

**ETHYLENE MEDIATED RESISTANCE AGAINST
BACTERIAL BLIGHT IN POMEGRANATE AND ITS
INFLUENCE ON PHENOLOGICAL CHARACTERS**

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**ETHYLENE MEDIATED RESISTANCE AGAINST
BACTERIAL BLIGHT IN POMEGRANATE AND ITS
INFLUENCE ON PHENOLOGICAL CHARACTERS**

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By

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CERTIFICATE

This is to certify that the thesis entitled **“ETHYLENE MEDIATED RESISTANCE AGAINST BACTERIAL BLIGHT IN POMEGRANATE AND ITS INFLUENCE ON PHENOLOGICAL CHARACTERS”** submitted by **Mr. MAHESH S. DASHYAL, UHS17PGD220** for the degree of **DOCTOR OF PHILOSOPHY** in **PLANT PATHOLOGY** to the University of Horticultural Sciences, Bagalkot, is a record of bonafied research work done by him during the period of his study at University of Horticultural Sciences, Bagalkot under my guidance and supervision and the thesis has not previously been formed the basis of the award of any other degree, diploma, associateship, fellowship or similar titles.

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(MAHESH S. DASHYAL)



***Affectionately Dedicated
to***

***My Everloving Parents
Farming Community and
Teachers***



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LIST OF ABBREVIATIONS

Sl. No.	Abbreviations	
1	%	Per cent
2	mm	Millimetre
3	^o C	Degree Celsius
4	µg	Microgram
5	ppm	Parts per million
6	cm	Centimetre
7	ml	Millilitre
8	mg	Milligram
9	CD	Critical difference
10	S.Em	Standard error of mean
11	µL	Microlitre
12	pH	Potential Hydrogen
13	ha	Hectare
14	H	Hours

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1. INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to family Lythraceae, an ancient favorite fruit of tropical and sub-tropical regions of the world. The fruit rind, seeds and pulp comprises of rich medicinal properties (Kirankumar *et al.*, 2018). It is a native of Iran and it is widely cultivated throughout India, Iran, China, Turkey, USA, Spain, Azerbaijan, Armenia, Afghanistan, Uzbekistan, the Middle East, Pakistan, Tunisia, Israel, dry regions of Southeast Asia, Peninsular Malaysia, the East Indies and tropical Africa (Sharma *et al.*, 2014).

The name *Punica granatum* derived from two Latin words ‘*Pommum*’ (apple) and ‘*granatus*’ (grainy) literally means ‘apple with many seeds’. *Punica granatum* with chromosome number $2n=16$ or 18 is the only available cultivated species for commercial production (Shilkina, 1973).

Pomegranate is considered as fruit of paradise because of its attractive colour, delicacy and has important dietary components (Ed Stover and Mercure, 2007). It is a good source of carbohydrates and minerals like calcium, iron and potassium and possess high pharmaceutical and therapeutic value. About 100 g of aril possess 80.97 g water, 0.95 g of protein, 17.17 g of carbohydrates, 259 mg of potassium, 3 mg of calcium, 0.3 mg of iron, 6.1 mg of vitamin C and 0.3 g of niacin, 0.03 g of thiamine and riboflavin each (Dhinesh and Ramasamy, 2016). Pomegranate has a wide consumer preference due to its attractive, juicy, refreshing arils and also for its different value added products like juice, jam, jelly, syrup, squash, anar-rub, wine and an acidulant known as anardhana (Saxena *et al.*, 1984; Dhinesh and Ramasamy, 2016).

In India's ancient Ayurveda system of traditional medicine, the pomegranate is frequently described as an ingredient in remedies (Jindal and Sharma, 2004). Due to its multipurpose medicinal uses, it is also known as “Dadima” in Ayurveda (Paranjpe, 2001) and as “Super fruit” in the global functional food industry (Martins *et al.*, 2006). Numerous studies reported that phytochemical have been identified from many parts of the pomegranate tree (peel, juice and seeds of pomegranate fruit) (Elfalleh *et al.*, 2011). Some of the beneficial health effects of pomegranate are due to the phenolic compounds contained in this fruit. Pomegranate is rich in antioxidants such

as phenolic acids, tannins, flavonols and anthocyanins. Hydrolyzable tannins including ellagitannins and gallotannins constitute the most prevalent compounds present in pomegranate and the predominant hydrolyzable tannin in pomegranates is known as punicalagin (Gómez-Caravaca *et al.*, 2013). Ellagic acid and punicalagin makes pomegranate fruit a potent source of antioxidants and also exhibits anti-bacterial, anti-tumor, anti-diabetic, anti-inflammatory, anti-cancer and anti-aging property. These are used to cure harmful diseases like atherosclerosis and skin cancer. In addition, the fruit also help in lowering blood pressure and protects skin from ultraviolet radiation (Viuda-Martos *et al.*, 2010). Fruit extract is used to treat jaundice, diarrhoea, dyspepsia and also found beneficial in treating Leprosy. Peel extract of pomegranate has hepatoprotective potential and is used to reduce obesity and to cure infant brain ischemia (Middha *et al.*, 2013). Dried and pulverised flower buds are used against bronchitis and its decoction is known to relieve oral and throat inflammations (Bhowmik *et al.*, 2013).

India is the major pomegranate growing country in terms of area and production. India contributes in the world pomegranate production with around 2.79 MT from the area of 0.25 M ha (Anonymous, 2018-19). There has been a steady increase in area and production in the country and by the year 2025, the area under pomegranate is projected to increase to 7.5 lakhs ha, from 1.25 lakhs ha at present (Pawar and Dingre, 2020). The leading pomegranate growing states of India are Maharashtra (70.2%), Karnataka (10%), Gujarat (7.4%), Andhra Pradesh (6.7%), Telangana (1.9%), Madhya Pradesh (1.9%), Tamil Nadu (1.0%) and Rajasthan (0.4%) (Khushboo and Desai, 2018).

Pomegranate crop has adaptability to a wide range of climatic conditions, hardy nature, low water requirement and good response to hi-tech practices, higher yield and returns on investment than many crops of dry regions of the world. Further, its therapeutic values and an increasing demand for table and processed products in local and export markets have made pomegranate a popular fruit of tropical and sub-tropical regions in the recent times (Sharma *et al.*, 2014).

India is the largest producer of pomegranates in the world, but accounts only 7 per cent share of total world exports when compared with other countries (Ganeshkumar *et al.*, 2016). Lack of adoption of modern technologies, improper

training and pruning methods, nutrient and water management and problems like fruit cracking and pest, diseases are major bottlenecks for production of quality fruits.

Earlier pomegranate was less prone to pest and diseases, but due to recent advancements like mono culturing *i.e.*, cultivation of pomegranate in large areas worldwide lead to emergence of new pest and diseases in severe form. Among them, bacterial and fungal diseases are prominent in pomegranate production regions, leading to considerable fruit losses throughout the growing season. Worldwide more than 55 pathogens have been reported to cause around 37 different diseases on pomegranate. The major ones in India are bacterial blight of pomegranate (caused by *Xanthomonas axonopodis* pv. *punicae*), wilt diseases (caused by *Ceratocystis fimbriata* alone and/or root-knot nematode *Meloidogyne incognita*), Phytophthora blight (*Phytophthora* spp.), fruit and leaf spots caused by fungal pathogens (*Colletotrichum gloeosporioides*, *Cercospora punicae*, *Alternaria alternate*) and fruit rots (including those caused by *Aspergillus* sp., *Penicillium* sp.) (Sharma *et al.*, 2020).

Among many diseases and pests, bacterial blight (*Xanthomonas axonopodis* pv. *punicae*) is very devastating and has become an increasingly serious threat for pomegranate. For the first time in India, bacterial blight of pomegranate was reported by Hingorani and Mehta (1952). Later on during 1959, Hingorani and Singh took a thorough investigation on the disease and causal organism and designated the pathogen as *Xanthomonas punicae* sp. Nov. Chand and Kishun (1991) reported the epidemic form of disease in Bangalore caused 60 – 80 per cent loss during 1991. Later, on the basis of DNA homology, the pathogen has been renamed as *Xanthomonas axonopodis* pv. *punicae* (Vauterin *et al.*, 1995). Presently it has a serious outbreak in all major pomegranate-growing states resulting in a major economic loss. The plant is susceptible to blight during all stages of growth and results in huge economic loss. In India, bacterial blight alone reduces the yield between 60 to 80 percent (Prasannakumar *et al.*, 2020).

Natural openings like stomata, hydathods, lenticels and nectarines become portals of entry to the bacterial pathogens. Small (2-5 mm) irregular water soaked lesions appear on leaves, later become dark brownish black with necrotic centre surrounded by yellow hallow. In advanced stages yellow hallow might not be visible; lesions may coalesce and often extend to veins and the midrib leads to premature leaf

fall. Lesions on stem may girdle or break at the point of infection which prominently occur at nodes called as nodal blight. Water soaked symptoms appear at initial stage of fruit later increases its size, turn dark brownish black and cause necrosis. Later 'L' or 'Y' shape cracking leads to splitting of fruit makes it unfit for consumption and for market (Benagi and Kumar, 2011).

Pomegranate requires careful diagnosis and timely handling to protect the crops from heavy losses. Due to unavailability of resistant cultivar management of bacterial disease is becoming great challenge day by day.

Bacterial blight is deadly disease and management is really challenging and only option for this is integrated disease management. Most of the studies on pomegranate are aiming at management of pathogen with poor attention has been given to understand plant resistance. Further in plant array of signals, hormones, growth regulators are implicated in disease resistance.

During the co-evolution of plants and their associated microbes, plants developed a complicated immune system against microbial pathogens, which genetically coordinates various defense pathways that lead to different outcomes. Plant immune responses to pathogens rely on rapid sensing of pathogen-associated molecular patterns (PAMPs) by defined pattern recognition receptors (PRRs) that initiate PAMP-triggered immunity (PTI) or resistance (R) proteins that recognize effector proteins secreted by microbial pathogens to activate a strong disease-resistance response (effector-triggered immunity, ETI). ETI is usually associated with hypersensitive response (HR, a form of programmed cell death or autophagy) at sites of infection (Block and Alfano, 2011) and may activate secondary immune responses in distal uninfected tissues, resulting in so-called systemic acquired resistance (SAR). During these immune responses, plant hormones such as salicylic acid (SA), jasmonates (JAs) and ethylene (ET) act as signals to trigger and mediate a diverse array of defense responses (Spoel and Dong, 2008). However ethylene is one hormone has been used for different developmental and reproductive parameter manipulations. On other hand ethylene is implicated as hormone of disease resistance.

The gaseous hormone ethylene is known to regulate multiple physiological and developmental processes in plants, such as seedling emergence, leaf and flower

senescence, ripening and organ abscission and is also involved in the reactions of plants to abiotic and biotic stresses. Ethylene has dual action, sometimes it acts as a virulence factor of the pathogen but, other times, the activity of other pathogens is affected negatively (Van Loon *et al.*, 2006). Ethylene often modulates defense signaling pathways, including those regulated by jasmonic acid and salicylic acid. Hormonal signal integrations with ethylene steer the defense signaling network to activate specific defenses that can have direct effects on attackers or systemically prime distant plant parts for enhanced defense against future attack. Conversely, ethylene signaling can also be exploited by attackers to hijack the defense signaling network to suppress effective defenses (Broekgaarden *et al.*, 2015).

Management of disease involves the spraying of expensive chemicals which in turn increases the cost of pomegranate production and these chemicals create residue in plant and soil thus, act as hazardous to environment. Antibiotics are becoming ineffective to the pathogen. Therefore, there is a need for development of an alternative eco-friendly measures and novel strategies are very much needed. Keeping all these aspects in view, the present investigation was undertaken with the following objectives:

1. Understanding the role of ethylene concentration on pathogen/disease development in plant.
2. Microbe induced ethylene regulation in pomegranate and its effect on disease development.
3. Bio efficacy of ethylene against bacterial blight and phenological characters in field conditions.
4. Elucidation of resistance with other hormones.

2. REVIEW OF LITERATURE

In pomegranate production, farmers use ethylene for leaf shedding and which in turn increase flower production. But, the information is not available or the effect of ethylene is unknown on bacterial blight incidence and also effect of ethylene application at low concentration on production of productive flowers is unknown. To know the effect of ethylene on pomegranate phenological characters and disease resistance present study was undertaken. Research findings in these areas are summarized and presented here under.

2.1 Disease occurrence

Oily spot disease was first reported in India from IARI, Delhi on leaf (Hingorani and Mehta, 1952). The disease incidence was also recorded from Bangalore, Karnataka and causative organism was identified as *Xanthomonas punicae* (Hingorani and Singh, 1959). Hingorani and Metha (1952) and Chand and Kishun (1991) isolated the causal organism and based on morphological, cultural, biochemical and physiological characters, later based on homology it was renamed as *Xanthomonas axonopodis* pv. *punicae* by Vauterin *et al.* (1995). The appearance of oily spot on pomegranate leaves was reported from Annamalainagar, Tamil Nadu state of India (Rangaswamy, 1962). The incidence of disease on leaves and fruits from Himachal Pradesh was recorded by Sohi *et al.* (1964). The disease was also recorded from various parts of Haryana (Kanwar, 1976), Karnataka (Chand and Kishun, 1987), Punjab (Rani and Verma, 2002), Rajasthan (Sharma *et al.*, 2010). The disease was also observed in South Africa (Petersen, 2010) and Turkey (Icoz *et al.*, 2014).

2.2 Its incidence and severity

Bacterial blight which was known as minor disease, turned into major disease in 1991 leading towards 60-70 per cent yield loss at IIHR experimental plots, Bangalore (Chand and Kishun (1991). Benagi and Kumar (2011) reported that the highest disease severity (74.80%) was observed in Bagalkot whereas minimum (6.73%) was recorded in Bellary district in Karnataka state. Further, sudden outbreak of disease was observed after 2002, leading to severe yield loss both in terms of quality and quantity. During 2008-09, disease has reached an alarming stage, bringing

substantial damage to the crop and caused heavy loss for pomegranate growers. According to a survey data, 41.50 per cent of disease incidence was noticed in Solapur, Sangli, Pune, Ahmednagar, Nasik, Osmanabad, Aurangabad, Jalna and Latur districts of Maharashtra (Sharma *et al.*, 2015). In Karnataka, major pomegranate growing districts like Bagalkot, Bijapur, Gadag and Koppal districts reported 58.33 per cent of disease incidence whereas Anantapur district of Andhra Pradesh having 75 per cent of pomegranate area recorded 43.47 per cent disease incidence (Sharma *et al.*, 2015).

Disease severity may vary upon the season. Sharma *et al.* (2010) noticed that, its severity was greater in rainy season mainly from July to September (48.90%) compared to autumn which lies between December to February (10.50%). Raju *et al.* (2011) revealed that, maximum severity was observed in Mrig bahar crop (44.20%) compared to Hasta bahar (17.90%) and Ambe bahar (29.30%).

2.3 Symptomatology

Hingorani and Mehta (1952) described the symptoms of oily spot disease as irregular lesions varying from 2 to 5 mm in size, initially appearing on the leaves, adjoining spots integrate and cover larger areas. Initially the spots were light brown in colour, surrounded with water soaked margin, later turned into deep brown as the disease progressed. The appearance of several spots on leaf lead to shedding of leaves later. Spots were not observed on twigs, branches and fruits at initial stages.

Rangaswamy (1962) recorded the symptoms of bacterial blight on leaves as necrotic spots or lesions surrounded by chlorotic halos or yellowing with translucent water-soaked appearance. As the severity of infection increased, leaves became distorted and shed off. Small, brown, water soaked spots were observed on leaves, flowers and fruits of pomegranate in different orchards of Haryana (Kanwar, 1976; Kishun, 1993 and Manjula and Khan, 2002). Pomegranate fruits were also found infected with water soaked lesions in the initial stages followed with dark brown, slightly raised surface with oily appearance, finally leads to necrotic symptoms on fruit. At severe stage of infection it leads to 'L' or 'Y' shape cracking on fruits (Sharma *et al.*, 2010). In the early stages, spots on leaves were small, circular or irregular with light yellow border and brownish in centre. Later on, number of neighboring spots mixed with each other and formed elongated and irregular lesions.

2.4 Favourable factors for disease incidence

2.4.1 Initial inoculum and secondary spread

Infected parts of pomegranate like fruits, leaves, twigs act as primary source of inoculums. Secondary spread may occur through planting materials, windblown rain splash, through runoff water or irrigation water. Contaminated tools and insects also may favour movement of disease from one place to another. Moreover, natural openings like stomata, lenticels, hydathodes or mechanical damage act as main portal for entry of pathogen (Ramesh and Ram, 1991).

2.4.2 Environmental factors

Bacterial blight pathogen may survive in temperature varied from 9-43°C and at lower humidity. Temperature of 30°C with relative humidity between 30-70 per cent or beyond 80 per cent is favourable for bacterial development (Sharma *et al.*, 2010; Sharma *et al.*, 2015). According to Jadhav and Sharma (2011), its infection may become severe under high humid condition where its relative humidity goes beyond 80 per cent.

Sharma *et al.* (2015) indicate that moderate temperatures (23.1-37.3°C), high humidity (>80%), and frequent rains were highly favorable for blight build-up and spread during May to July.

2.5 Pathogenicity test

Chand and Kishun (1991) performed pathogenicity test by inoculating the bacterial culture (2×10^8 CFU/ml) on 40 days old leaves by pinprick, rubber block pressure, leaf cut, atomization methods. They reported that leaf cut was effective method with 100 per cent infection covering 70-90 per cent leaf area within 21 days while atomization induced lowest infection of 6-7.5 per cent with incubation period of 17-40 days.

Raghuwanshi *et al.* (2013) used healthy seedlings of pomegranate cultivar Bhagwa and kept in the humid chamber for about 2 to 3 days before inoculation. Then inoculated veinlets from one set of plant leaves and a set of fruits were injected with 48 hours old bacterial culture with the help of hypodermal syringes. The plants and fruits were kept for incubation in moist chamber. A set of control was maintained for

each set which were sprayed with distilled water only. Warm and humid conditions were maintained in the chamber by spraying sterilized water. The organism was re-isolated on sucrose containing nutrient agar medium from artificially inoculated leaves and fruits developed typical symptoms of disease after 5 days.

Bora and Kataki (2014) performed pathogenicity test by spraying 48 hours old bacterial culture (2×10^8 CFU/ml) on pinpricked 40 days old healthy pomegranate leaves. Infection occurred within 23 days of inoculation and produced identical symptoms observed on infected plant showing symptoms of bacterial blight on pomegranate plant. The organism re-isolated from artificially inoculated plant yielded an organism similar to one used for inoculation experiments.

Patel *et al.* (2015) performed pathogenicity test based on Koch's postulates to prove pathogenic nature of *Xanthomonas* isolates by spraying of 48 hours old bacterial culture onto the injured and uninjured leaves, stem and fruits of healthy pomegranate plants. The characteristics symptoms were observed on pomegranate leaves after four days of inoculation as small water soaked lesions, which turned brown to black colored after six days, later developed into angular to irregular shaped spots along the margins, veins and veinlets of the leaf lamina. Re-isolation was carried out from those lesions for each isolate and comparisons were made with original culture to confirm the identity of the pathogen. The re-isolated culture resembled the original mother culture and thus pathogenicity test was confirmed.

