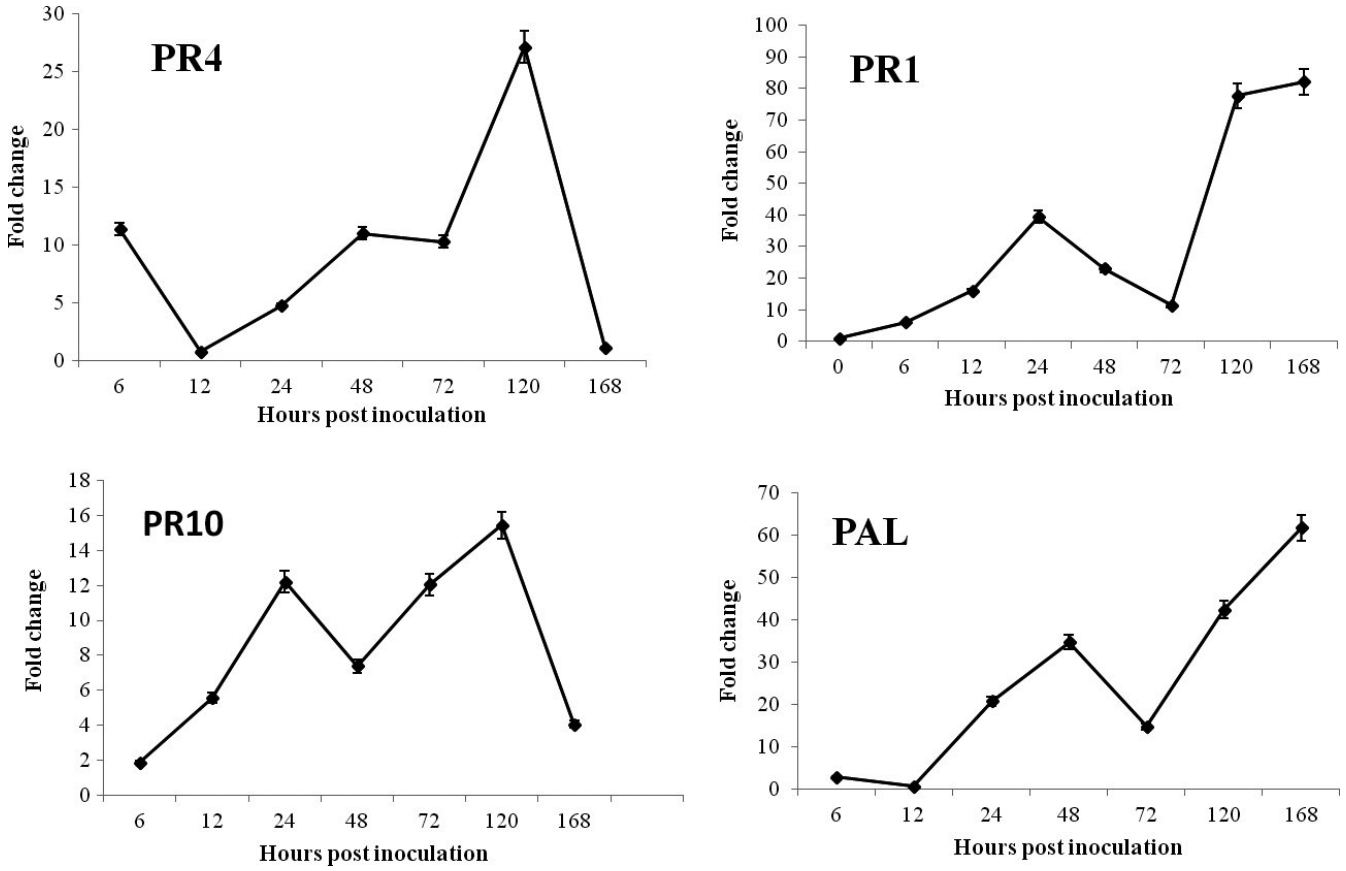

FIGURE 6 Relative expression of pathogenesis-related genes (*PR-1*, *PR-10*, *PR-4*) and PAL gene using qRT-PCR in clove oil-treated plants. Total RNA isolated from clove oil-treated plants and RNA was reverse-transcribed into cDNA and used as a template for qRT-PCR as described in materials and method. The bar indicates the standard error