2.6 Cultural characteristics of the pathogen

Chand and Kishun (1991) and Manjula and Khan (2002) collected infected leaves and fruit samples showing typical symptoms of bacterial blight disease from suspected pomegranate plants and subjected for isolation of the pathogen within 48 hours of sample collection on nutrient agar medium by streak plate method. All the diseased samples yielded yellow pigmented, mucoid, circular, convex, rounded, glistening, raised colonies after incubation at $27 \pm 1^\circ\text{C}$ temperature. Chopade *et al.* (2014) isolated *Xanthomonas axonopodis* pv. *punicae* on nutrient glucose agar medium (peptone -5 g, dextrose-10 g, beef extract powder -3 g, agar -20 g, distilled water- 1 L).

Nine isolates of *Xanthomonas axonopodis* pv. *punicae* were revived from stored culture by streaking on Yeast Peptone agar (yeast extract 5 g, peptone 10 g, agar 20 g and water 1 L, pH 7.0) plate and incubated at 28°C for 72 to 96 hours. The well separated fluidal colony of *Xanthomonas axonopodis* pv. *punicae* isolates were selected and single colony of each isolate was stored in 50 per cent glycerol at -70°C for further study by Mondal *et al.* (2012a).

The bacteria were isolated from the infected leaves, small twigs and fruits of pomegranate on sterile nutrient agar medium with sucrose (NAS). The inoculated plates were incubated at 28±2°C for 24-72 hours. After 2-3 days, incubated plates were observed for the presence of typical pale yellow, glistening colonies which were transferred to nutrient agar medium with sucrose slants and maintained on yeast extract glucose agar with charcoal slant for further study by Raghuwanshi *et al.* (2013) and Digvijay *et al.* (2014).

Bora and Kataki (2014) carried out isolation of the pathogen from infected leaves, fruits of pomegranate and observed yellow pigmented mucoid, circular, convex, rounded, glistening, raised colonies after 3 days of incubation at 27±1°C temperature on nutrient agar medium by streak plate method.

2.7 Molecular identification of the pathogen

Petersen *et al.* (2010) performed direct polymerase chain reaction for analysis of typical yellow colored colonies of pathogen of bacterial blight on pomegranate using *Xanthomonas*- specific primers for *gyrB* gene, Xgyrconpcr F1 and Xgyrconrpcr1.

Kale *et al.* (2012) reported that restriction digestion of plasmids confirmed that this bacterium has mega plasmid of size around 200 kbp which is typical of genus *Xanthomonas*.

Mondal *et al.* (2012b) reported the identity of the *Xanthomonas axonopodis punicae* (Xap) isolates that was confirmed through 16S rRNA gene based universal primers, Forward 5'-AGAGTTTGATCCTGGCTAG-3' and reverse 5'-AGGAG GTGAT CCAGCCGCA-3'. The pathogenicity of the Xap isolates was confirmed following Koch's postulates. Further they have developed a polymerase chain

reaction (PCR) based detection technique for this pathogen using primers designed from *gyrB* gene. A primer set KKM5 and KKM6 was synthesized based on sequence alignment of 530 nucleotides of C-terminus region in the *gyrB* genes from 15 different bacterial strains. The primer set was validated for amplification of 491bp of *gyrB* gene. No amplification was observed in other phytopathogenic Xanthomonads including *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *mangiferaeindicae*, *Xanthomonas campestris* pv. *manihotis*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas axonopodis* pv. *axonopodis* and *Pantoea agglomerans*. They developed a technique that could detect the pathogen in infected pomegranate plant samples including leaf, fruit and stem within 3 hrs at a detection limit 0.1 ng μL^{-1} template DNA.

Mondal and Shanmugam (2013) mentioned various methods for routine diagnosis of bacterial pathogens, integrated approaches including 16S rDNA sequencing, multi locus sequence typing (MLST), Biolog-phenotyping, fatty acid methyl esterase (FAME) profiling and pathogenicity assay following Koch's postulates. They have described different methods of identification of plant pathogen based on the recent advancement in the area of use of PCR based approaches. These are real-time PCR, multiplex PCR, nested PCR, Bio-PCR, repetitive PCR, Loop-mediated isothermal amplification (LAMP) are among the detection options that provides rapid data analysis with specificity. However, they further stated that one has to choose various serology-based techniques consisting of both poly and monoclonal antibodies that are used for the detection of phytopathogenic bacteria using ELISA assay for the diagnosis of bacterial plant pathogens.

2.8 Understanding the role of ethylene concentration on pathogen/disease development in plant

Exogenously applied ethylene at 5 to 100 $\mu\text{L/litre}$ had increased β -1, 3-glucanase activity in soybean hypocotyls approximately 2-fold $\mu\text{L/litre}$ 2 days after the treatment. Inoculation of ethylene-pretreated plants with a compatible race of *Phytophthora megasperma* f. sp. *glycinea* resulted in partial resistance to the fungus as revealed by decreased rates of hyphal colonization in host tissue and higher levels of glyceollin. Increase of β -1,3-glucanase activity in ethylene-treated plants were

noticeable after 12 h and glucanase activity further increased for 2 to 3 days. Partial resistance to the fungus was expressed when hypocotyls were inoculated at 24 h after ethylene treatment (Yoshikawa *et al.*, 1990).

Treatment of sweet oranges with lower concentration before five days of pathogen inoculation showed resistance towards disease development (citrus stem end rot) when compared to fruits inoculated with pathogen (*Diplodia natalensis*) two days after ethylene treatment (Brown and Lee, 1993).

A tomato mutant impaired in ethylene perception *Never ripe* exhibited a significant reduction in disease symptoms in comparison to the wild type after inoculations of both genotypes with virulent bacterial (*Xanthomonas campestris* pv *vesicatoria* and *Pseudomonas syringae* pv *tomato*) and fungal (*Fusarium oxysporum* f sp *lycopersici*) pathogens. Bacterial spot disease symptoms were also reduced in tomato genotypes impaired in ethylene synthesis (1-aminocyclopropane-1-carboxylic acid deaminase) and perception, thereby corroborating a reducing effect for ethylene insensitivity on foliar disease development (Lund *et al.*, 1998).

Disease resistance or susceptibility induced by ethylene is mainly dependent on time of application or on its application level. Ethylene levels were manipulated in *V. dahliae*-infected tomato plants by the application of an ethylene synthesis inhibitor aminoethoxy vinyl glycine (AVG) and/or ethylene's biosynthetic precursor 1-aminocyclopropane-1-carboxylate (ACC) and the effects on disease severity were examined. Statistically significant reductions in disease severity were consistently obtained for AVG-treated plants that had ACC added at the time of inoculation. A model is therefore proposed in which ethylene treatment at post-infection enhances *Verticillium* wilt development in tomato whereas its presence at the time of infection inhibits disease development (Robison *et al.*, 2001).

Ethylene play important roles in plant defense responses to pathogens. To investigate the contributions of ethylene in resistance of tomato (*Lycopersicon esculentum*) to the fungal pathogen *Botrytis cinerea*, experiment was conducted. Plants pretreated with ethylene showed a decreased susceptibility toward *B. cinerea*, whereas pretreatment with 1-methylcyclopropene, an inhibitor of ethylene perception, resulted in increased susceptibility. Ethylene pretreatment induced expression of

several pathogenesis-related protein genes before *B. cinerea* infection. Proteinase inhibitor I expression was repressed by ethylene and induced by 1-methylcyclopropene. Ethylene also induced resistance in the mutant *Never ripe*. The results confirmed that ethylene responses are important for resistance in tomato to *B. cinerea* (Diaz *et al.*, 2002).

Treatment of *Arabidopsis* seedling with 1 mM ACC (1-aminocyclopropane-1-carboxylic acid) enhanced resistance against *Pseudomonas syringae* pv. *tomato* and this is regarded as induced systemic resistance (ISR) which is elicited by specific strains of nonpathogenic, root-colonizing bacteria, such as *Pseudomonas fluorescens* strain WCS417 (Hase *et al.*, 2003).

Pathogenesis related gene expression in rice was found to be tightly regulated in a dose-dependent manner at very low ethylene concentrations but was significantly decreased with higher concentrations. Treatment with 0.25 mM ethephon (applied as drench, foliar, or foliar-drench), which released ethylene and lowered blast index of cultivars Newbonnet, LaGrue and Cypress grown in upland. If flooded, blast index of ethephon-treated cultivars were decreased by drench and foliar-drench applications (Singh *et al.*, 2004).

The fungus *Botrytis cinerea* infects the tomato and bean plants. In this study the effect of ethylene on disease development due to *B. cinerea* on tomato and bean plant was studied. The role of exogenously applied ethylene (Ethephon) in the interaction between *B. cinerea* and tomato and bean plants was studied *in vitro* and *in vivo*. Ethephon® has not significantly affected the fungus mycelium growth rate on potato dextrose agar (PDA), while it significantly increased disease severity by 50.4 per cent to 55.8 per cent on tomato plants and by 27.6 per cent to 33.8 per cent on bean plants at concentration 200-600 µg/ml. Aminoethoxyvinylglycine (AVG) significantly decreased mycelium growth *in vitro* at 200 µg/ml by 47 per cent and it decreased disease severity by 45 per cent to 49 per cent at concentrations 200 -300 µg/ml on tomato plants and 55 per cent to 75 per cent at 100-300 µg/ml on bean plants (Hardan *et al.*, 2005).

Pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus*, is the most serious disease of pine tree with great economic losses. Ethylene acts as a signal transduction material to cause terpenoid produce in pine

trees. Ethylene product (0.1–1% ethrel) was inoculated into seedlings which increased several times greater in the disease development which began a few days earlier than the water potential decrease and chlorosis in needles. Ethylene increase occurred synchronously with cambial death and they were followed by water deficiency in leaves (Wang *et al.*, 2010).

One of the most dangerous diseases of corn is *Ustilago maydis* (DC.) *corda*, which damages the maize yield every year. Aim of this research was to examine the possible compensation effects of gibberellin (GA3) and ethylene (as ethrel). Ethylene treatment increased while gibberellin treatment reduced the impact of the infection (Szóke *et al.*, 2020).

A possible role for ethylene in the interaction of *Arabidopsis thaliana* and the obligate biotrophic protist *Plasmodiophora brassicae*, causing clubroot disease, has been investigated. The ethylene (ET) precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was lower in infected compared to control roots during early time points of the interaction. One gene for ACC oxidase was consistently upregulated in infected roots, whereas transcriptional regulation of other ET biosynthesis genes varied according to the time point during infection. The ET-overproducing mutant *eto2* was slightly less susceptible to the clubroot pathogen. The analysis of mutants within the ET signaling pathway showed that they were more susceptible to the clubroot pathogen than wild-type Columbia, where in formed larger galls (Knaust and Ludwig-Müller, 2013).

Ethylene pretreatment exhibited enhanced disease resistance with significantly less fungal biomass than the mock control plants. However, plants pretreated with 1-Methylcyclopropene (1-MCP) were more susceptible to *Magnaporthe oryzae*, as revealed by the higher fungal biomass in the treated leaves than in the control leaves. These results imply that activation of ethylene signaling is indispensable for resistance to *M. oryzae* in rice (Yang *et al.*, 2017).

Phytohormones, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA), play key roles in plant defence following pathogen attack. Here, experiment was conducted to investigate their involvement by the analysis of the expression of ET marker genes following *Fusarium oxysporum* f. sp. *lycopersici* (Fol) infection and by bioassays of tomato mutants affected in either hormone production or perception. Fol

inoculation triggered the expression of SA and ET marker genes, showing the activation of these pathways. In ACD and Never ripe (Nr) mutants, in which ET biosynthesis and perception, respectively, are impaired, showed decreased disease symptoms and reduced fungal colonization on infection. Results shows that SA is a negative and ET a positive regulator of Fol susceptibility (Di *et al.*, 2017).

Considering wheat as an important staple food crop and the great impact of blast, caused by *Pyricularia oryzae*, to decrease yield, this study aimed to investigate the involvement of ethylene on wheat susceptibility to blast based on its basal and induced levels in plant tissues. Plants from cultivars BRS Guamirim and BRS 220 (susceptible and partially resistant to blast, respectively) were non-sprayed or sprayed with Ethrel (0.0001 M) 24 h before inoculation with *P. oryzae*. Blast severity was higher on ethylene-sprayed plants regardless of the cultivar. High foliar ethylene concentration for inoculated plants from cultivars BRS Guamirim and BRS 220 was associated with an increase in leaf tissues susceptibility to blast. Ethylene biosynthesis on wheat leaves was triggered by *P. oryzae* infection (Rodrigues *et al.*, 2020).

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum* (Foc), is a severe disease affecting cucumber (*Cucumis sativus* L.). To better understand of the defense mechanisms elicited in response to Foc inoculation, RNA sequencing-based transcriptomic profiling of responses of the Fusarium wilt-resistant cucumber line ‘Rijiecheng’. Results shown that 4116 genes that were differentially expressed between 0 h and other time points after inoculation. Real-time PCR analysis showed that ethylene-related genes were induced in response to Foc infection. After Foc infection and exogenous application of ethephon, the ethylene-related genes were highly expressed. In response to exogenous ethephon treatment in conjunction with Foc inoculation, the infection resistance of cucumber seedlings was enhanced. Ethylene signaling pathways play a positive role in regulating the defense response of cucumber to Foc infection (Dong *et al.*, 2020).

2.9 Microbe induced ethylene regulation in pomegranate and its effect on disease development

Pseudomonas fluorescens strain CHA0, a root colonizing bacterium, has a broad spectrum of biocontrol activity against plant diseases. An ACC deaminase gene, together with its regulatory region, was transferred into *P. fluorescens* strains

CHA0 and CHA96, a global regulatory *gacA* mutant of CHA0. ACC deaminase activity was expressed in both CHA0 and CHA96. Introduction of ACC deaminase genes into strain CHA0 improved its ability to protect cucumber against *Pythium* damping-off and potato tubers against *Erwinia* soft rot in small hermetically sealed containers. In contrast, ACC deaminase activity had no significant effect on the ability of CHA0 to protect tomato against *Fusarium* crown and root rot and potato tubers against soft rot in large hermetically sealed containers. These results suggest that ACC deaminase activity may have lowered the level of plant ethylene. The role of stress-generated plant ethylene in susceptibility or resistance depends on the host-pathogen system and on the experimental conditions used (Wang *et al.*, 2000).

Ethylene evolved during compatible or susceptible disease interactions may hasten and/or worsen disease symptom development. Study was conducted to examine the effect of reduced ethylene synthesis in ACC deaminase gene transformed tomato plant infected with by *Verticilium dahliae*. In ACC deaminase transformed tomato plant, pathogen was detected in stem sections of plants with reduced symptoms, suggesting that reduced ethylene synthesis results in increased disease tolerance (Robison *et al.*, 2001).

Indiragandhi *et al.* (2008) studied the induction of defense responses in tomato elicited by *Methylobacterium oryzae* CBMB20. Significantly increased activities of pathogenesis-related (PR) proteins and defense enzymes such as β -1,3-glucanase, phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase were noted in *M. oryzae* CBMB20 pretreated and challenged with *Pseudomonas syringae* pv. *tomato* (Pst) compared to either control or *M. oryzae*-treated tomato plants. Increased PR proteins and defense enzyme activities were correlated with the reduction of stress ethylene level. Reduced ethylene level led to reduction of the disease severity (26%) in *M. oryzae* CBMB20-treated plants challenged with pathogen.

In addition to the well-known roles of indoleacetic acid and cytokinin in crown gall formation, the plant hormone ethylene also plays an important role in this process. Many plant growth-promoting bacteria (PGPB) encode the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can degrade ACC. To study the effect of ACC deaminase on crown gall development, an ACC deaminase gene from the PGPB *Pseudomonas putida* UW4 was introduced into *Agrobacterium*

tumefaciens C58, so that the effect of ACC deaminase activity on tumour formation in tomato and castor bean plants could be assessed. Plants were also coinoculated with *A.tumefaciens* C58 and *P.putida* UW4 or *P.putida* UW4-acdS (an ACC deaminase minus mutant strain). In both types of experiments, it was observed that the presence of ACC deaminase generally inhibited tumour development on both tomato and castor bean plants (Hao *et al.*, 2007).

The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase cleaves the ethylene precursor ACC into alpha-ketobutyrate and ammonia. The decreased level of ethylene allows the plant to be more resistant to a wide environmental stress including plant pathogens. Present study focused on to characterize the ACC deaminase activity of a *Pseudomonas entomophila* strain PS-PJH isolated from the red pepper rhizosphere region grown at Jinan, Korea. The isolate produced 23.8 +/- 0.4 micromol of alpha-ketobutyrate/mg of protein/h during ACC deamination under *in vitro* conditions. Results of the multiple sequence alignment revealed more than 99 per cent identity (nucleotide and amino acid) with acdS gene of *Pseudomonas putida* strains AM15 and UW4. The isolated bacteria promoted 43.3 and 34.1 per cent of growth in *Raphanus sativus* and *Lactuca sativa* plants, respectively (Kamala-Kannan *et al.*, 2010).

Viterbo *et al.* (2010) evaluated the 1-Aminocyclopropane-1-carboxylate deaminase (ACCD) activity in the biocontrol and plant growth-promoting fungus *Trichoderma asperellum* T203. Fungal cultures grown with ACC as the sole nitrogen source showed high enzymatic activity. The enzyme encoding gene (*Tas-acdS*) was isolated and an average 3.5-fold induction of the gene by 3 mM ACC was detected by real-time PCR. RNAi silencing of the ACCD gene in *T. asperellum* showed decreased ability of the mutants to promote root elongation of canola seedlings.

Treating roots of tomato (*Solanum lycopersicum*) seedlings in a suspension of the ACC deaminase-producing *Pseudomonas putida* UW4, *Burkholderia phytofirmans* PsJN or *Azospirillum brasilense* Cd1843 transformed by plasmid pRKTACC carrying the ACC deaminase-encoding gene acdS from UW4, significantly reduced the development of tumours and ethylene level (four to five fold) on tomato plants injected with pathogenic *Agrobacterium* (Toklikishvili *et al.*, 2010).

Salas-Marina *et al.* (2011) studied role of *Trichoderma atroviride* in Arabidopsis growth promotion and systemic disease resistance. Results shown expression levels of JA/ET and pathogen-induced genes like PDF1.2 and LOX-1 up regulated. PDF1.2 was up regulated at 96 h post inoculation with *Trichoderma atroviride* in both roots and leaves, whereas LOX-1 underwent no significant changes in roots, but reached four- times the level of expression in leaves. The gene expression data of JA/ ethylene defense related genes suggest that *T. atroviride* induces systemic resistance in Arabidopsis through JA/ET pathway.

Amutharaj *et al.* (2012) studied bioinoculation effect of ACC deaminase positive wild strains of *Pseudomonas* and *Paenibacillus*, as coaggregates, on the maximization of ISR (Induced Systemic Resistance) against *Pyricularia oryzae* in upland rice cv. asd-19. It was observed that the application of acid positive, wild strains of *Pseudomonas* and *Paenibacillus*, together with challenge inoculation of *Pyricularia oryzae* positively altered the biochemical constituents viz., increasing total phenol content, reduction in reducing sugar and non-reducing sugar level and increased starch content of the host plant.

The use of ACC deaminase-producing plant growth promoting bacteria shown to be a useful strategy in order to reduce biotic and abiotic constraints that affect plant health and development. An inoculation assay was performed on 3-4 months *Pinus pinaster* (maritime pine) seedlings obtained from a nursery in Portugal. The bacteria *Pseudomonas putida* UW4 wild-type and ACC deaminase mutant strains were inoculated in the roots of pine seedlings followed by stem inoculation of *Bursaphelenchus xylophilus*. The inoculation of the *Pseudomonas putida* UW4 wild-type strain lead to a significant reduction of *B. xylophilus* induced symptoms. In addition, *P. putida* UW4 ACC deaminase knockout mutant was unable to promote pine seedling growth or to decrease *B. xylophilus* induced symptoms (Nascimento *et al.*, 2013).

Study conducted to test the induction of defense responses in tomato against bacterial wilt by stress ethylene level reduction mediated by the ACC deaminase activity of *Methylobacterium* strains. Under greenhouse conditions, the disease index value in *Methylobacterium* sp. inoculated tomato plants was lower than control plants. Plants treated with *Methylobacterium* sp. challenge inoculated with *Ralstonia*

solanacearum (RS) showed significantly reduced disease symptoms and lowered ethylene emission under greenhouse condition. The ACC and ACO (1-aminocyclopropane-1-carboxylate oxidase) accumulation in tomato leaves were significantly reduced with *Methylobacterium* strains inoculation. While ACC oxidase gene expression was found higher in plants treated with *R. solanacearum* than *Methylobacterium* sp. This study confirms the activity of *Methylobacterium* sp. in increasing the defense enzymes by modulating the ethylene biosynthesis pathway (Yim *et al.*, 2013).

Study aimed to reduce the bacterial spot disease incidence in tomato plants caused by *Xanthomonas campestris* pv. *vesicatoria* (XCV) by modulating stress ethylene with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity of *Methylobacterium* strains. Under greenhouse condition, *Methylobacterium* strains inoculated and pathogen challenged tomato plants had low ethylene emission compared to pathogen infected ones. ACC accumulation and ACC oxidase (ACO) activity with ACO related gene expression increased in XCV infected tomato plants over *Methylobacterium* strains inoculated plants. *Methylobacterium* spp., resulted lowest ACO related gene expression (1.46 Normalized Fold Expression) in pathogen challenged tomato. But a significant increase in ACO gene expression (7.09-folds) was observed in the bacterial pathogen infected plants (Yim *et al.*, 2014).

Contreras-Cornejo *et al.* (2015) conducted experiment to study ethylene production by *Trichoderma atroviridae*. Arabidopsis seedlings cocultivated with *T. atroviride* have an altered root architecture and greater biomass compared with axenically grown seedlings. These effects correlate with ethylene production by *T. atroviridae*. Results revealed that *T. atroviridae* grown on media supplemented with 1mM/L methionine shown increased ethylene production after four to five days of fungal growth on media. Ethylene production was confirmed by GC-mass spectrometry analysis.

Flavescence dorée is an epidemic yellows disease of grapevine, caused by a phytoplasma (*Candidatus Phytoplasma vitis*). Study was conducted to test whether the endophyte *Pseudomonas migulae* 8R6, able to synthesize 1-aminocyclopropane-1-carboxylate (ACC) deaminase, can limit the phytoplasma-induced damages in periwinkle, a model plant hosting FDP. Plant protection induced by 8R6 and its

mutant, impaired in ACC deaminase synthesis. The strain 8R6 significantly reduced the number of symptomatic plants (53%). The ACC deaminase activity of the endophytic bacteria *P. migulae* 8R6 helps the plant to regulate the level of the stress-related hormone ethylene, leading to significantly improved resistance to phytoplasma infection (Gamalero *et al.*, 2016).

Tomato cultivation is highly susceptible for soil borne diseases and among them southern blight disease caused by *Scelerotium rolfsii* is very common. As an alternative eco-friendly approach to control the disease antagonistic microbes are being characterized. Among them plant growth promoting rhizobacteria *Paenibacillus lentimorbus* B-30488 (B-30488) with antagonistic properties, multiple PGP attributes stress tolerance and ACC deaminase enzyme activity is characterized. Results were obtained from *in vivo* experiments where B-30488 inoculation has efficiently controlled the disease caused by *S. rolfsii* and improve the plant growth. Deleterious enhanced ethylene level in *S. rolfsii* infected plants was also ameliorated by inoculation of ACC deaminase producing B-30488. The ACC accumulation, ACO and ACS activities were also modulated in *S. rolfsii* infected plants (Dixit *et al.*, 2016).

Leonetti *et al.* (2017) studied ethylene biosynthesis and systemic acquired resistance (SAR) induction by treating *Trichoderma harzianum* (Th) strains ITEM 908 (T908) and T908-5 in tomato plant infected with root-knot nematode. Result revealed that colonization by Th-primed tomato roots showed higher expression of ACO (ACC-oxidase) gene and activation of ethylene biosynthesis. Induction of SAR and ethylene biosynthesis upon nematode invasion shown significant decrease of development and reproduction of the inoculated J2s and similar inhibition was reported on the infection of the second J2 generation.

Study was conducted to determine whether ACC (1-aminocyclopropane-1-carboxylic acid) deaminase-containing bacterial treatments could enhance the tolerance of poppy (*Papaver somniferum* L.) plants against biotic stress of downy mildew caused by *Peronospora* sp. Three different genotypes of *P. somniferum*, that is, Sampada, J-16 and I-14 were included in the experiment. The ACC deaminase-

containing bacteria *Pseudomonas putida* (WPTe) reduced the downy mildew disease severity and significantly improved the growth and yield of *P. somniferum* plants. The chlorophyll content, photosynthetic rate, stomatal conductance and transpiration rate were modulated upon WPTe treatments in the poppy plants. Result shown reduced synthesis of ethylene precursor (ACC) and abscisic acid (ABA) and enhanced production of indole acetic acid (IAA) in *P. somniferum* plants upon WPTe treatments. These results highlight that the ACC deaminase-containing plant growth-promoting rhizobacteria (PGPR) enhance the tolerance of *P. somniferum* plant against downy mildew (Barnawal *et al.*, 2017).

An isolate, named *Trichoderma harzianum* T-soybean, showed growth-promoting for soybean seedlings and induced resistance to *Fusarium oxysporum* under greenhouse. T-soybean treatment obviously reduced disease severity and induced quickly the H₂O₂ and O₂⁻ burst as well as pathogenesis related protein gene (PR3) expression after *F. oxysporum* inoculation and subsequently diminished the cell damage in soybean caused by the pathogen challenge. Reactive oxygen species (ROS) scavenging enzymes activity analysis showed that the activities of peroxidase (POD), polyphenol oxidase (PPO) and superoxide dismutase (SOD) increased significantly in T-soybean pretreated plants. These results suggested that T-soybean treatment induced resistance in soybean seedlings to *F. oxysporum* by accompanying the production of ROS and the increasing of ROS scavenging enzymes activity as well as PR3 expression (Zhang *et al.*, 2017).

2.10 Bio efficacy of ethylene against bacterial blight and phenological characters in field conditions

2.10.1 Bio efficacy of ethylene against bacterial blight in field condition

Leaves inoculated with *Xanthomonas campestris* pv. *citri* (Xc) had a higher endogenous 1-aminocyclopropane-1-carboxylic acid (ACC) content than the leaves treated with aminoethoxy vinyl glycine (AVG) or streptomycin before inoculation of pathogen on citrus leaves. All the inhibitors of ethylene biosynthesis tested were effective in suppression of ethylene production from Xc inoculated leaves. The inhibitors also suppressed the rate of disease progression (Dutta and Biggs, 1991).

The ethylene-insensitive *Never ripe (Nr)* mutant displays increased tolerance to the virulent strain, while maintaining resistance to the avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* in tomato. Transgenic plants overexpressing a wild-type NR cDNA were infected with virulent *X. campestris* pv. *vesicatoria* (Ciardi *et al.*, 2000).

Ethylene also played crucial role in defense responses against various biotic stresses. Pretreatment of tomato plants with ethylene (1 µl/L) resulted in an increased, yet partial, resistance to *Botrytis cinerea*, both at the level of the percentage of expanding lesions and, in some cases, the lesion expansion rate. The severity of symptom development was well correlated with the extent of fungal biomass, reflected by the *B. cinerea* actin mRNA level. Pretreatments with the strong, irreversible ethylene perception inhibitor 1-MCP resulted in a significant increase of disease (Diaz *et al.*, 2002).

Ethylene also leads susceptibility to various plant diseases. Arabidopsis mutants with reduced ethylene signalling or perception were more resistant to *Fusarium graminearum* than wild-type, while mutants with enhanced ethylene production were more susceptible. Attenuation of expression of EIN2 in wheat, a gene encoding a core component of ethylene signalling, reduced both disease symptoms and myco-toxin contamination of grain (Chen *et al.*, 2009).

Twenty-four hours later treating with ethylene, seedlings were inoculated with a virulent strain of *Phytophthora capsici*. Treatment with 5 mM ethephon prevented disease symptoms in 30 per cent of the pepper seedlings. Interestingly, blocking the ethylene receptor with application of 300 µM silver nitrate impeded the protective effects of ethephon. Transgenic pepper seedlings overexpressing endogenous PR10 or esterase genes, which are induced by the ethylene treatment, completely resisted the infection (Núñez-Pastrana *et al.*, 2011).

Ethrel sprayed at 50, 100 and 150 ppm in minimizing the damage of *Alternaria alternata* in faba bean compared to Tridex-80 per cent fungicide. In faba bean ethrel sprayed at 150 ppm shown reduction of disease incidence by 76.37 per cent compared to control (El-Hai *et al.*, 2015).

Fusarium graminearum, a causative agent in Fusarium head blight (FHB) of wheat, is a hemibiotrophic pathogen, meaning it has both biotrophic and necrotrophic phases during the course of infection. However, the role of ethylene (ET) signalling in the host response to *Fusarium* spp. is unclear; some studies indicate that ET mediates resistance, while others have shown that it is associated with susceptibility. To investigate whether wheat genotype can influence the ET-mediated response to FHB, the effect of chemical treatments affecting the ET pathway was studied in six wheat genotypes in detached-head assays. ET-inhibitor treatments broke down resistance to both initial infection and disease spread in three resistant wheat genotypes, whereas ET-enhancer treatments resulted in reduced susceptibility in three susceptible genotypes (Foroud *et al.*, 2019).

2.10.2 Bio efficacy of ethylene for phenological characters in field conditions.

Dutcher (1972) reported early flowering in ‘Carabao’ mango in the Philippines in response to traditional smudging methods which was due to the ethylene gas produced by the smudge fires. This effect was duplicated by weekly or twice monthly foliar spray applications of 125-250 ppm ethephon.

Chacko *et al.* (1972) investigated the effect of ethrel on flower induction in ‘off’ year in Langra mango trees. Result of investigation showed that large number of panicles emerged even from dormant buds situated in the woody branches of the ethrel treated trees, indicating the extreme floriferous condition induced by ethrel treatment. Whereas ethrel could induce flowering in the ‘off’ year Langra trees without any new growth.

Singh and Dhillon (1986) noted reduction in incidence of floral malformation in mango plants treated with NAA 100 ppm, IBA 200 ppm, 2,4-D 100 ppm, ethrel 200 ppm and cycocel 3000 ppm treatments applied at flower bud differentiation. The per cent of hermaphrodite flowers in healthy panicles were the highest with ethrel 300 ppm of all other treatments.

Chaudari and Desai (1993) reported that application of Ethrel (250 ppm) on pomegranate ‘Ganesh’ one month before expected flowering produces the highest percentage of hermaphrodite flowers, intermediate flowers and reduces the percentage of male flowers.

Brahmachari *et al.* (1995) reported that in guava the application of ethrel at 25 or 50 ppm enhanced quality of fruit, fruit set percentage, weight, while, reduced number and weight of seeds thereby increased pulp / seed ratio.

Yamasaki *et al.* (2003) conducted experiment to study the effects of AVG and ethephon on sex expression in the cucumber. Result shown application of 1.0 mM of AVG at the 4-leaf stage, the incidence of male flowers was increased from node 8 to node 13 on the main stem compared to the controls. When 0.35 mM of ethephon was applied to the shoot apices of monoecious cucumbers at the 4-leaf stage, the incidence of female flowers was increased from node 10 to node 14 compared to the control. The gynoeceous controls produced only female flowers from node 3 to 20 under the experimental conditions.

Shaban (2004) reported that C/N ratio increased with application of ethrel during the period of flower bud differentiation that flowering increased in mango trees. Shyamal *et al.* (2010) studied the plant growth substances on vegetative growth, flowering and fruit quality of papaya and revealed that TIBA (100 and 150 ppm) and ethrel (200 and 300 ppm) proved to be the best particularly in the number of days taken for sex differentiation and promotion of female ness, respectively. Karim *et al.* (2007) studied that ethrel at 1 per cent resulted in greater number of panicles as compared with ethrel at 0.5 per cent and 1.5 per cent in young mango trees.

Supe and Marbhal *et al.* (2008) studied the effect of plant growth substances on fruit quality of pomegranate. They were observed that application of ethrel at 500 ppm at 90th days after first irrigation resulted in reduced rind thickness (0.64mm) and acidity (0.40%).

Experiment was carried out to study the effects of precursor-microbial interactions on induction of flowering in pomegranate. Precursor + microbial inoculum (*Pseudomonas fluorescens* and *Trichoderma viride*) treatments recorded significantly superior results on various parameters. Application of precursor + inoculum significantly increased the leaf ethylene levels over precursors used alone. Application of methionine 2.5 ppm + inoculum and FeSO₄ 250 ppm recorded significantly higher percentage of hermaphrodite flowers and intermediate flowers and reduced the male flower percentage. Chemicals alone could not increase the fruits

per tree, but with inoculum, the number of fruits and yield per tree were increased significantly (Reddy *et al.*, 2011a).

Reddy *et al.* (2011b) conducted experiment to study the effect of ethylene inhibiting chemicals on yield and quality of pomegranate. Result shown application of various ethylene inhibiting chemicals proved to be superior to reduce the ethylene levels in leaves of pomegranate. Spraying of cobalt chloride 2.5 ppm recorded significantly lower levels of ethylene in leaves of pomegranate. Numbers of fruits per tree, yield per tree and average fruit weight were recorded highest in plants sprayed with cobalt chloride 2.5 ppm in pomegranate.

The yield attributes of phalsa were enhanced with the application of ethrel 1000 ppm in terms of advancement of harvesting and also reduction in number of pickings. Hence ethrel 1000 ppm may be applied to improve the fruit quality parameters as well as yield parameters and also to fetch the premium price in the market due to their attractive colour appearance than control (Kaur *et al.*, 2018). The quality of fruits in terms of total soluble solids, reducing sugar and total sugar were significantly higher in treatment ethrel 1000 ppm. Further, ethrel 1000 ppm also significantly reduced the span of harvesting and number of picking (Kacha *et al.*, 2012).

Seven traits related to flowering and sex expression in melon were studied and their reaction to application of two growth regulators (ethrel and gibberellic acid) was observed. Four monoecious genotypes (Sesame, ED-3, ED-4, Pobeditel) and four andromonoecious genotypes (Chinese muskmelon, Anannas, Fiata, A2-3lb) had been used for experiments. Results revealed that ethrel had higher effects on the investigated traits than gibberellic acid. Ethrel increased the 7.18 number of perfect flowers per plant (31.42%), reduced the 21.47 number of male flowers per plant for (17.98%) (Girek *et al.*, 2013).

Goswami *et al.* (2013) studied the importance of synthetic plant growth regulators in achieving higher yield and better quality of fruits. Application of ethrel 200 ppm has improved the TSS, reducing sugar, non-reducing sugar, total sugar and ascorbic acid content of pomegranate when compared with control.

The effect of plant growth regulators on flowering and yield of muskmelon (*Cucumis melo* L.) was carried. Two sprays of plant growth regulators *i.e.* Ethrel, Naphthalene acetic acid, Gibberellic acid, SADH and Kinetin were given at 2nd and 4th leaf stages in the treatment NAA 150 ppm + Ethrel 250 ppm. The yield characters such as fruit diameter (12.97 cm) and yield per plant (7.01 kg) were observed maximum in the treatment NAA 150 ppm + Ethrel 250 ppm. The application of NAA 150 ppm + ethrel 250 ppm observed the best treatment, while compared to other treatment and control (Devi and Madhanakumari, 2015).

Exogenous ethylene, or ethephon, has been widely used to induce pineapple flowering. The relative expression of ethylene receptor genes AcERS1b, AcETR2a and AcETR2b was significantly increased during the earlier period of pineapple inflorescence formation, especially at 1 to 9 days after ethylene (200mg/L) treatment these genes play important roles during pineapple flowering induced by exogenous ethephon (Li *et al.*, 2016).

Lamo *et al.* (2017) studied the influence of pre harvest and pre flower sprays of ethrel on flowering behaviour, fruit yield of phalsa cultivar purple round under jammu- sub tropics. Pre flowering and pre harvest spray of 1250 ppm ethrel resulted in early flowering. Ethrel 1250 ppm resulted into early end date of flowering.

Efficacy of two flower induction chemicals, potassium nitrate (KNO₃) and ethephon on reproductive growth parameters and yield components were evaluated on two mango varieties: 'Apple' and 'Ngowe'. KNO₃ was applied at two concentrations (2 and 4%) and ethephon (600 and 1000 ppm) then compared to control (water). They were applied to trees which failed to flower/set fruit in 2014 season. KNO₃ (4%) and ethephon increased percentage flowering in both 'Ngowe' and 'Apple' significantly ($p < 0.05$) shortened time to flowering and increased fruit set. The findings show that KNO₃ and ethephon can be used to induce flowering/fruitleting in mango fruits. These technologies can therefore be applied to induce off-season mango production (Maloba *et al.*, 2017).

Solutions to increasing flowering rate on lychee in warm winter conditions have been identified as mechanized and chemical measures. Application of ethrel at different concentrations in warm, rainy winter season inhibited vegetative growth, hence, improving flowering rate on lychee in comparison to the control. Application

of ethrel at 600 ppm was recorded with the flowering rate of 62.3 per cent, meanwhile the value obtained in the control was only 36.7 per cent (Nghie *et al.*, 2017).

Sex expression is a complex process and in-depth knowledge of its mechanism in pumpkin is important. Shoot apices at the 4-leaf stage were treated with the ethylene-releasing agent 2-chloroethylphosphonic acid (Ethrel), aminoethoxyvinyl glycine (AVG), AgNO₃ and indoleacetic acid (IAA). The number of female flowers up to node 20 on the main stem of '2013-12' increased significantly after Ethrel and IAA treatment and decreased significantly after AVG and AgNO₃ treatment. Transcriptome data and chemical treatment results indicated that IAA might affect pumpkin sex expression by inducing CmaACS7 expression and indirectly affecting ethylene production and its synthesis and signal transduction pathways play crucial roles in pumpkin flower sex expression (Wang *et al.*, 2019).