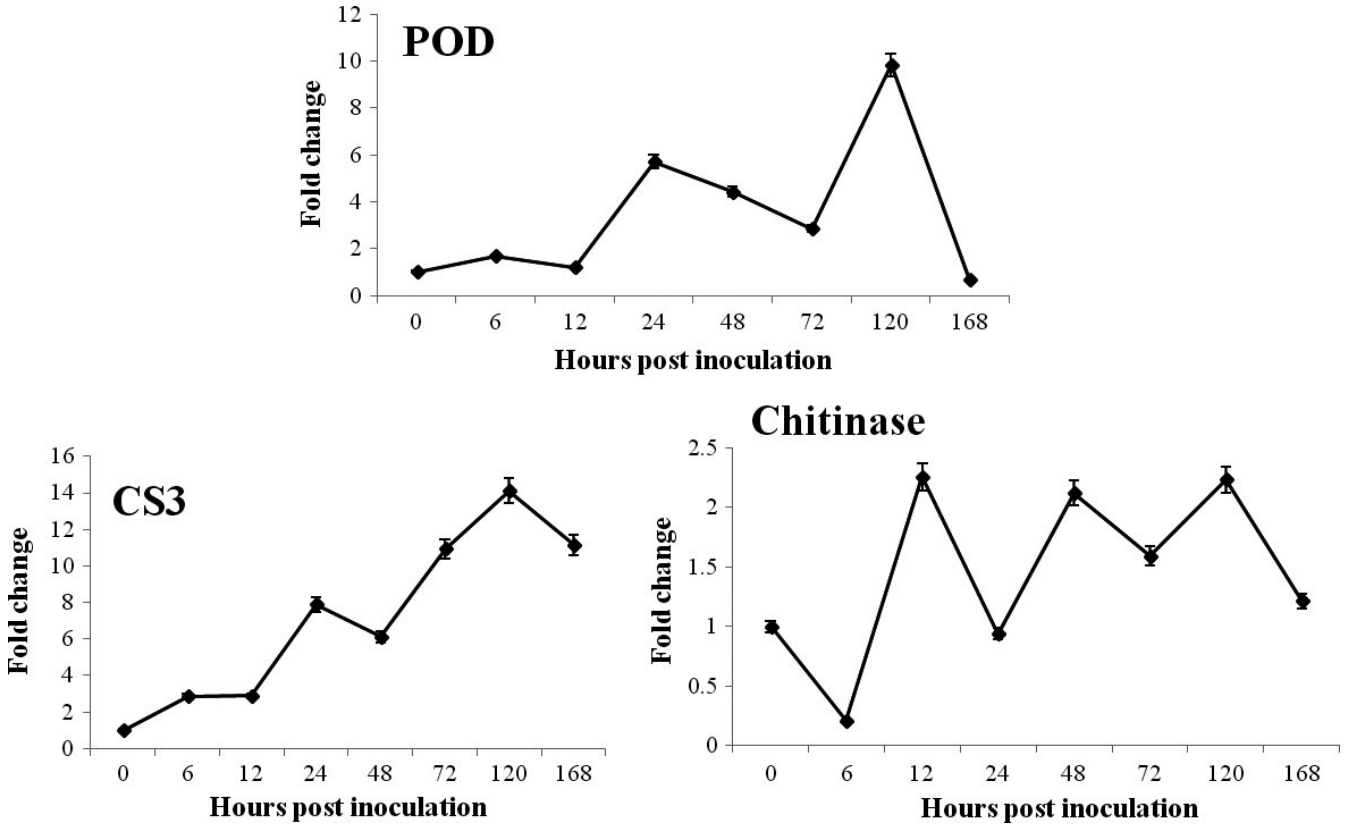

FIGURE 7 Relative expression of defense-related genes—chitinase, POD, and CS3 gene using qRT-PCR in clove oil-treated plants. Total RNA isolated from clove oil-treated plants and RNA was reverse-transcribed into cDNA and used as a template for RT PCR as described in materials and method. The bar indicates the standard error

response to XAP and very interestingly clove oil application along with XAP significantly reduced the H_2O_2 levels (Figure 8a). Further, the total ROS levels were measured by using cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). The total ROS levels were low under controlled conditions and these were further reduced in clove oil applied leaves. XAP infection accelerated ROS and interestingly, the clove oil application in XAP infected leaves suppressed the ROS (Figure 8a), suggesting that clove oil alone can reduce ROS and it can also reduce ROS in response to XAP infection.

4.7 | Clove oil mediated reduction of disease severity in fruits and increased total yield

Bio-efficacy of clove oil was evaluated in 2 seasons for its effect against disease severity and total yield. Pooled data of both leaf blight severity and fruit blight incidence of 2 seasons are represented (Table 3). In the average of 2 seasons, clove oil alone recorded 13.14% of disease severity, whereas in combination with COC recorded 3.25%, followed by streptocycline (7.09%) and COC (11.56%). A similar trend was also recorded in fruit blight incidence (Table 3). Fruit blight of 6.14% was recorded in the clove oil treatment, and in combination with COC, 1.84% disease severity was recorded which is statistically superior to streptocycline with disease severity of leaf and fruit blight. The control plants recorded 61.23% and 60.38% of leaf and fruit blight, respectively.

Treatment of clove oil and COC also recorded the highest fruit of 37.97 fruits per plant followed by standard control streptocycline with 31.03 fruits per plant (Table 3). Similarly, total yield was high in the combined treatment of clove oil and COC with 14.04 tonnes per acre followed by 11.12 in streptocycline (Figure 9). The data have evidently revealed that clove oil in combination with COC is an alternative over streptocycline, which has innumerable repercussions.

4.8 | Benefit: Cost ratio

Maximum yield and high gross returns were obtained in combined treatment of clove and COC with ₹ 1:5.67, followed by streptocycline (1; 4.85), COC (1; 3.93), and clove oil (1; 3.42) compared to control plants (1; 1.85; Table 4). Indicating the use of clove oil and COC is the most profitable option in BB management in pomegranate.