Study was conducted to induce flowering at the desired period with higher percentage of bisexual flowers and fruit set to improve yield and minimize fruit cracking through withholding of irrigation, pruning and ethrel application. Among interactions, withholding of irrigation during June + pruning and thinning + ethrel 2 ml/l + DAP 5 g/l treatment induced flowering during optimal climatic conditions and significantly improved sex ratio and fruit set of pomegranate (Kumar *et al.*, 2020).

2.11 Elucidation of resistance with other hormones

The ethylene (ET) receptors ETR1, ETR2, ERS1, ERS2 and EIN4 are kinases which act as signaling repressors until ET binding occurs. The negative regulation occurs through the activity of putative MAP3K, CTR1 which phosphorylates EIN2 (Hua and Meyerowitz, 1998). EIN2 is a central component in ET signaling that in the phosphorylated form is located in endoplasmic reticulum. It is likely that dephosphorylation results in EIN2 translocation to the nucleus (Ju *et al.*, 2012). Within the nucleus transcriptional activation involves components such as EIN2 and EIN3 regulating the expression of key transcription factors ORA59 and ERF1 (Stepanova and Alonso, 2005).

Plants lacking EIN3 and EIL1 display enhanced PAMP defenses and heightened resistance to *Pseudomonas syringae* bacteria infecting Arabidopsis. Conversely, plants over accumulating EIN3 are compromised in PAMP defenses and

exhibit enhanced disease susceptibility to *P. syringae*. Microarray analysis revealed that EIN3 and EIL1 negatively control PAMP response genes. Further analyses indicated that SALICYLIC ACID INDUCTION DEFICIENT2 (SID2), which encodes isochorismate synthase required for pathogen-induced biosynthesis of salicylic acid (SA), is a key target of EIN3 and EIL1 (Chen *et al.*, 2009).

Cross-talk between jasmonate (JA), ethylene (ET) and Salicylic acid (SA) signaling is thought to operate as a mechanism to fine-tune induced defenses that are activated in response to multiple attackers. Strong activation of JA and ET responses by the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola* prior to SA treatment counteracted the ability of SA to suppress the JA response. The APETALA2/ETHYLENE RESPONSE FACTOR transcription factor ORA59, which regulates JA/ET-responsive genes such as PDF1.2, emerged as a potential mediator in this process (Leon-Reyes *et al.*, 2010).

The hypersensitive interaction between Tobacco mosaic virus (TMV) and tobacco results in accumulation of salicylic acid (SA), defense gene expression and development of systemic acquired resistance (SAR) in uninfected leaves. The plant hormones SA and ethylene have been implicated in SAR. From a study with ethylene-insensitive (Tetr) tobacco, concluded that ethylene perception is required to generate the systemic signal molecules in TMV-infected leaves that trigger SA accumulation, defense gene expression and SAR development in uninfected leaves (Verberne *et al.*, 2003). Ethylene acts positively on the level of SA-mediated resistance against the pathogen *Leptosphaeria maculans* in *Brassica napus* (Sašek *et al.*, 2012).

3. MATERIAL AND METHODS

The present investigation was carried out during 2018-20 at Department of Plant Pathology, College of Horticulture, Bagalkot and Bio-control laboratory, UHS, Bagalkot. Pot experiments were performed in the green house where minimum and maximum temperatures ranged from 25 and 28°C; minimum and maximum relative humidity (RH) ranged from 40 and 70 per cent, respectively. Field trial was conducted in three farmer's fields at Tulasigeri village situated at 16.1351° N latitude and 75.3213° E longitude (red soil), 16.1302° N latitude and 75.3141° E longitude (red soil), 16.1127° N latitude and 75.3500° E longitude (red soil) in Bagalkot district.

3.1 Cleaning of glass wares and sterilization of media

All the plastics and glass wares were kept in cleaning solution for overnight. Cleaning solution was prepared by dissolving 60 g of potassium dichromate, 60 ml of concentrated sulphuric acid in one litre of water. Each of these chemicals dissolved separately in 500 ml of water and finally mixed. All the materials were washed thoroughly with sterile distilled water and then dried in hot air oven at 160°C for one hour. The cleaned and dried petriplates were wrapped with paper and autoclaved. Test tubes and flasks containing both solid and liquid media respectively plugged with non-absorbent cotton, these were covered with paper and autoclaved at 121.6°C and 15 PSI for 20 min. The blades, forceps, nichrome, scalpel, wire loop were dipped in ethanol and then sterilized by heating to flame.

3.2 Isolation of pathogen and confirmation

3.2.1 Collection of diseased plant parts of pomegranate tree

Pomegranate plants were observed for presence of symptoms of bacterial blight. Leaves of pomegranate plants showing black colored, oily spots on them were collected from pomegranate field located at near Bagalkot District, Karnataka, India. The sterile scissor used to cut the branches containing infected leaves. Then the plant leaves were kept in polythene bag and closed it securely. It was brought to laboratory and kept it in shade, cool place and used for further study.

3.2.2 Methodology

Pure colony of *Xanthomonas axonopodis* pv. *punicae* (Xap) was isolated from bacterial blight infected fruits. Leaves infected with bacterial blight were surface sterilized with one per cent sodium hypochlorite and washed twice with sterile water and approximately 2 mm diameter lesions were cut and macerated in sterile water and streaked on nutrient glucose agar (NGA) medium and incubated at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 3–4 days. Single colonies were sub-cultured on NGA medium and incubated at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$; pinheaded, yellowish colonies, which appeared after 48 h of incubation, were selected for inoculum preparation and streaked on fresh NGA medium.

Composition of NGA media: g/L (All chemicals are from Thermo Fisher Scientific India Pvt. Ltd.).

Media components	Quantity g/L
Peptone	5 g
Dextrose	10 g
Beef extract	3 g
Agar	18 g

3.3 Molecular identification of *Xanthomonas axonopodis* pv. *punicae* (Xap)

3.3.1 Extraction of total genomic DNA from bacteria

The extraction of total genomic DNA was carried out following a modified protocol as described by Adachi and Takashi (2002). 200 μl NA broth grown culture was centrifuged for 5 min at 10,000 rpm and the bacterial cells were resuspended in 50 μl of proteinase K solution (50 $\mu\text{g ml}^{-1}$ in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and kept at 56°C for 15 min, then at 80°C for 15 min to denature the proteinase, then placed on ice to cool the samples for 5 min. After centrifugation for 5 min at 13000 rpm, the supernatant was transferred to a new eppendorf tube. The supernatant was treated with RNase A (100 $\mu\text{g ml}^{-1}$) for 30 min at 37°C followed by extraction with phenol : chloroform : isoamylalcohol (25:24:1) and precipitation by adding 0.1 volume of ammonium acetate and 2 volumes of chilled ethanol. The DNA

pellet was washed with 70 per cent ethanol and resuspended in 50 µl sterile water. The DNA samples were quantified using Nano-drop Spectrophotometer. For PCR amplification the concentration of DNA used was 100 ngµl⁻¹.

3.3.2 PCR and amplification conditions

Total genomic DNA from *Xap* was isolated from the single colony inoculated in NG broth culture grown for 72 hr at 28±0.5°C. Isolated DNA was quantified using the NanoDrop spectrophotometer (ND-1000, ThermoFisher, MA, USA). A primer set, KKM-5 Forward 5'GTTGATGCTGTTCCACCAGCG3' and KKM-6 Reverse 5'CATTTCATTTGCCCCAAGCCC3' were designed from the C terminus region of *gyrB* gene by Monda *et al.* (2012b) was used for PCR-based identification of *Xap*. PCR amplification was performed in a 15 µl reaction mix containing 100 ng of DNA, 1X PCR Buffer, 200 µM of dNTPs, 0.2 µMoles each of forward and reverse primer and 1 U *Taq* DNA polymerase (Merck, Bangalore, India). Primers were amplified with PCR cycle of initial denaturation at 94°C for 4 min, then 30 cycle denaturation at 94 °C for 60 sec, annealing for 45 sec at 55°C and extension 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes in a thermocycler (Eppendorf vepo protect Germany). PCR products were resolved on 1.4 per cent agarose gel using a horizontal electrophoresis system (Bio-Rad, Hercules, California, USA). The amplified products were stained using ethidium bromide (0.001 mg/ml) and gel images were photographed using a Gel Logic 212 Pro imaging system (Gel Logic 212 PRO, Carestream, USA).

3.4 Testing the pathogenicity of *Xanthomonas axonopodis* pv. *Punicae*

Six month old Bhagwa plants raised in greenhouse conditions (28 ±0.5 °C, 60-70% relative humidity) were used for pathogenicity tests and for all other studies. Preparation of bacterial inoculum: Bacterial inoculum was prepared by inoculating the pure and single colony of *Xap* in NG broth, culture flasks were incubated at 28 ±0.5 °C for 72 hr and bacterial inoculum with a minimum concentration of 0.25 OD₆₀₀ nm (10⁸ CFU/ml) was used for pathogenicity test. To check the pathogenicity of the isolate's protocols reported by Sharma *et al.* (2017) was followed.

3.5 Understanding the role of ethylene concentration on pathogen/disease development in plant

In order to assess the role of ethylene at different dosage, treatment of ethylene for different cultivars of pomegranate viz. Daru, Bhagwa, Wonderful, Ganesh, Nana was carried out in 4 different concentrations viz. 100, 150, 200, 300 ppm under greenhouse condition. Single spray of different concentrations was applied, covering to the foliage uniformly. After 24 hours of ethylene treatment, all the plants were inoculated with pathogen. After appearance of the symptoms one hundred leaves were collected randomly and graded using the severity scale given by Singh *et al.* (2015) and calculated the severity of bacterial blight. For each treatment three replications were maintained.

3.5.1 Plants and growth conditions

Experiments were conducted on six month old plants of cultivar Bhagwa in pots, filled with equal proportion of fine sand: garden soil: well decomposed farmyard manure. Experiments were performed in the green house where minimum and maximum temperatures ranged from 24° C and 29° C; minimum and maximum relative humidity (RH) ranged from 30 per cent and 70 per cent, respectively (Plate 1).

3.5.2 Bacterial strain and culture conditions

X. axonopodis pv. *punicae* was multiplied on nutrient glucose agar and was grown at $28 \pm 1^\circ\text{C}$ for 48-72 h. As described in materials and methods under section 3.4.

3.5.3 Pathogen Inoculation

Three plants per treatment were spray inoculated with suspension of *X. axonopodis* pv. *punicae* (0.2 OD at 600 nm) and incubated further, under high humidity in polythene bags for 48 h. The plants were observed at regular interval and data on incidence and severity of bacterial blight on leaves were recorded.

The disease severity on leaves were recorded using the formula:

$$\text{Severity (\%)} = \frac{\text{No. of infected leaves} \times \text{Disease grade}}{\text{Total number of leaves} \times \text{Max grade}} \times 100$$



Plate 1: Representative picture of greenhouse evaluation of different concentrations of ethrel against bacterial blight of pomegranate

Data analysis was done by using two factorial complete randomized block design (CRD).

Scoring systems for Severity Grade (Bacterial Blight)			
Severity Grade to be allotted	Severity (%)	Observation Method on different units	
		Leaves and fruits	Stems (No. of cankers or stem lesions /plant)
0	0	Disease not seen	0
1	1-10	Disease not easily visible, very few units/plant found diseased.	1
2	11-25	Disease visible easily in each direction, but most (75%) of the units look healthy	2-3
3	26-50	Both disease and healthy units are equally observed	4 – 6
4	51-75	Disease seen very easily, with only some healthy units	7-10
5	76-100	All most all units are diseased with few healthy units.	≥11

(Singh *et al.*, 2015)

3.5.4 Pathogen quantification

3.5.4.1 DNA Extraction

In order to detect the Xap in the plant tissue, the inoculated pomegranate leaves (100 mg) were homogenized in 1 to 2 ml of extraction buffer (100 mM TrisHCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 3.5 per cent CTAB, 0.2 per cent β mercaptoethanol) in sterile mortar and pastel. Then the extract was incubated at 60°C for 30 min and vortexed for 5 min. The suspension was mixed with 1 volume of chloroform/isoamyl alcohol (24:1) and centrifuged for 10 min at 6000 rpm. The aqueous phases were transferred into fresh tube and add 0.8 to equal volume of isopropanol and centrifuged 20 min at 13000 rpm. The supernatant was discarded and the pellet was washed twice with 70 per cent ethanol by spinning at 10,000 rpm for 2 min and dry for 5 to 10 min at room temperature. The pellet was suspended in 20 to

30 µl sterile distilled water and 1µl was used for PCR amplification. 5 µl of PCR product was loaded on agarose gel (1%) with 0.5 µg ml⁻¹ of ethidium bromide and run in 1X TAE buffer at 100 volt for 30 min. DNA was quantified using a NanoDrop 1000 spectrophotometer (ND-1000, ThermoFisher, MA, USA).

3.5.4.2 Quantitative real-time PCR (qPCR)

qPCR was evaluated for rapid diagnosis of bacterial blight infection using DNA extracted from blight-infected pomegranate leaves, with Xap DNA as the positive control. qPCR was performed in a 10 µl reaction mixture comprising 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 µM each of forward and reverse primer and 20 ng of DNA isolated by a modified CTAB method used as the template. qPCR was performed in a Step OnePlus Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using XopQ as the target gene and GAPDH (KF856731, forward primer 5'-AGCCTACAACCAAA CATCAAGC-3' and reverse primer 5'-GGTGCCGAGTTCATTGTGGA-3' as the reference gene to normalize sample-to-sample variation. The following PCR conditions were used: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60 °C for 1 min. Following PCR amplification, melt-curve analysis of the amplicons was conducted from 60°C to 95°C and data were collected at 0.3°C intervals. DNA from all the five varieties were extracted on 12th day and quantified.

3.6 Microbe induced ethylene regulation in pomegranate and its effect on disease development

Treatment	Treatment details
T ₁	<i>Pseudomonas putida</i> (soil application) at 4 x 10 ⁸ CFU/ml
T ₂	<i>Pseudomonas putida</i> (foliar spray) at 4 x 10 ⁸ CFU/ml
T ₃	<i>Pseudomonas putida</i> (foliar spray + soil application) at 4 x 10 ⁸ CFU/ml
T ₄	<i>Trichoderma asperellum</i> (soil application) at 4 x 10 ⁶ CFU/ml
T ₅	<i>Trichoderma asperellum</i> (foliar spray) at 4 x 10 ⁶ CFU/ml
T ₆	<i>Trichoderma asperellum</i> (foliar spray + soil application) at 4 x 10 ⁶ CFU/ml
T ₇	Control



(a)



(b)



(c)



(d)

Plate 2: Representative picture of microbe induced ethylene regulation in pomegranate and its effect on disease development.

- a) Plants under imposition of treatments b) Bioagents broth culture
c) Spray inoculation of bioagents d) Sample collection for gene expression

In order to assess the regulation of ethylene level by bio agents, plants were treated with *Pseudomonas putida* as soil application (T1), foliar spray (T2) and combination of both soil and foliar spray (T3) and similarly, *Trichoderma asperellum* applied as soil treatment (T4), foliar spray (T5) and combination of both soil and foliar spray (T6) with non-inoculated plant as control (T7). After 48 hours of bioagents treatment, all the plants were inoculated with pathogen. After appearance of the symptoms one hundred leaves were collected randomly and graded using the severity scale given by Singh *et al.* (2015) and calculated the severity of bacterial blight and for each treatment three replications were maintained.

3.6.1 Plants and growth conditions

Experiments were conducted on six month old plants of cultivar Bhagwa in pots, filled with equal proportion of fine sand: garden soil: well decomposed farmyard manure. Conditions as described previously in materials and methods under section 3.5.1 (Plate 2).

3.6.2 Bacterial strain and culture conditions

As described previously in materials and methods under section 3.4

3.6.3 Pathogen Inoculation

Three plants per treatment were spray inoculated with suspension of *X. axonopodis* pv. *punicae* as described previously in materials and methods under section 3.4. Disease severity was measured and data analysis was done by using completely randomized block design (CRD).

3.6.4 Gene expression

3.6.4.1 Collection of the sample

Plants were inoculated with bio agents and after 24 hours pathogen was inoculated and later samples were collected. Samples (leaves) were collected in different time points (0, 6, 24, 48, 72 hours, day 6 and day 9) after pathogen inoculation and stored in deep freezer until use.

3.6.4.2 Isolation of RNA

Total RNA isolation was carried out using the Spectrum plant total RNA kit (Sigma, USA) by following the instruction provided in the kit. Following RNA isolation quantification of RNA was done using NanoDrop (Thermo Scientific) by measuring the OD value at 260 and 280 nm. To purify the RNA from genomic DNA contaminations the whole RNA was treated with DNase I enzyme (RNase free, Thermo Scientific).

3.6.4.3 DNase treatment

DNase treatment for all the RNA preparations was performed using DNase I enzyme (RNase free) (Thermo Scientific). For removal of DNA, 1 µg of RNA was incubated in a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 0.1 mM CaCl₂ and 1 U DNase I at 37°C for 30 min. The reaction was stopped by heating the reaction mixture at 65°C for 10 min in presence of 2.5 mM EDTA. The DNA free RNA was further used for first strand cDNA synthesis.

3.6.4.4 First strand cDNA synthesis

A total of 1000 ng of RNA was used for cDNA synthesis using RevertAid H-minus M-MuLV Reverse Transcriptase (Thermo Scientific) according to the manufacturer's protocol. The first strand cDNA synthesis was performed for a total RNA samples extracted using First strand cDNA synthesis kit, Thermo Scientific. cDNA synthesis kit (Thermo Scientific). 0.5 µg of total RNA, 1 µL each of 50 Mm oligo (dT) 18 and 60 µM random hexamer and nuclease-free water to make up the volume to 8 µL were added, mixed by gentle pipetting. The reaction mixture was heated at 65°C for 5 min followed by rapid chilling on ice. To the reaction mixture, 12 µL of master mix containing 10 µL of M-MuLV Reaction Mix (2X) buffer and 2 µL of M-MuLV Reaction Mix (10X) buffer was added, mixed gently and centrifuged briefly and incubated at 25°C for 5 min, 42°C for 60 min. The reaction was stopped by heating the mixture at 75°C for 5 minutes and chilling on ice.