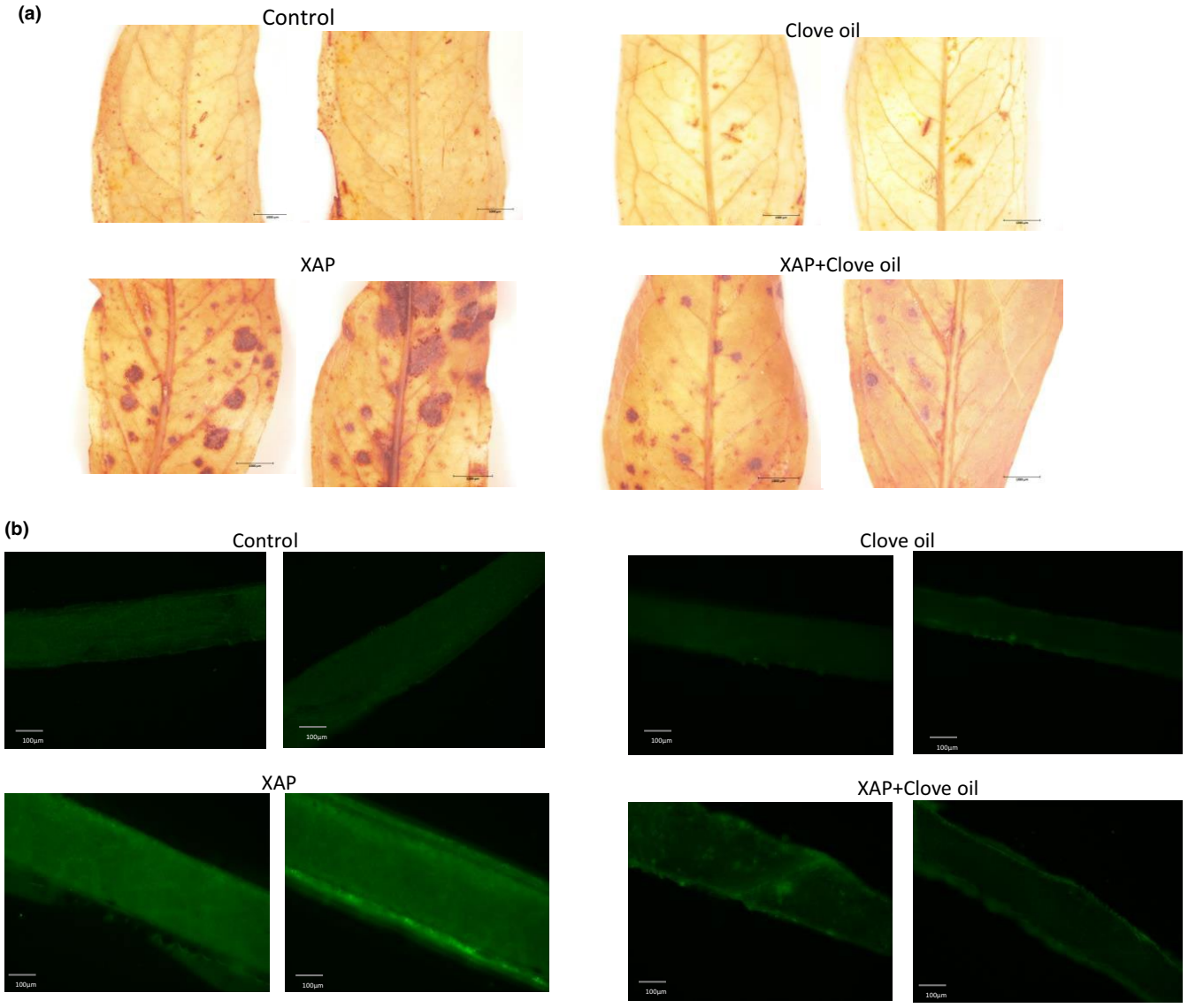

FIGURE 8 ROS levels in response to clove oil, XAP, and XAP + clove oil. (a) Hydrogen peroxide levels in response to clove oil, XAP, and XAP + clove oil and control as indicated in the figure. Image is representative example of six independent replicates. (b) Total ROS levels (DCF fluorescence) measured by H2DCFDA fluorescence. Image is representative example of five independent replicates

5 | DISCUSSION

Bacterial blight caused by XAP is a devastating pathogen, and it is one of the major constraints for successful pomegranate cultivation (Benagi & Ravi Kumar, 2009; Sharma & Sharma, 2011). Currently, bacterial blight (BB) disease management is largely relying on the wide use of synthetic antibiotics. Due to the increased development of resistance via antibiotics and other synthetic chemicals, it is essential and recommended to reduce the use of these options (Doddaraju et al., 2019; Kumar et al., 2021; McManus & Stockwell, 2000). Induction of local and systemic resistance in plants via application of natural extracts is one of the important strategies in integrated disease management and to reduce the use of antibiotics in field conditions (Srivastava et al., 2011). Previously, Chowdappa et al. (2018) tested various oils such as clove, eucalyptus, lemongrass, pungam, peppermint, wintergreen, and citronella and their preliminary findings suggested that clove oil is among them which can be effective against XAP but molecular mechanism of induction of plant defenses by clove oil and its combination with COC was not yet unraveled. Hence in this present study, eugenol and clove oil were used to counter bacterial blight caused by XAP.

Clove oil recorded an antibacterial property at the concentration of 0.1%–0.5% and maximum inhibition was recorded at 0.5% followed by 0.3% similarly eugenol recorded maximum zone of inhibition at 0.2% (Figure S1). Previously, it was demonstrated that clove oil and eugenol have antimicrobial properties against various fungi such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Aspergillus* sp.,

Phomopsis viticola, *lasiodiplodia theobromae*, and *Rhizopus* sp. and bacteria such as *Pseudomonas syringae* (Sukatta et al., 2008), *Ralstonia solacearum*, *X. compestris* pv. *pelargonii*, *Streptomyces* spp., and *Rhodococcus fascians* (Huang & Lakshman, 2010), and *Erwinia caratovora* (Deans & Ritchie, 1987). But the role of these compounds against XAP was identified upon confirming the antimicrobial effect of the tested concentrations of clove oil and eugenol were evaluated for their phytotoxicity effects. Clove oil at 0.3% and 0.5% and eugenol at 0.2% recorded phytotoxic symptoms such as scorching, browning of leaves, and finally dropping. Hence, 0.2% (2 ml L^{-1}) of clove oil and 0.1% of eugenol were used as an optimum dose for testing under greenhouse conditions.

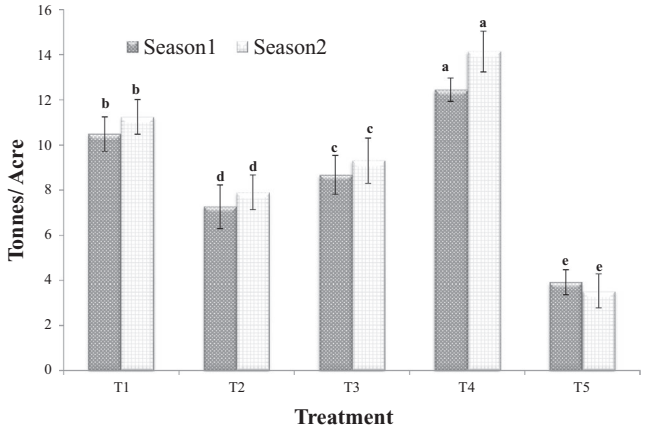

FIGURE 9 Effect of clove oil, streptocycline, and combined treatment of clove oil and COC in two seasons, represented as tonnes per acre area. T1—streptocycline 0.5 g L^{-1}, T2—clove oil 2 ml L^{-1}, T3—COC 2.5 g L^{-1}, T4—clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}, and T5—water control. The bar represents the standard error of mean value, and the letter indicates the statistical significance at Duncan's multiple range test TP < 0.05 between treatments

Under the greenhouse condition, the treatment of eugenol was not much effective as it recorded maximum disease severity (14.56%) when compared to clove oil (7.34%). The reduced efficiency of eugenol under the greenhouse condition could be due to the volatile nature of eugenol (Nurdjannah & Bermawie, 2012). Similar observations were recorded by Kishore et al. (2007), where foliar treatment of clove oil was more effective than eugenol in the management of crown rot and late leaf Spot and diseases of Peanut. However, promising results were observed in the combination treatment of clove oil and COC that recorded only 2.38% disease severity, whereas chemical treatment streptocycline recorded 3.65%.

Relative quantification of pathogenic DNA is an approach in determining plant disease severity in the commercial crop which was successfully employed in the current research (Doddaraju et al., 2019). In our study, the relative quantification of XAP DNA by using qRT-PCR analysis revealed that treatment with clove oil and combination inhibits the accumulation of pathogen DNA to the great extent in the host when compared to streptocycline (Figure 4).

Several molecules are effective under a controlled environment, but they fail to reproduce the results under field conditions. To test the efficiency of clove oil and COC combination, field evaluation was carried out for two seasons. Under field conditions, also clove oil at 0.2% markedly

TABLE 4 Effect of clove oil on average fruit yield per plant under field condition for two successive seasons and cost-to-benefit ratio for each treatment

	Total yield			
	Season1	Season2	Pooled	
Treatments	Average fruit per plant (kg)	Average fruit per plant (kg)	Average fruit per plant (kg)	Cost–benefit ratio in Indian rupee (C: B)
T1	29.96^{b} (0.68)	32.1^{b} (0.69)	31.03^{b} (0.53)	1:4.85
T2	20.78^{d} (0.87)	22.58^{d} (0.68)	21.68^{d} (0.58)	1:3.42
T3	24.78^{c} (0.78)	26.6^{c} (0.89)	25.66^{c} (0.61)	1:3.93
T4	35.55^{a} (0.46)	40.39^{a} (0.83)	37.97^{a} (0.72)	1:5.67
T5	11.18^{e} (0.50)	10.1^{e} (0.67)	10.64^{e} (0.42)	1:1.85
C.D @ 0.05	1.87	2.28	1.56	
CV	8.39	9.521	9.75	
SE(m) ±	0.64	0.79	0.55	
SE(d)	0.918	1.122	0.78	