3.6.4.5 Gene Expression analysis

cDNA was diluted to a ratio of 1:5 and used for qPCR analysis. The samples were subjected to transcript analysis of ethylene pathway gene ACC oxidase and phenylpropanoid pathway gene phenylalanine ammonia lyase (PAL). The observation were made for regulation of ethylene responsive gene upon above treatment.

qPCR was performed using the Step-OnePlus Real-Time PCR System (Applied Biosystems), with three biological and two technical replicates. The housekeeping gene GAPDH was used for normalizing the expressed data. The primer pairs used in the present study were listed in Table 1. The qPCR analysis was performed in a 10 µl reaction mixture containing SYBR Green master mix of 1X concentration (Applied Biosystems, Foster City, California), 0.25 µM of primer, 2 µl of cDNA dilution (ten-fold dilution). The qPCR cycle was performed as following initial activation 50°C for 2 min, 2 min at 94°C followed 40 cycles of by denaturation 94°C and 1 min annealing and extension at 60°C. Post completion of qPCR cycle, melt curve analysis of the amplified products was set between 60 to 95°C and data were collected at every 0.3°C to determine the primer specificity. The relative gene expression patterns were calculated in terms of fold change as $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

3.7 Bio efficacy of ethylene against bacterial blight and phenological characters in field conditions

Ethrel at different concentration 0, 100, 150, 200ppm were selected for its bio efficacy testing under field condition (Plate 3). Ethrel was sprayed in field to know the impact towards minimizing disease incidence and phenological characters. Ethrel was sprayed at 15 days after pruning, 60 and 90 days after complete flowering (Plate 4). Observations for severity was recorded at 60, 90, 120 days after Bahar treatment and for phenological characters reading was recorded at flowering stage, fruit setting stage and at the time of harvesting. Observation pertaining to its disease severity on leaves, fruits and on twigs and phenological characters were described as follows. Experiment was conducted in farmers field.

Table 1: Gene specific primers used for PCR and qPCR experiments

Sl. No.	Gene	Primer (5'-3')	Amplified fragment size (bp)
1	gyrB (gyrase B)	KKM5F-GTTGATGCTGTT CACCAGCG KKM5R-CATTCATTTCGCCCAAGCCC	491
2	XopQ (effector gene)	XopQF-GCGAGGAACTTGGAATGCTC XopQR-AGGTCTGAAGGCTTTTTCGCG	190
3	ACC deaminase from <i>Pseudomonas putida</i>	PacdF- CATGGCTACCAACTGCACGA PacdR- GAGCCTTGCCAGTGTAGAGG	130
4	ACC deaminase from <i>Trichoderma asperellum</i>	TacdF-CACCAACCCACCACAACTCT TacdR- GCGCGGGTATGGTTTGATTG	141
5	Phenylalanine ammonia-lyase	PALF-TCGGGAAGCTGATGTTTGC PALR-CCCCTTGAAGCCATAGTCC	116
6	ACC oxidase	ACCOF-CGTCGGAGATGTTAGAGAC ACCOR-CGAAGGAGCACTACAAGAG	150
7	GAPDH	GAPDHF- AAGGGTGGTGCWAAGAAGG GAPDHR- CCCCACTCRTTGTCTACC	230

3.7.1 Observations recorded

3.7.1.1 Per cent disease severity on leaves

Five shoots were selected randomly and disease severity was recorded from beginning of imposition of treatment and calculated by using formula.

$$\text{Per cent severity} = \frac{\text{No. of infected leaves} \times \text{Disease grade}}{\text{Total number of leaves} \times \text{Max grade}} \times 100$$

3.7.1.2 Per cent disease severity on fruits

Infected fruits were counted from beginning of imposition of treatment and severity was calculated by using formula.

$$\text{Per cent severity} = \frac{\text{No. of infected fruits} \times \text{Disease grade}}{\text{Total number of fruits} \times \text{Max grade}} \times 100$$

3.7.1.3 Twig blight severity (%)

Infected twigs were counted from beginning of imposition of treatment and severity was calculated using formula.

$$\text{Per cent severity} = \frac{\text{No. of infected twigs} \times \text{Disease grade}}{\text{Total number of twigs} \times \text{Max grade}} \times 100$$

Data analysis was done by using two factorial Randomized Complete Block Design (RCBD).

3.7.1.4 Flower characters

There are three types of flowers mainly bisexual, male and intermediate. For visual observation two types of flowers viz., hermaphrodite and male flowers were taken into count (Plate 5). Hermaphrodite flowers are usually vase shaped and male flowers are bell shaped (Hollard *et al.*, 2009). Data analysis was done by using two factorial Randomized Complete Block Design (RCBD).

3.7.1.4.1 Number of hermaphrodite, male flower per plant

For hermaphrodite flowers, fully opened vase shaped flowers were counted by visual observation. For male flowers, fully opened bell shaped flowers were counted.

3.7.1.4.2 Percentage of hermaphrodite flowers

$$\text{Hermaphrodite flowers/plant} = \frac{\text{Number of hermaphrodite flowers/plant}}{\text{Total number of flowers/plant}} \times 100$$

3.7.1.4.3 Percentage of male flowers

$$\text{Male flowers/plant} = \frac{\text{Number of male flowers/plant}}{\text{Total number of flowers/plant}} \times 100$$

3.7.1.4.4 Sex ratio

$$\text{Sex ratio} = \frac{\text{Number of male flowers/plant}}{\text{Number of hermaphrodite flowers/plant}} \times 100$$

3.7.1.5 Fruit characters

3.7.1.5.1 Percentage of fruit set

$$\text{Per cent fruit set} = \frac{\text{Total No. of fruits per plant after complete flowering}}{\text{Total number of flowers produced per plant}} \times 100$$

3.7.1.5.2 Percentage of fruit retention

$$\text{Per cent fruit retention} = \frac{\text{Total number of fruits at harvest}}{\text{Total number of flowers produced per plant}} \times 100$$

3.7.1.5.3 Colour of fruit

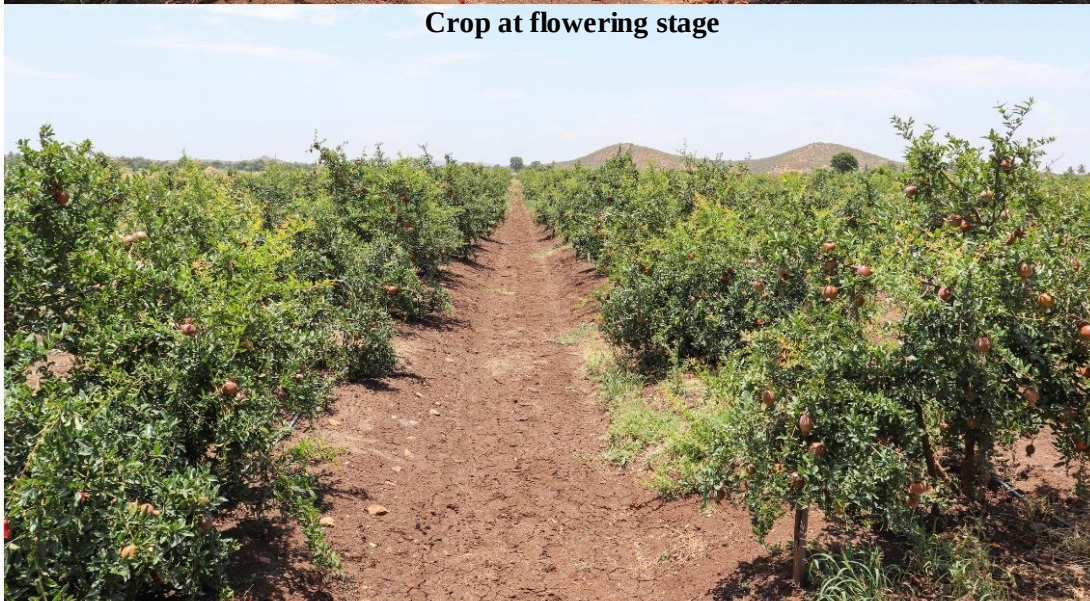
Colour of the sample was measured using Hunter colour meter (Model: Color Flex® EZ Standard Box) fitted with 8 mm diameter aperture (Plate 6). The instrument was calibrated using black and white tiles provided. Colour was expressed in units L* (lightness/darkness), a* (greenness/redness), b* (blueness/yellowness). Pomegranate samples were placed across the aperture of the colour meter. Three measurements were performed and values of the samples were averaged.



Emergence of new flush after ethrel spray



Crop at flowering stage



Crop at fruit setting stage

Plate 3: Overview of field experimental plot at different crop stages



Plate 4: Spray application of ethrel under field condition



Male flower



Hermaphrodite flower

Plate 5: Flower types in pomegranate observed under field condition

3.7.1.5.4 Fruit weight (g)

Fruits were harvested after full maturity and average fruit weight was taken by using weighing balance.

3.7.1.5.5 Time of harvest

Number of days recorded from days of complete flowering to harvesting.

3.7.1.5.6 Fruit yield per plant (kg)

Fully matured fruits were harvested and expressed in terms of kilograms.

3.7.1.5.7 Fruit yield

Fruit yield per plant was recorded and these were converted into yield per hectare by multiplying yield obtained per plant into total number of plants accommodated per hectare and expressed in tonnes.

3.8 Elucidation of resistance with other hormones

In order to assess effect of different hormones, plants were sprayed with different hormones viz., ethylene 300ppm, salicylic acid 300ppm and jasmonic acid 300ppm (Plate 7). After 24 hours of hormonal spray plants were inoculated with pathogen. After appearance of the symptoms one hundred leaves were collected randomly and graded using the severity scale given by Singh *et al.* (2015) and calculated the severity of bacterial blight and for each treatment three replications were maintained.

Treatment	Disease Severity
Treatments	Concentration
T1-Ethrel	300 ppm
T2-Methyl jasmonate	300 ppm
T3-Salicylic acid	300 ppm
T4-Water Control	-
T5-Ethanol Control	0.5 ml/litre

3.8.1 Plants and growth conditions

Experiments were conducted on six month old plants of cultivar Bhagwa in pots, filled with equal proportion of fine sand: garden soil: well decomposed farmyard manure. Experiments were performed in the green house where minimum and maximum temperatures ranged from 24° C and 29° C; minimum and maximum relative humidity (RH) ranged from 30 per cent and 70 per cent, respectively.

3.8.2 Bacterial strain and culture conditions

X. axonopodis pv. *punicae* culture was used and multiplied as described in materials and methods under section 3.4.

3.8.3 Pathogen Inoculation

Three plants per treatment were spray inoculated with suspension of *X. axonopodis* pv. *punicae* as described in materials and methods under section 3.4.

3.8.4 Observations

Disease severity was measured as mentioned previously in materials and methods under section 3.7.1.1. Data analysis was done using completely randomized design (CRD).

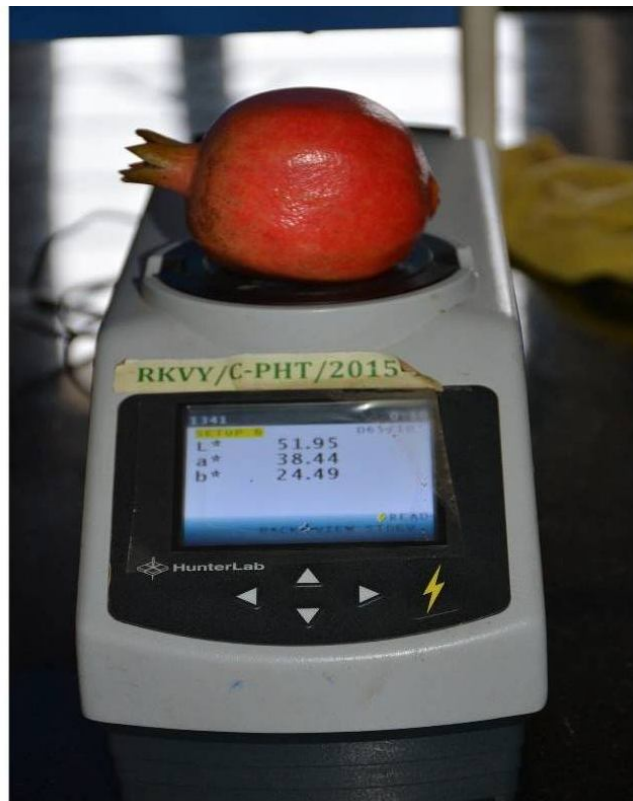


Plate 6: Measuring Colour of the sample using Hunter colorimeter



Plants under imposition of treatments



Spray application of hormones

Plate 7: Representative picture of elucidation of resistance with other hormones

4. EXPERIMENTAL RESULTS

The present investigation was carried out to know the “Effect of ethylene on bacterial blight disease resistance and its effect on phenological characters of pomegranate” under field condition as well as under greenhouse conditions. Results obtained during investigation are presented in this chapter.

4.1 Identification of the pathogen of bacterial blight of pomegranate

4.1.1 Symptomatology

Symptoms of bacterial blight observed on above ground parts of the plant. On leaves, one to several, small, regular to irregular grayish water soaked lesion appeared on abaxial surface that looked transparent yellow when observed against light. The lesions further enlarged and turned dark brown to black, eventually becoming visible on both leaf surfaces. Lesions coalesced to form larger lesions which resulted in blighted appearance followed by defoliation. Nearly identical symptoms observed on spray inoculated plantlets. On midrib of a leaf lamina, the bacterial inoculation led to extensive chlorosis of leaf lamina, browning of the blade, blackening of the veins and eventual defoliation. On twigs and branches, the symptoms began as brown to black water soaked lesions around the nodes or injuries. The lesions under high humid conditions were sticky, enlarged and led to girdling and cracking of the nodes on slight pressure. It was further observed that when lesions appeared on young new twigs, all the leaves above the lesion turned intense yellow and fell off. On floral parts the bacterium could neither cause any visible symptoms nor was it ever observed in naturally infected plants. On fruits, water soaked lesions initially appeared on fruit surface that later turned dark brown. These lesions enlarged and coalesced to cover large areas (Plate 8). Thin shining white encrustation of dried bacterial ooze or droplets of glue like bacterial ooze was often observed on lesions. Under high humidity, blight lesions were sticky or slimy. Formation of L or Y cracks on necrotic fruit spots and splitting of fruits was invariably observed. Interestingly, infection was restricted only to the rind.

4.1.2 Isolation of the pathogen

The different parts of the pomegranate plant showing characteristic symptoms of bacterial infection were collected for isolation. Pathogen was isolated by streak plate method on NGA (nutrient glucose agar) media at 28°C. After 48 hours of incubation individual colonies are picked and sub cultured on media. Later pure culture is stored in refrigerator at 4° C for further use.

4.1.3 Morphological characters of pathogen

The morphological characters like its fuscan development, unique dark brown fuscan pigmentation was observed in *Xap* inoculated NGA medium. Its intensity increased after 7 days of incubation. Production of pigmentation is a unique character of *Xap*, helps in differentiating with other non-pathogenic bacteria. Colonies on NGA plates are circular, convex, mucoid, shiny and yellow were observed (Plate 9).

4.1.4 Molecular identification of pathogen

The molecular identification of the pathogen was established using a primer set, KKM-5 Forward and KKM-6 Reverse primers amplify gyraseB (GyrB) gene designed by Mondal *et al.* (2012b) (primer sequences are mentioned in Table 1). The primer set gave amplicon size of 491 bp, which is specific to only *X. axonopodis* pv. *punicae* (Plate 9).

4.1.5 Testing the pathogenicity of pathogen

Six month old Bhagwa plants raised in greenhouse conditions (28 ±0.5 °C, 60-70% relative humidity) were used for pathogenicity tests and for all other studies. Results revealed that, Symptoms were observed 9 days after inoculation. Initially plants shown water soaked legions on the lower leaf surface and later turned into dark brown spots surrounded by yellow halo on leaf. From these inoculated leaves pathogen was re-isolated and compared with original culture and also confirmed using specific primers.

a)



b)



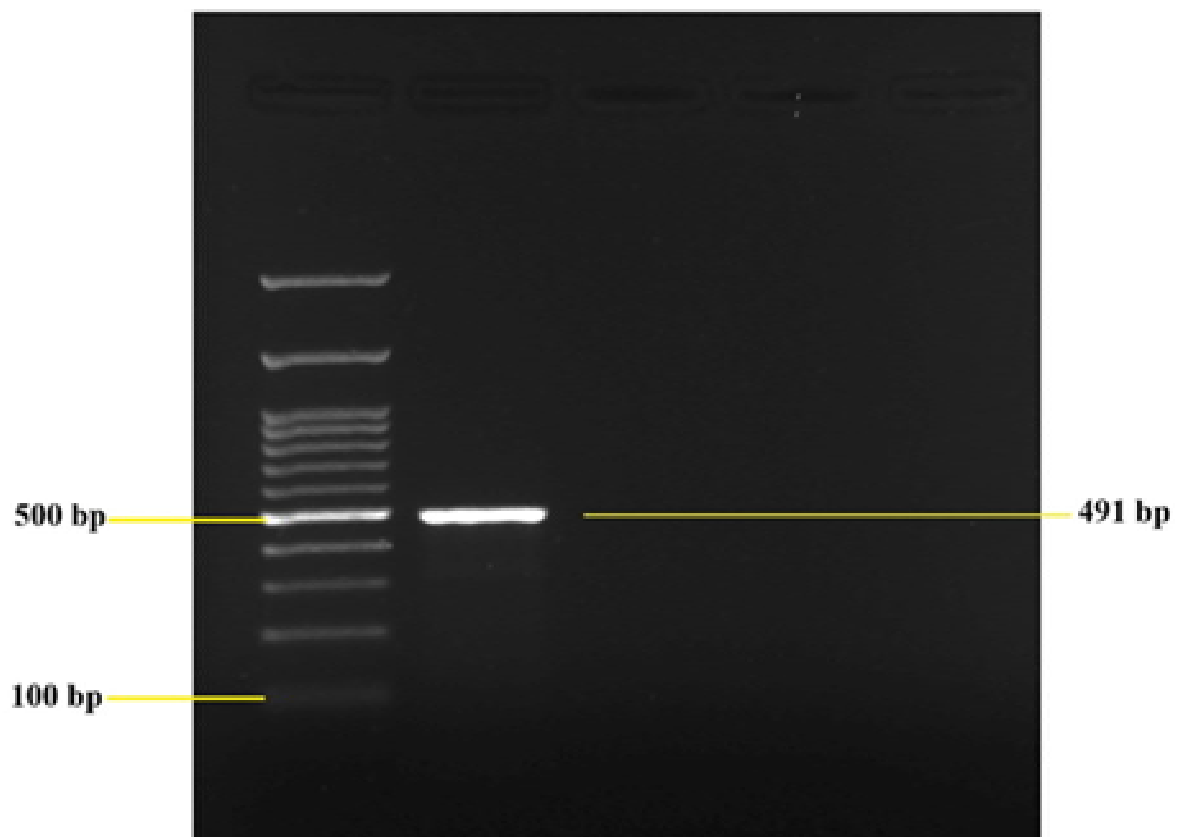
c)



Plate 8: Symptoms of bacterial blight on a) Leaves b) Fruit c) Twigs



Pure culture of *Xap* on NGA media



Confirmation of *Xap* using primer set KKM-5 and KKM-6 from bacterial DNA

Plate 9: Identification of *Xanthomonas axonopodis* pv. *punicae*



(a)



(b)



(c)



(d)

Plate 10: Proving pathogenicity of *Xanthomonas axonopodis* pv. *Punicae*

- a) Pathogen inoculation by spray method b) Incubation under high humidity in polythene bags for 48 hours after spray inoculation c) Visible symptoms on spray inoculated plant d) Re-isolated pathogen

4.2 Understanding the role of ethylene concentration on pathogen/disease development in plant

4.2.1 Disease severity

Foliar application of ethrel showed higher disease severity whereas control plants showed lower disease severity in all the varieties (Table 2). Among the different treatments, ethrel at 300ppm (E4) showed significantly higher disease severity of 28.64 per cent followed by 200ppm (26.47%) (E3) and 150ppm (24.7%) (E2) whereas control plant showed lower disease severity (20.94%).

Among different varieties, Bhagwa (V1) showed higher disease severity of 31.57 per cent followed by Wonderful and ruby with disease severity of 28.64 per cent and 25 per cent respectively whereas least disease severity was noted in Nana with 15 per cent.

There was non-significant difference observed in interaction between ethrel concentration (T) and varieties (V) in all possible combination with respect to disease severity.

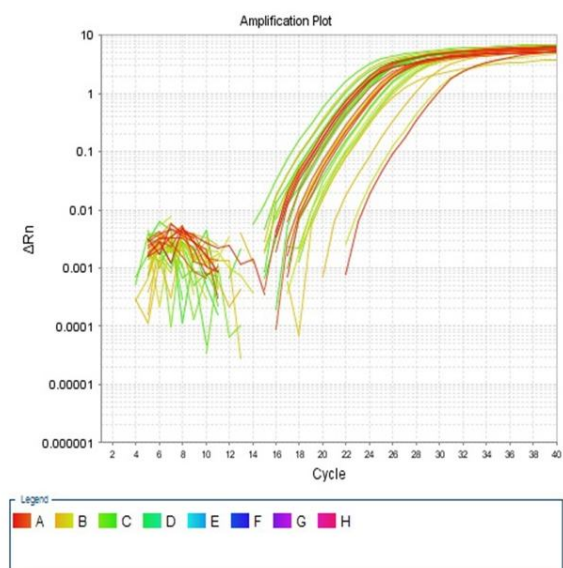
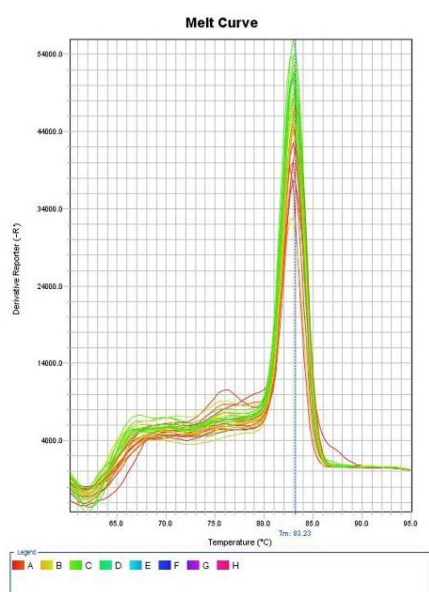
4.2.3 Pathogen quantification

In this study *Xanthomonas axonopodis* pv. *punicae* (*Xap*) infecting pomegranate was quantified using qPCR in 5 different varieties viz., Bhagwa, Wonderful, Ruby, Ganesh and Nana treated with different concentrations of ethylene (100 ppm (T1), 150 ppm (T2) and 200ppm (T3), 300 ppm (T4), Control (T5)) and after 24 hours plants were challenge inoculated with pathogen under greenhouse condition. After appearance of clear symptoms on plant, leaf samples were collected and frozen in liquid nitrogen and stored in -80° C and then leaf samples were used for DNA extraction and quantification of pathogen. *Xap* specific primer XopQ was used to amplify the genomic DNA of *Xap* using *xopQ* primer and Quantification is expressed in terms of fold change by comparing with housekeeping gene GAPDH and melt curve, Amplification plots are depicted in Fig. 1. Results obtained during investigation are presented in Table 3 and depicted in Figure 2.

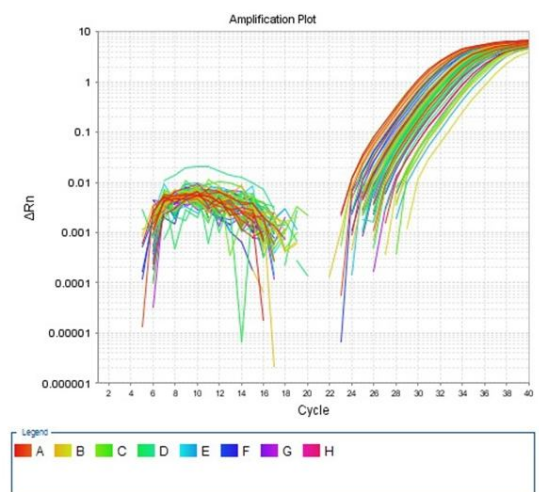
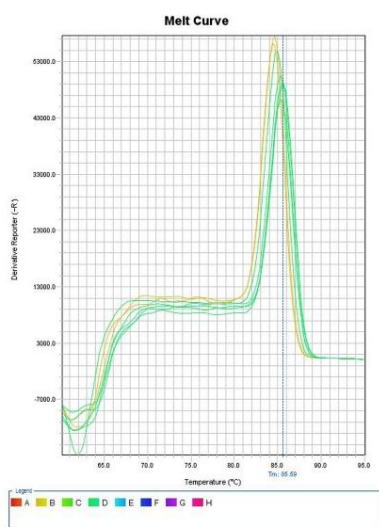
Table 2: Effect of ethylene on severity of bacterial blight on different varieties of pomegranate under greenhouse condition

Treatment	Disease Severity (%)
Ethylene concentration	
E1-100 ppm	23.00 (28.45)
E2-150 ppm	24.7 (29.64)
E3-200 ppm	26.47 (30.79)
E4-300 ppm	28.64 (32.21)
E5-Control	20.94 (27.02)
S. Em $\pm$	0.258
CD at 5%	0.725
Variety	
V1-Bhagwa	31.57 (34.15)
V2-Wonderful	28.64 (32.31)
V3-Ruby	25.00 (29.94)
V4-Ganesh	23.52 (28.97)
V5-Nana	15.00 (22.72)
S. Em $\pm$	0.258
CD at 5%	0.725
Interaction (Variety x Concentration)	
V1E1	29.21 (32.68)
V1E2	30.77 (33.68)
V1E3	34.29 (35.84)
V1E4	35.87 (36.79)
V1E5	27.73 (31.77)
V2E1	27.05 (31.32)
V2E2	28.36 (32.17)
V2E3	30.02 (33.22)
V2E4	33.18 (35.16)
V2E5	24.59 (29.70)
V3E1	23.46 (28.91)
V3E2	25.19 (30.07)
V3E3	26.49 (30.96)
V3E4	28.91 (32.52)
V3E5	20.97 (27.24)
V4E1	21.97 (27.94)
V4E2	23.83 (29.21)
V4E3	25.36 (30.23)
V4E4	27.08 (31.35)
V4E5	19.39 (26.11)
V5E1	13.31 (21.38)
V5E2	15.33 (23.04)
V5E3	16.19 (23.71)
V5E4	18.16 (25.21)
V5E5	12.03 (20.25)
S. Em $\pm$	0.57
CD at 5%	NS

Note: Figure in parenthesis indicate Arc sign transformed values



GAPDH



XopQ

Fig. 1: Melt curve and amplification plot of GAPDH, *XopQ* genes as determined by qRT-PCR in different varieties of pomegranate

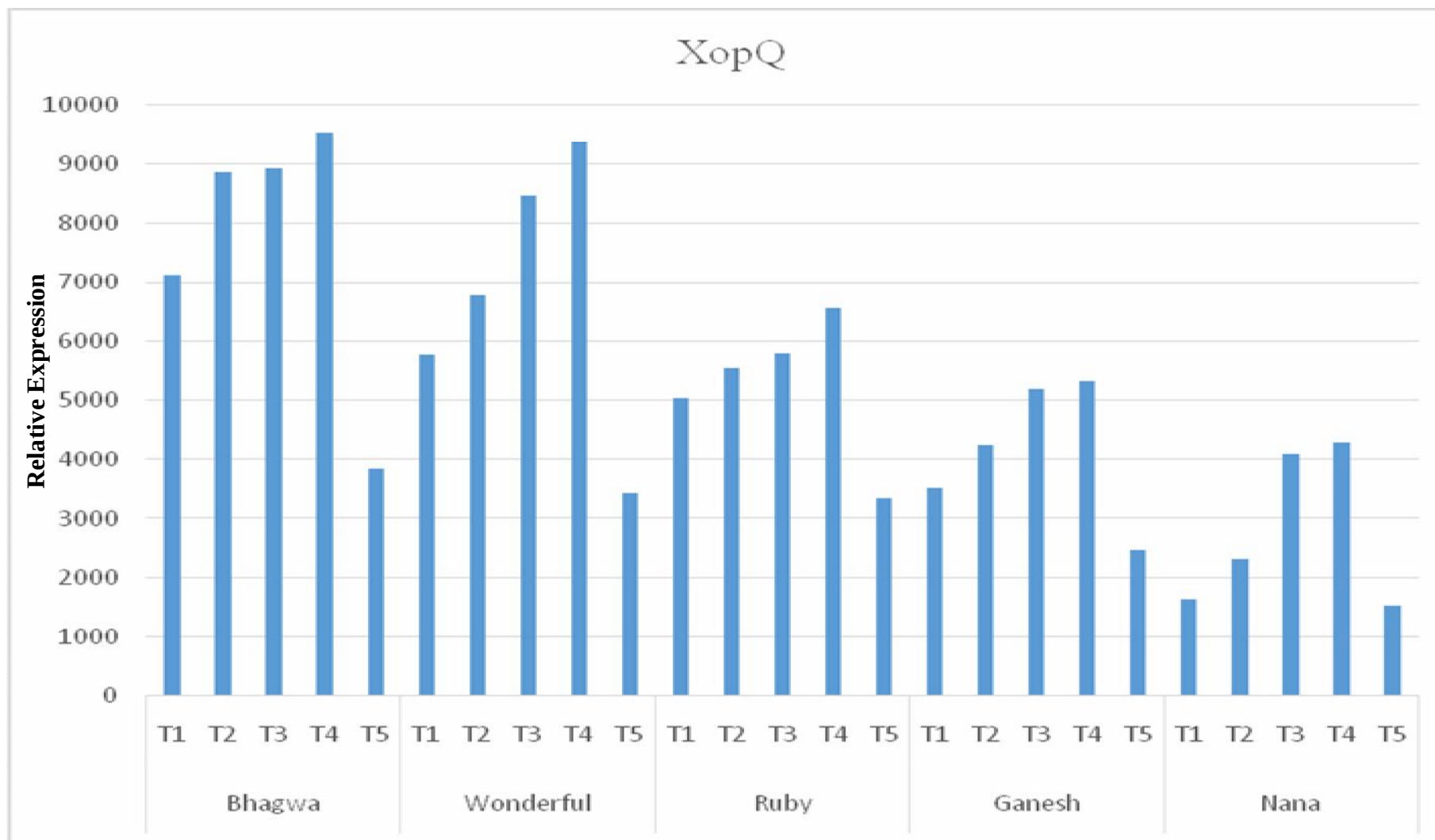


Fig. 2: qPCR quantification of pathogen in pomegranate in response to application of different concentrations of ethrel

Table 3: Quantification of pathogen in pomegranate varieties inoculated with *Xanthomonas axonopodis* pv. *punicae* in response to different concentrations of ethrel treatment under greenhouse condition

Sl. No.	Variety	Concentration	<i>XopQ</i> gene CT Value	<i>XopQ</i> gene Fold expression	Log10 ratio
1	Bhagwa	T1-100 ppm	20.00	7117.45	3.85
		T2-150 ppm	19.61	8856.36	3.95
		T3-200 ppm	19.49	8916.98	3.95
		T4-300 ppm	19.35	9522.67	3.98
		T5-Control	20.55	3838.21	3.58
2	Wonderful	T1-100 ppm	21.20	5780.54	3.76
		T2-150 ppm	21.15	6779.59	3.83
		T3-200 ppm	20.24	8445.19	3.93
		T4-300 ppm	19.86	9371.62	3.97
		T5-Control	21.74	3426.59	3.53
3	Ruby	T1-100 ppm	24.55	5032.81	3.70
		T2-150 ppm	22.48	5542.45	3.74
		T3-200 ppm	22.00	5802.99	3.76
		T4-300 ppm	21.98	6571.90	3.82
		T5-Control	25.02	3346.96	3.52
4	Ganesh	T1-100 ppm	24.88	3525.70	3.55
		T2-150 ppm	24.28	4244.41	3.63
		T3-200 ppm	23.57	5192.03	3.72
		T4-300 ppm	22.07	5323.49	3.73
		T5-Control	25.20	2475.51	3.39
5	Nana	T1-100 ppm	24.57	1622.01	3.21
		T2-150 ppm	24.55	2316.21	3.36
		T3-200 ppm	24.38	4095.65	3.61
		T4-300 ppm	24.20	4301.75	3.63
		T5-Control	25.67	1520.90	3.18

The qPCR analysis showed, in Baghawa (V1) plants sprayed with ethrel at 300ppm (T4) has higher pathogen load by exhibiting higher copies of *xopQ* gene accumulation with fold change of 9522.67. Followed by ethrel spray at 200ppm (T3) shown higher pathogen load (8916.98-folds), 150ppm (8856.36-folds) and 100ppm (7117.45-folds). Least fold change in *xopQ* gene expression was recorded in control (3838.21-folds).

Wonderful (V2) plants treated with Ethrel at 300ppm (T4) shown higher pathogen load when compared with other concentrations of Ethrel sprayed by exhibiting 9371.62 fold change. Followed by ethrel sprayed at 200ppm (T3) shown higher pathogen load (8445.19-folds). Least pathogen load was observed in control (T5) plants (3426.59-folds) and followed by plants sprayed with ethrel at 100ppm (T1) by exhibiting fold change of 5780.54.

Experimental analysis revealed that, Ruby (V3) variety shown higher pathogen load at 300ppm by exhibiting fold change of 6571.90 and followed by ethrel sprayed at 200ppm (T3) (5802.99-folds). Among all treatment, T5 (control) shown least load of pathogen (3346.96-folds) and followed by plants sprayed with ethrel at 100ppm (T1) by exhibiting fold change of 5032.81.

Ganesh variety (V4) shown higher pathogen load at 300ppm (T4) by exhibiting 5323.49 fold change and followed by ethrel sprayed at 200ppm (T3) (5192.03-folds). In contrast, lower pathogen load was observed in T5 (control) by exhibiting 2475.51 fold change and followed by plants sprayed with ethrel at 100ppm (T1) by exhibiting fold change of 3525.70.

Similar trend was observed in Nana Variety (V5) which showed higher pathogen load at 300ppm (T4) (4301.75-folds), followed by ethrel sprayed at 200ppm (T2) (4095.65-folds). In contrast, lower pathogen load was observed in T4 (control) by exhibiting 1520.90 fold change and followed by plants sprayed with ethrel at 100ppm (T1) by exhibiting fold change of 1622.01.

Among all the genotypes higher pathogen load was observed on Baghawa variety (V1) sprayed with ethrel at 300ppm (9522.67-folds) and followed by Wonderful variety (V2) sprayed with ethrel at 300ppm with fold change of 9522.67. In contrast, Nana (V5) variety shown least load of pathogen when sprayed with ethrel at

300ppm with fold change of 4301.75. Followed by Ganesh variety (V4) showed lower pathogen load at 300ppm by exhibiting fold change of 5323.49. Among different concentrations, pathogen load in all genotypes increased with increased concentration. Least pathogen in all genotypes recorded in control plants (T5). Maximum pathogen load in control plants was observed in Bhagwa (V1) with fold change of 3838.21 and least pathogen load in control plants was observed in Nana variety (V5) with fold change of 1520.90.

4.3 Microbe induced ethylene regulation in pomegranate and its effect on disease development

4.3.1 Primer design and PCR amplification of ACC deaminase producing gene in bio-control agents used in the experiment

ACC deaminase primers, PacdF/R (accession: CP007620.1) and TacdF/R (accession: FJ751936.1) were designed for *Pseudomonas putida* and *Trichoderma asperellum* respectively (Primer sequences are listed in Table 1). DNA from both biocontrol agent was extracted using standard protocol, PCR amplification using designed primers was done. Predicted product size of 130 bp was obtained for *Pseudomonas putida* and 141bp for *Trichoderma asperellum* was obtained hence, amplification shown the presence of ACC deaminase gene in both the biocontrol agents (Plate 11).

4.3.2 Greenhouse experiment

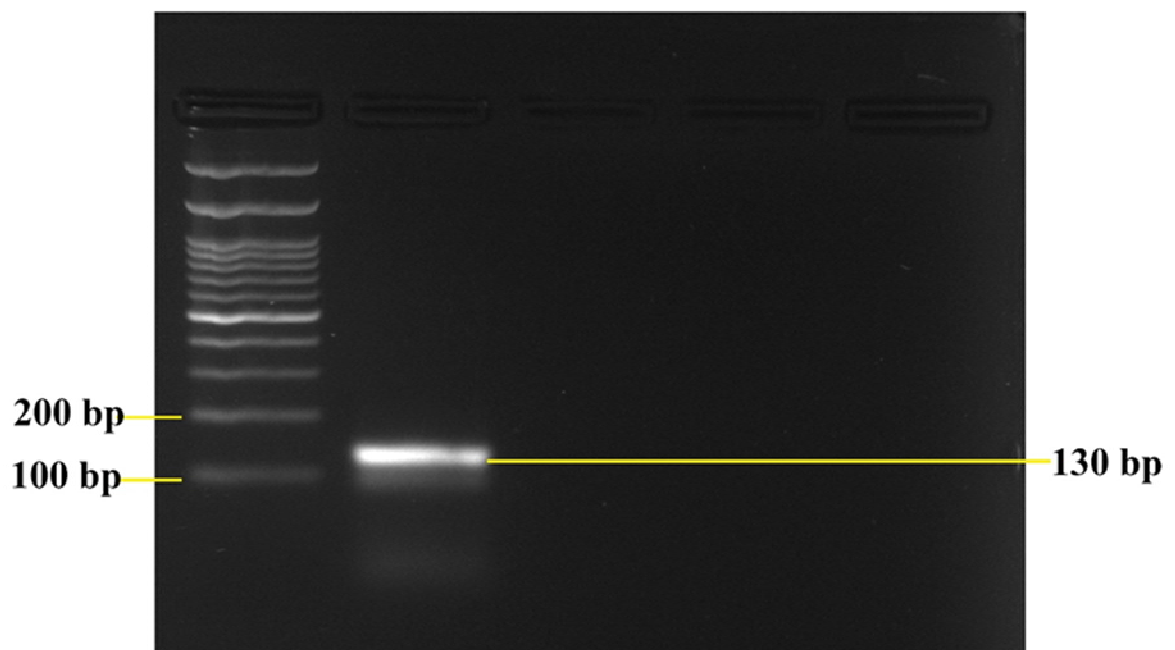
4.3.2.1 Disease severity

Application of bioagents shown greater reduction in disease severity when compared to untreated control plants (Table 4). Treatment 3 (combination of both soil and foliar spray of *Pseudomonas putida*) shown lower disease severity (4.24%) followed by T2 (foliar spray of *Pseudomonas putida*) and T6 (combination of both soil and foliar spray of *Trichoderma asperellum*) with the disease severity of 5.79 per cent and 8.78 per cent respectively whereas control plant shown higher disease severity of 28.2 per cent.