Notes: All the figure numbers in parentheses represent the standard error and the letters indicate the significant difference between mean values at $p < 0.05$ according to Duncan's new multiple range tests. Pooled data represent the average value of two seasons. T1—streptocycline 0.5 g L^{-1}, T2—clove oil 2 ml L^{-1}, T3—COC 2.5 g L^{-1}, T4—clove oil 2 ml L^{-1} + COC 2.5 g L^{-1}, and T5—water control. Results are indicated as average mean data.

Abbreviations: CD, critical difference; CV, critical variance; SE(d), standard deviation; SE(m), standard error mean.

improved the disease tolerance and suppressed the disease by recording only 13.14% disease severity over the control with 61.23% disease (Table 3). Further, the combination of clove oil and COC was compatible as a spraying solution and recorded 3.25% disease severity with substantial yield improvement over streptocycline (Figure 9). Previously, clove oil use was reported as effective against bacterial spot caused by *X. vesicatoria* and was found to induce disease tolerance in tomato (Lucas et al., 2012). Clove oil principally containing eugenol acts by disrupting the cytoplasmic membrane of the pathogen and thereby increasing non-specific cell permeability leading to pathogen death (Devi et al., 2010). Recent reports reveal that the hydroxyl group in eugenol binds to cellular proteins, preventing enzyme action in *Enterobacter aerogenes* (Burt, 2004). Moreover, the hydrophobic nature of eugenol enables it to penetrate the lipopolysaccharide of the gram-negative cell membrane of a bacterium and alters the cell structure, which subsequently results in the leakage of intracellular constituents thereby inhibiting the pathogen (Sikkema et al., 1994). These prospects in other horticultural crops demonstrated that results of the present study indicated that clove oil could greatly influence pomegranate resistance to *X. axonopodis* pv. *punicae* by inducing systemic resistance against the pathogen and inhibiting the pathogen in the host system.

Copper-based molecules are also widely used against bacteria including *Xanthomonas* species (Araújo et al., 2012), and uses of copper-based molecules are acceptable under the organic horticultural system (Jeyaraman & Robert, 2018). Hence, exploring the idea of combining plant derivative and prominent plant protecting molecules like COC is essential in pomegranate being a former ISR agent and the antimicrobial nature as well. To our knowledge, this is the first report on the combined use of essential oil and COC compounds successfully used in the management of major diseases in a commercial crop like pomegranate. The combination of clove and COC also resulted in high gross returns of 1: 5.67, compared to generally used streptocycline (1;4.85). This indicates that the application of clove oil is not only an eco-friendly approach for disease management but it also a highly profitable approach to the farmer.

Understanding the molecular events during the induction of systemic resistance and the expression of defense responses in response to clove oil treatment is essential.

Nitric oxide is a free radical signal molecule known to play a role in induction of plant defense responses (Klessig et al., 2000) and clove oil-induced plant defense responses and reduction of disease probably also due to increased NO production. Our result suggest that clove oil application can induce nitrate reductase as the activity was elevated in response to clove oil and activity was further enhanced in combination of clove oil + XAP (Figure 5a). The molecular mechanism of induction of NR activity in response to clove oil needs further investigation. NR can induce NO production (Rockel et al., 2002). Our results of chemiluminescence suggest that clove oil can enhance NO production which can directly affect the bacterial survival at the site of infection and the produced NO can also activate various defense responses. This can explain the induction of (Figures 6 and 7) genes coding PR proteins such as *PR1*, *PR4*, and *PR10* in post-pathogen infection stages. PR proteins are the major defense responsive genes extensively studied in plant disease resistance as these are the first line of resistance responsive genes expressed during pathogen attack. PR protein expression is an integral part of the salicylic acid pathway and contributes to strengthening immune systems against the pathogen (Srinivasan et al., 2009). Previously, it was reported that these PR proteins were components of defense in the crops like carrot, rice, tobacco, apple, and many more crops against the pathogens (Dubos et al., 2010; Malnoy et al., 2007; Wally et al., 2009). PAL, a defense enzyme in the phenylpropanoid pathway, was found activated by clove oil treatment, and its increased expression contributed to enhanced activity of phytoalexins, flavonoids, and lignin which are directly involved in disease resistance (Dubos et al., 2010). Significant upregulation of the *PAL* gene at 120 and 168 hpi indicates the resistance inducing property of clove oil and thus plays a crucial role during defense response (Figure 6). Our results are in line with Wang and fan (2014) where they found increased expression of *PAL* and *PR* genes when treated with eugenol during infection of yellow leaf curl virus and stated that eugenol plays a key role during disease resistance. Treatment of tomato plant with eugenol recorded the high expression level of *PAL* and *PR* genes against yellow leaf curl virus suggesting a key role of eugenol during disease resistance.