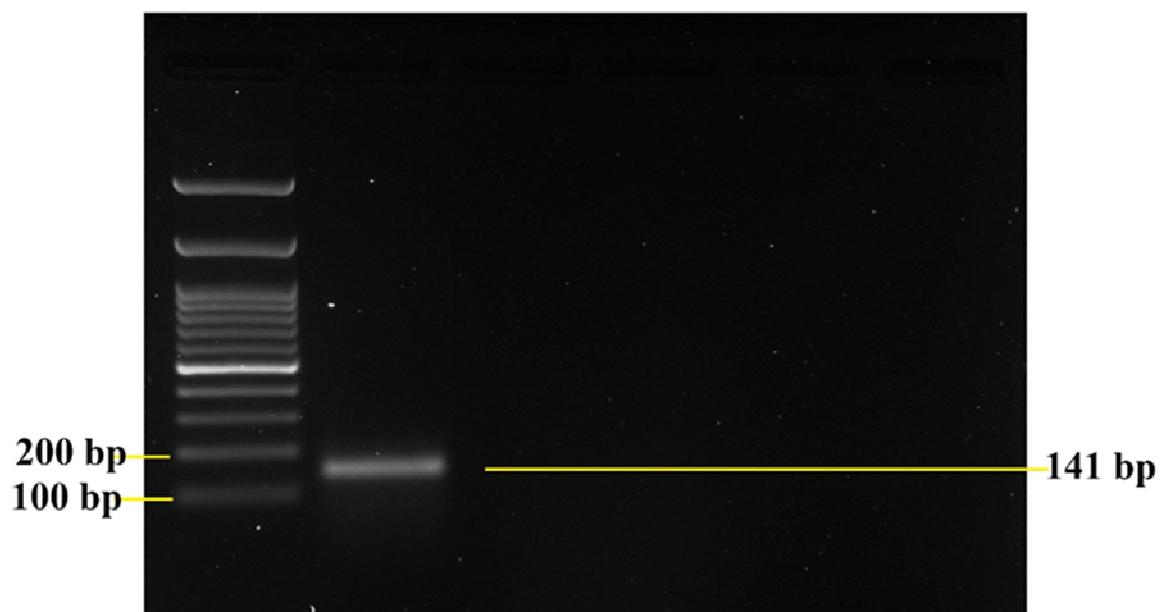
Table 4: Effect of application of bio-agents on severity of bacterial blight on pomegranate under greenhouse condition

Treatment	Disease severity (%)
T1- <i>Pseudomonas putida</i> (soil application) 4×10^8 CFU/ml	22.88 (28.54)
T2- <i>Pseudomonas putida</i> (foliar spray) 4×10^8 CFU/ml	5.79 (13.9)
T3- <i>Pseudomonas putida</i> (foliar spray + soil application) 4×10^8 CFU/ml	4.24 (11.78)
T4- <i>Trichoderma asperellum</i> (soil application) 4×10^6 CFU/ml	24.79 (29.85)
T5- <i>Trichoderma asperellum</i> (foliar spray) 4×10^6 CFU/ml	10.89 (19.22)
T6- <i>Trichoderma asperellum</i> (foliar spray + soil application) 4×10^6 CFU/ml	8.78 (17.21)
T7-Control	28.2 (32.05)
S. Em $\pm$	0.649
CD at 5%	1.889

Note: Figure in parenthesis indicate Arc sign transformed values

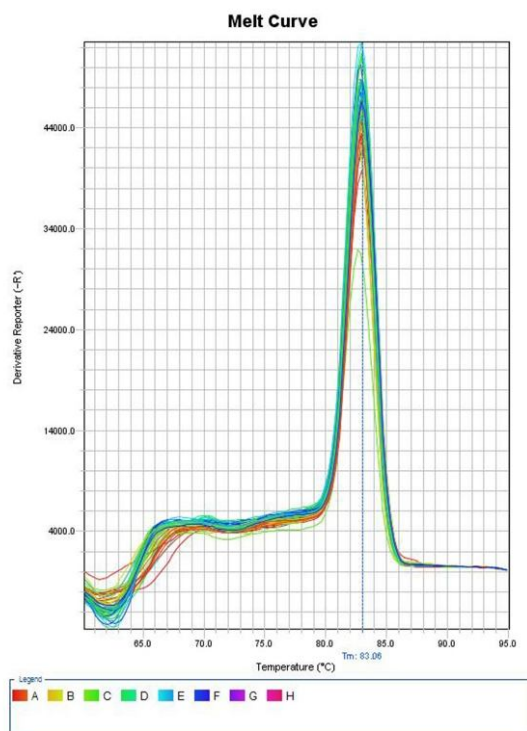


Amplification of ACC deaminase gene in *Pseudomonas putida*

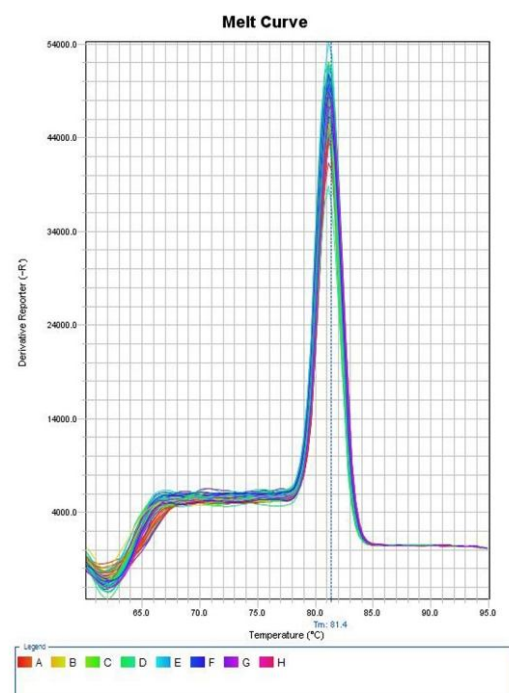


Amplification of ACC deaminase gene in *Trichoderma asperellum*

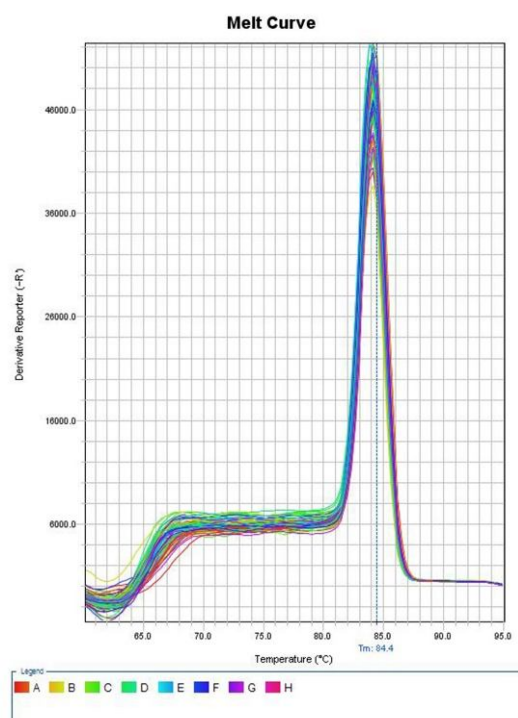
Plate 11: PCR amplification of ACC deaminase producing gene in bio-control agents



GAPDH



ACC Oxidase



PAL

Fig 3: Melt curve of GAPDH, ACC Oxidase, PAL genes as determined by qRT-PCR in various bio-agent treatments

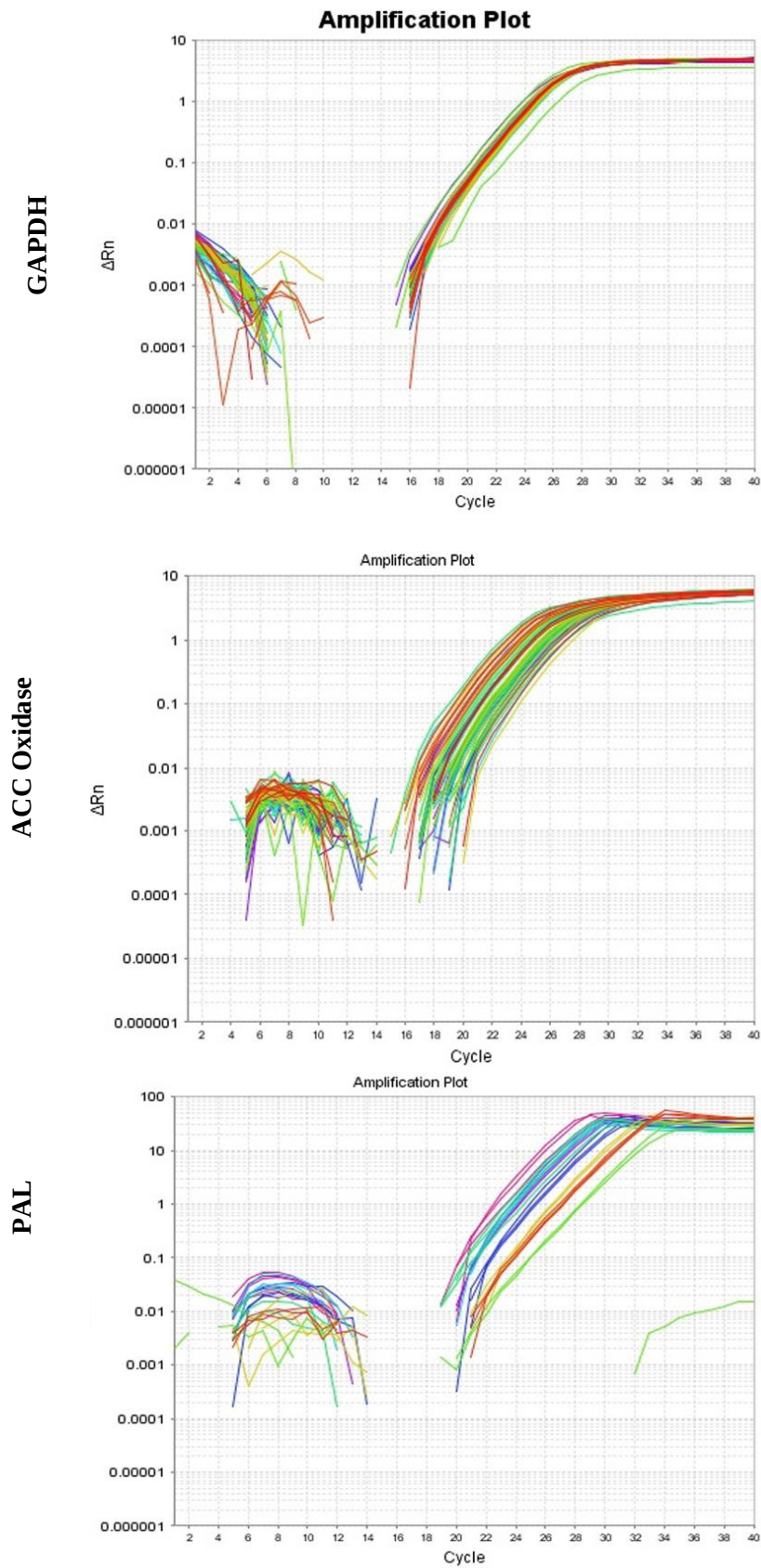


Fig 4: Amplification plot of GAPDH, ACC Oxidase, PAL genes as determined by qRT-PCR in various bioagent treatments

4.3.3 Gene expression

Activity of defence related genes and their expression rate gives clear understanding of defence mechanism of action behind application of any hormones towards controlling harmful pathogens. Thus in the present study, transcript level of 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACC oxidase) and Phenylalanine ammonia lyase (PAL) defence response genes expressed during disease resistance were studied using qPCR. Results obtained were compiled and interpreted in following paragraphs.

The transcripts of the gene ACC oxidase and PAL expression at different time points were detected in all the seven treatments after challenge inoculation with pathogen. Gene expression was studied in different time points viz., 0, 24, 48, 72, 144, 216 hours after pathogen inoculation. GAPDH gene is used to normalize the gene expression values. Melt curve and amplification plots of GAPDH, ACC oxidase, PAL are depicted in figure 3 and figure 4 respectively.

4.3.3.1 Leaf

Table 5 and 6 depict that relative expression of ACC oxidase and PAL in pomegranate leaf. The induction of synthesis of ethylene is mainly done by enhancing the synthesis of *ACC oxidase* (enzyme responsible for synthesis of ethylene). The gene, ACC oxidase, had shown similar trend in gene expression in all the treatment but at different levels, but decreased expression level was seen in initial hours of post-inoculation (hpi) with pathogen and expression gradually increased from 0 hpi to 216 hpi (Fig. 5). PAL is the key enzyme in the phenol synthesis pathway and the accumulation of phenols that act as phytoalexins is considered the primary inducible response in plants against a number of biotic and abiotic stresses. PAL expression profile in pomegranate leaf revealed that, PAL gene *mRNA* transcript levels were high in early time points between 24 to 48 hpi (Fig. 6).

Soil application of *Pseudomonas putida* (T1), shown higher up-regulation of ACC oxidase gene at 216 hpi with 3.65-folds when compared with other time points. Expression was increased from 0 hpi (1.12-folds) to 216 hpi except at 6 hpi (0.68-folds) and 72 hpi (1.23-folds). PAL gene expression was gradually increased from 0 hpi to 6 hpi (1.51- and 1.63-folds, respectively), sudden increase in PAL was seen at 48 hpi with 4.53-folds, then onwards gradually decreased.

Table 5: Relative gene expression of ACC oxidase gene in leaves of pomegranate plants inoculated with *Xanthomonas axonopodis* pv. *punicae* and treated with bio-agents

Treatment	Hours after inoculation (HAI)	ACC Oxidase Fold expression	Log10 ratio
T1- <i>Pseudomonas putida</i> (soil application) 4 x 10 ⁸ CFU/ml	0h	1.12	0.05
	6h	0.68	-0.17
	24h	1.71	0.23
	48h	1.80	0.26
	72h	1.23	0.09
	144h	2.38	0.38
	216h	3.65	0.56
T2- <i>Pseudomonas putida</i> (foliar spray) 4 x 10 ⁸ CFU/ml	0h	0.54	-0.27
	6h	0.57	-0.24
	24h	1.54	0.19
	48h	1.31	0.12
	72h	1.71	0.23
	144h	2.20	0.34
	216h	2.53	0.40
T3- <i>Pseudomonas putida</i> (foliar spray + soil application) 4 x 10 ⁸ CFU/ml	0h	0.41	-0.39
	6h	0.56	-0.25
	24h	1.44	0.16
	48h	1.54	0.19
	72h	1.63	0.21
	144h	1.93	0.29
	216h	2.13	0.33
T4- <i>Trichoderma asperellum</i> (soil application) 4 x 10 ⁶ CFU/ml	0h	1.10	0.04
	6h	0.61	-0.21
	24h	1.72	0.24
	48h	2.26	0.35
	72h	1.99	0.30
	144h	2.74	0.44
	216h	3.80	0.58

Contd.....

Treatment	Hours after inoculation (HAI)	ACC Oxidase Fold expression	Log10 ratio
T5- <i>Trichoderma asperellum</i> (foliar spray) 4 x 10 ⁶ CFU/ml	0h	0.60	-0.22
	6h	0.55	-0.26
	24h	1.68	0.23
	48h	1.93	0.29
	72h	1.82	0.26
	144	2.57	0.41
	216	3.17	0.50
T6- <i>Trichoderma asperellum</i> (foliar spray + soil application) 4 x 10 ⁶ CFU/ml	0h	0.47	-0.33
	6h	0.60	-0.22
	24h	1.56	0.19
	48h	1.47	0.17
	72h	1.82	0.26
	144h	2.21	0.34
	216h	2.85	0.45
T7- Control	0h	1.00	0.00
	6h	0.75	-0.13
	24h	2.21	0.34
	48h	2.14	0.33
	72h	3.12	0.49
	144h	4.23	0.63
	216h	6.16	0.79

Table 6: Relative gene expression of PAL gene in leaves of pomegranate plants inoculated with *Xanthomonas axonopodis* pv. *punicae* and treated with bio-agents

Treatment	Hours after inoculation (HAI)	PAL Gene Fold expression	Log10 ratio
T1- <i>Pseudomonas putida</i> (soil application) 4×10^8 CFU/ml	0h	1.51	0.18
	6h	1.63	0.21
	24h	4.53	0.66
	48h	3.52	0.55
	72h	2.52	0.40
	144h	2.55	0.41
	216h	2.10	0.32
T2- <i>Pseudomonas putida</i> (foliar spray) 4×10^8 CFU/ml	0h	2.53	0.40
	6h	2.63	0.42
	24h	6.58	0.82
	48h	4.47	0.65
	72h	4.21	0.62
	144h	3.58	0.55
	216h	2.84	0.45
T3- <i>Pseudomonas putida</i> (foliar spray + soil application) 4×10^8 CFU/ml	0h	2.52	0.40
	6h	2.32	0.37
	24h	7.14	0.85
	48h	5.48	0.74
	72h	5.18	0.71
	144h	4.53	0.66
	216h	3.14	0.50
T4- <i>Trichoderma asperellum</i> (soil application) 4×10^6 CFU/ml	0h	1.46	0.16
	6h	1.71	0.23
	24h	4.13	0.62
	48h	3.58	0.55
	72h	2.13	0.33
	144h	2.44	0.39
	216h	1.55	0.19

Contd.....

Treatment	Hours after inoculation (HAI)	PAL Gene Fold expression	Log10 ratio
T5- <i>Trichoderma asperellum</i> (foliar spray) 4 x 10 ⁶ CFU/ml	0h	1.82	0.26
	6h	1.96	0.29
	24h	4.29	0.56
	48h	2.79	0.45
	72h	2.41	0.38
	144h	2.18	0.34
	216h	2.14	0.33
T6- <i>Trichoderma asperellum</i> (foliar spray + soil application) 4 x 10 ⁶ CFU/ml	0h	2.14	0.33
	6h	2.22	0.35
	24h	4.42	0.65
	48h	3.75	0.57
	72h	2.64	0.42
	144h	3.27	0.51
	216h	2.54	0.40
T7- Control	0h	1.00	0.00
	6h	0.90	-0.05
	24h	2.54	0.41
	48h	1.85	0.27
	72h	1.36	0.13
	144h	1.68	0.22
	216h	1.28	0.11

Spray application of *Pseudomonas putida* (T2), shown lower expression of ACC oxidase gene but, slightly higher enhancement in the gene expression of ACC oxidase gene at 216 hpi with 2.53-folds. From 0 hpi (0.54-folds) to 6 hpi (0.57-folds) gene expression was low but shown gradual increase in gene expression from 24 hpi (1.54-folds) to 216 hpi except decline in gene expression at 48 hpi with 1.31-folds. Gene expression of PAL gene was higher at 24 and 48 hpi with fold change of 6.58 and 4.47 respectively. Further gene expression gradually decreased from 72 hpi (4.21-folds) to 216 hpi (2.84-folds). Lowest expression was seen in 0 hpi with 2.53-folds.