Peroxidase activity was high in the clove oil treatment which is known to catalyze the reactive oxygen species and other lignans to strengthen the antioxidant systems by reinforcing the cell wall through cross-linking with hydroxyproline-rich glycoprotein-like molecule. This can explain the reduction of hydrogen peroxide levels in response to clove oil application (Figure 8a). Interestingly, clove oil also reduced total ROS (Figure 8b) suggesting a link between clove oil application, antioxidant defenses, and reduction of ROS. Previously, it was shown that ROS and NO react and form peroxynitrite ($ONOO^-$) which leads to localized cell death (Bellin et al., 2013). In the case, clove oil application NO levels have increased but ROS levels were reduced. The specific ratio of ROS might play role in clove oil-induced plant defense responses but the determination of specific ratios of NO/ROS and peroxynitrite levels in future can give clues on molecular mechanism.

Treatment of clove oil reduced the bacterial leaf spot of tomato by activating defense response (Lucas et al., 2012). Interestingly, Wang and Fan (2014) stated that the treatment of tomato plants with eugenol induces hydrogen peroxide

burst which triggers different immune responsive genes resulting in systemic resistance in the host against the virus. In Newhall navel orange, treatment with clove extracts increases the activity of catalase and peroxidase during postharvest storage led to increased shelf life (Zeng et al., 2012).

Clove oil additionally triggers the activity of callose synthase, and deposition of callose recorded a gradual increase over the different time points inducing systemic resistance against pathogen. The deposition of callose acts as a physical barrier against the pathogen, and these depositions occur between the cell wall and plasma membrane during biotic stress. Treatment of clove extract in Newhall orange increased the activity of glucanase and chitinase gene during postharvest storage decreasing postharvest decay. Further, callose deposition also reported acting as a structural barrier in pomegranate against XAP (Kumar & Mondal, 2013). Earlier studies indicated that CS was also triggered by methyl salicylate, chitosan (Zhang et al., 2002), dichloroisonicotinic acid (Sparla et al., 2004), and jasmonic acid (Jaiti et al., 2009).

6 | CONCLUSION

From the above data, we provide unequivocal evidence that clove oil is an effective agent having both antimicrobial and plant immunity-inducing activities via activation of nitrate reductase and nitric oxide production, a novel result obtained in the study. Foliar application of clove oil at 0.2% and its use in combination with copper molecule effectively reduces bacterial blight disease incidence in pomegranate under greenhouse and field conditions; thus, it presents as an alternative option to synthetic antibiotics in disease management practice. We found the increased yield of pomegranate with clove oil application; hence, this can be a very good strategy to improve the yield of this fruit crop to enhance food security under bacterial blight disease prevailing conditions. In the future, application and testing of clove oil in wide range of crops can help in enhancing crop resistance to various pathogens and ultimately contribute for increasing yield to achieve the food security.

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CONFLICT OF INTERESTS

The authors declare no conflict interests.

AUTHOR CONTRIBUTIONS

GM and JS designed the experiments. PK, VL, and PD performed experiments. BSM guided PK. JGK designed the nitric oxide and ROS-related work. AK and PS performed experiments designed by JGK.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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