Both spray and soil application of *Pseudomonas putida* (T3), also shown lower expression of ACC oxidase gene but, shown slight up-regulation at 216 hpi with 2.13-folds. Lowest expression was seen at 0 hpi with 0.41-folds but shown gradual increase in gene expression from 0 hpi to 216 hpi. PAL transcript accumulation in leaves of pomegranate showed the strongest, 7.14-fold enhancement at 24 hpi. Followed by higher expression was seen at 48 hpi with 5.48 fold change. Gene expression then shown gradual decrease from 72 hpi (5.18-folds) to 216 hpi (3.14-folds). In contrast lowest transcript levels of PAL gene was observed at 0 hpi (2.52-folds).

Soil application of *Trichoderma asperellum* (T4) shown higher transcript levels of ACC oxidase gene at 216 hpi with 3.80-folds. Lowest expression was seen at 6 hpi and gradual increase in gene expression was observed from 0 to 216 hpi except at 6 hpi and 72 hpi with 0.61- and 1.99-folds respectively. The gene PAL transcript levels in leaves of pomegranate showed the higher, 4.13-fold enhancement at 24 hpi. Followed by higher expression was seen at 48 hpi with 3.58 fold change. Gene expression then shown gradual decrease from 72 hpi (2.13-folds) to 216 hpi (1.55-folds). In contrast, lowest transcript levels of PAL gene was observed at 0 hpi (1.46-folds).

Spray application of *Trichoderma asperellum* (T5) showed higher expression of ACC oxidase gene at 216 hpi with 3.17-folds activity. Lowest expression was seen at 6 hpi and showed gradual increase in gene expression from 0 to 216 hpi except at 6 hpi (0.55-folds) and 72 hpi (1.82-folds). While, PAL expression levels in leaves of pomegranate showed the higher, 4.29-fold enhancement at 24 hpi. Followed by higher

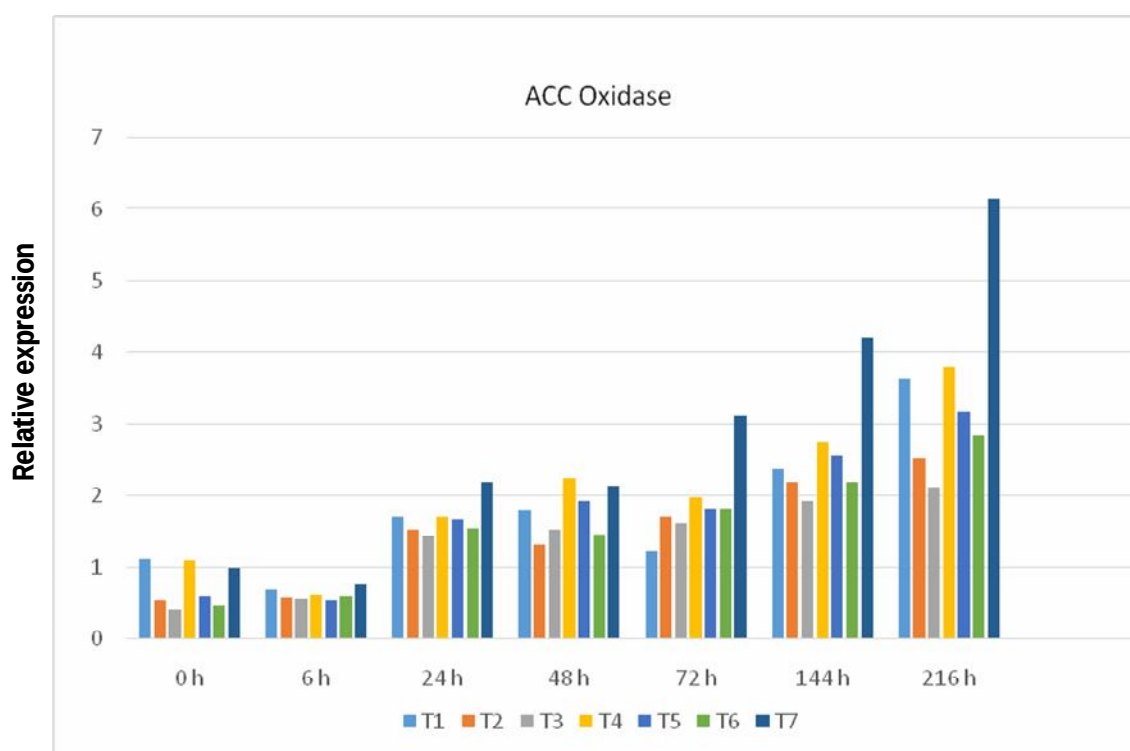


Fig. 5: Relative expression of ACC oxidase gene in leaves of pomegranate upon bio agent application

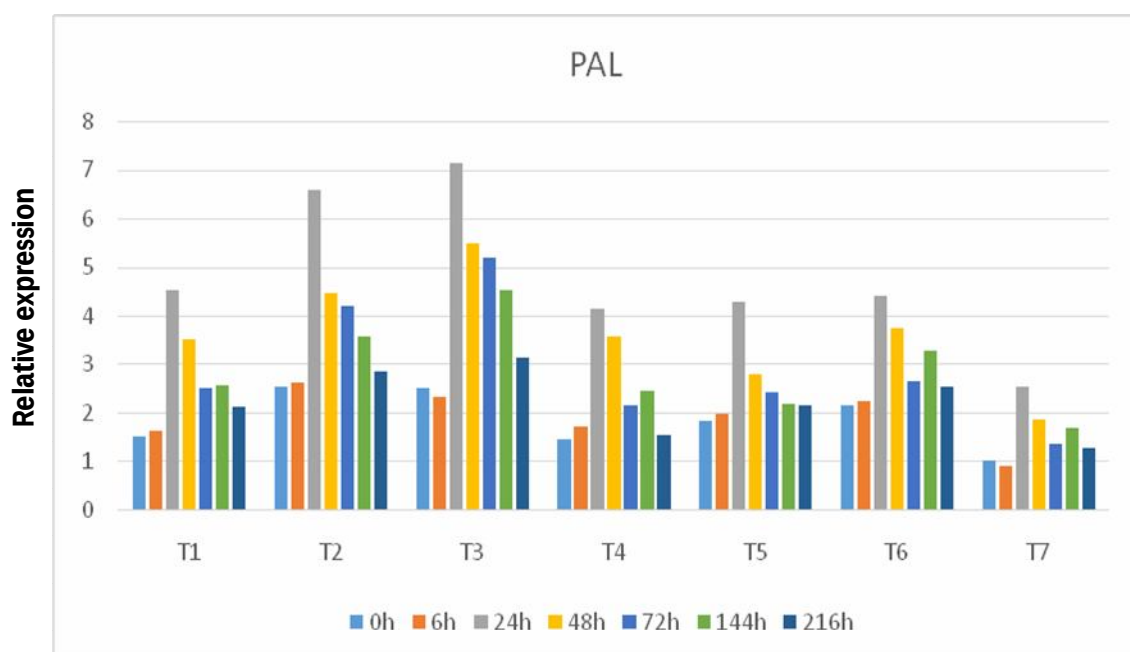


Fig. 6: Relative expression of PAL gene in leaves of pomegranate upon bio agent application

expression of PAL was seen at 48 hpi with 2.79 fold change. Gene expression thereafter shown gradual decrease from 72 hpi (2.41-folds) to 216 hpi (2.14-folds). In contrast, lowest transcript levels of PAL gene was observed at 0 hpi (1.82-folds).

Both spray and soil application of *Trichoderma asperellum* (T6) exhibited lower expression of ACC oxidase gene but shown higher expression at 216 hpi with fold change of 2.85. Lower transcript abundance was noticed at 0 hpi when compared with other time points within the treatment. Gene expression shown increase in fold changes from 0 hpi to 216 hpi except at 48 hpi (1.47-folds). Whereas, PAL expression levels in leaves of pomegranate showed the higher, 4.42-fold enhancement at 24 hpi. Followed by higher up-regulation of PAL gene was seen at 48 hpi with 3.75 fold change. Gene expression thereafter showed gradual decrease from 72 hpi (2.64-folds) to 216 hpi (2.54-folds). In contrast, lowest transcript levels of PAL gene was observed at 0 hpi (2.14-folds).

Control plants (T7) (without any bio-agent application) shown higher expression of ACC oxidase gene at 216 hpi by exhibiting 6.16-fold activity. Lowest expression was seen at 6 hpi but gradual increase in gene expression was observed from 0 to 216 hpi except at 6hpi (0.75-folds) and 72 hpi (3.12-folds). PAL transcript accumulation in leaves of pomegranate showed the higher, 2.54-fold enhancement at 24 hpi. Followed by higher expression was seen at 48 hpi with 1.85 fold change. Gene expression then shown gradual decrease from 72 hpi (1.36-folds) to 216 hpi (1.28-folds). In contrast lowest transcript levels of PAL gene was observed at 6 hpi (0.90-folds).

Overall the maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 6 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 6.16 fold change activity and least gene expression at 216 hpi was observed in T3 (2.13-folds) and T2 (2.53-folds). Lowest expression of ACC oxidase gene among all time points was observed in T3 and T6 at 0 hpi with fold change of 0.41 and 0.47 respectively. Meanwhile, the PAL gene expression was maximum at 24 hpi in all the treatments. Among all treatments maximum up-regulation of PAL gene was observed in T3 and T2 with fold change of 7.14 and 6.58 at 24 hpi respectively and at 24 hpi lowest gene expression of PAL was observed in

T7 (2.54-folds) and T4 (4.13-folds). In contrast, among all the time points, lowest transcript levels of PAL was observed in T7 at 6 hpi (0.90-folds) and followed by T4 at 0 hpi (1.46-folds).

4.3.3.2 Root

Table 7 and 8 revealed that relative expression of ACC oxidase and PAL in pomegranate root. The gene, ACC oxidase, shown decreased expression levels initial hours of post-inoculation from 0, 6, 24 hpi with pathogen and then gradually increased from 48 hpi to 216 hpi except at 72 hpi (Fig. 7). Similarly, PAL expression profile of pomegranate root shown, PAL gene *mRNA* transcript levels were high in early time points, shown increasing gene expression between 6 to 48 hpi and maximum expression was observed at 24 hpi (Fig. 8).

Soil application of *Pseudomonas putida* (T1), shown lower expression of ACC oxidase gene at different time interval, even then higher expression was at 216 hpi with 2.64-folds. Expression was peaked from 144 hpi (2.50-folds) to 216 hpi and lowest expression was seen at 6 hpi (0.38-folds). From 0 hpi (0.83-folds) to 72 hpi (0.73-folds) gene expression exhibited continuous up and down regulation of ACC oxidase gene. While, PAL expression levels in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 5.57 and 4.98 respectively. Thereafter the gene expression gradually decreased from 72 hpi (3.73-folds) to 216 hpi (1.26-folds). Lowest expression was observed at 216 hpi.

Spray application of *Pseudomonas putida* (T2), shown higher expression of ACC oxidase gene but, higher enhancement in the gene expression of ACC oxidase gene was peaked from 144 hpi (2.65-folds) to 216 hpi (3.0-folds). From 0 hpi (1.16-folds) to 72 hpi (1.27-folds) gene expression was low but shown continuous up and down regulation. In contrast, least expression was observed at 24 hpi (0.88-folds). While, PAL transcript levels in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 2.61 and 2.57 respectively. Thereafter the gene expression gradually decreased from 72 hpi (2.26-folds) to 216 hpi (0.59-folds). Lowest expression was observed at 216 hpi.

Table 7: Relative gene expression of ACC oxidase gene in roots of pomegranate plants inoculated with *Xanthomonas axonopodis* pv. *punicae* and treated with bio-agents

Treatment	Hours after inoculation (HAI)	ACC Oxidase Gene Fold expression	Log10 ratio
T1- <i>Pseudomonas putida</i> (soil application) 4 x 10 ⁸ CFU/ml	0h	0.83	-0.08
	6h	0.38	-0.42
	24h	0.39	-0.41
	48h	1.16	0.06
	72h	0.73	-0.14
	144h	2.50	0.40
	216h	2.64	0.42
T2- <i>Pseudomonas putida</i> (foliar spray) 4 x 10 ⁸ CFU/ml	0h	1.16	0.06
	6h	1.23	0.09
	24h	0.88	-0.05
	48h	1.41	0.15
	72h	1.27	0.10
	144h	2.65	0.42
	216h	3.00	0.48
T3- <i>Pseudomonas putida</i> (foliar spray + soil application) 4 x 10 ⁸ CFU/ml	0h	0.81	-0.09
	6h	0.29	-0.54
	24h	0.34	-0.47
	48h	0.90	-0.04
	72h	0.68	-0.17
	144h	2.32	0.37
	216h	2.56	0.41
T4- <i>Trichoderma asperellum</i> (soil application) 4 x 10 ⁶ CFU/ml	0h	0.91	-0.04
	6h	0.47	-0.33
	24h	0.80	-0.10
	48h	1.39	0.14
	72h	0.83	-0.08
	144h	2.73	0.44
	216h	2.88	0.46

Contd.....

Treatment	Hours after inoculation (HAI)	ACC Oxidase Gene Fold expression	Log10 ratio
T5- <i>Trichoderma asperellum</i> (foliar spray) 4 x 10 ⁶ CFU/ml	0h	1.12	0.05
	6h	1.03	0.01
	24h	1.27	0.10
	48h	1.33	0.13
	72h	1.07	0.03
	144h	2.75	0.44
	216h	3.24	0.51
T6- <i>Trichoderma asperellum</i> (foliar spray + soil application) 4 x 10 ⁶ CFU/ml	0h	0.95	-0.02
	6h	0.38	-0.42
	24h	0.46	-0.34
	48h	1.04	0.02
	72h	0.76	-0.12
	144h	2.59	0.41
	216h	2.78	0.44
T7- Control	0h	1.00	0.00
	6h	1.50	0.18
	24h	1.19	0.08
	48h	1.45	0.16
	72h	1.27	0.10
	144h	3.54	0.55
	216h	3.77	0.58

Table 8: Relative gene expression of PAL gene in roots of pomegranate plants inoculated with *Xanthomonas axonopodis* pv. *punicae* and treated with bio-agents

Treatment	Hours after inoculation (HAI)	PAL Gene Fold expression	Log10 ratio
T1- <i>Pseudomonas putida</i> (soil application) 4 x 10 ⁸ CFU/ml	0h	2.52	0.40
	6h	4.50	0.65
	24h	5.57	0.75
	48h	4.98	0.70
	72h	3.73	0.57
	144h	2.80	0.45
	216h	1.26	0.10
T2- <i>Pseudomonas putida</i> (foliar spray) 4 x 10 ⁸ CFU/ml	0h	2.11	0.32
	6h	2.45	0.39
	24h	2.61	0.42
	48h	2.57	0.41
	72h	2.26	0.35
	144h	1.92	0.28
	216h	0.59	-0.23
T3- <i>Pseudomonas putida</i> (foliar spray + soil application) 4 x 10 ⁸ CFU/ml	0h	2.70	0.43
	6h	4.61	0.66
	24h	5.75	0.76
	48h	5.11	0.71
	72h	3.84	0.58
	144h	2.92	0.47
	216h	1.31	0.12
T4- <i>Trichoderma asperellum</i> (soil application) 4 x 10 ⁶ CFU/ml	0h	2.42	0.38
	6h	4.12	0.61
	24h	4.63	0.67
	48h	4.21	0.62
	72h	3.15	0.50
	144h	2.50	0.40
	216h	0.95	-0.02

Contd.....

Treatment	Hours after inoculation (HAI)	PAL Gene Fold expression	Log10 ratio
T5- <i>Trichoderma asperellum</i> (foliar spray) 4 x 10 ⁶ CFU/ml	0h	1.90	0.28
	6h	2.13	0.33
	24h	2.83	0.45
	48h	2.25	0.35
	72h	2.18	0.34
	144h	1.93	0.29
	216h	0.48	-0.34
T6- <i>Trichoderma asperellum</i> (foliar spray + soil application) 4 x 10 ⁶ CFU/ml	0h	2.64	0.42
	6h	4.51	0.65
	24h	4.85	0.69
	48h	4.44	0.65
	72h	3.25	0.51
	D6	2.59	0.41
	D9	1.13	0.05
T7- Control	0h	1.00	0.00
	6h	2.17	0.34
	24h	2.21	0.34
	48h	2.31	0.36
	72h	2.15	0.33
	144h	1.76	0.24
	216h	0.46	0.34

Both spray and soil application of *Pseudomonas putida* (T3), shown lower transcript levels of ACC oxidase gene but, shown up-regulation from 144 hpi (2.32-folds) to 216 hpi (2.56-folds). From 0 hpi (0.81-folds) to 72 hpi (0.68-folds) gene expression was low but shown continuous up and down regulation. In contrast, least expression was observed at 6 hpi (0.29-folds). While, PAL transcript levels in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 5.75 and 5.11 respectively. Thereafter the gene expression gradually decreased from 72 hpi (3.84-folds) to 216 hpi (1.31-folds). Lowest expression was observed at 216 hpi.

Soil application of *Trichoderma asperellum* (T4), shown lower transcript levels of ACC oxidase gene but, shown up-regulation from 144 hpi (2.73-folds) to 216 hpi (2.88-folds). From 0 hpi (0.91-folds) to 72 hpi (0.83-folds) gene expression was low but shown continuous up and down regulation. In contrast, least expression was observed at 6 hpi (0.47-folds). While, PAL transcript levels in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 4.63 and 4.21 respectively. Thereafter the gene expression gradually decreased from 72 hpi (3.15-folds) to 216 hpi (0.95-folds). Lowest expression was observed at 216 hpi.

Spray application of *Trichoderma asperellum* (T5) shown higher expression of ACC oxidase gene from 144 hpi (2.75-folds) to 216 hpi (3.24-folds). From 0 hpi (1.12-folds) to 72 hpi (1.07-folds) gene expression was low but shown continuous up and down regulation. While, least expression was observed at 6 hpi (1.03-folds). While, PAL transcript levels in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 2.83 and 2.25 respectively. Thereafter the gene expression gradually decreased from 72 hpi (2.18-folds) to 216 hpi (0.48-folds). Lowest expression was observed at 216 hpi.

Both spray and soil application of *Trichoderma asperellum* (T6) exhibited lower transcript accumulation of ACC oxidase gene but, shown higher expression from 144 hpi (2.59-folds) to 216 hpi (2.78-folds). From 0 hpi (0.95-folds) to 72 hpi (0.76-folds) gene expression was low but shown continuous up and down regulation. While, least expression was observed at 6 hpi (0.38-folds). While, PAL transcript abundance in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 4.85 and 4.44 respectively. Thereafter the gene expression gradually decreased from 72 hpi (3.25-folds) to 216 hpi (1.13-folds). Lowest expression was observed at 216 hpi.

Control plants (T7) (without any bio-agent application) shown higher expression of ACC oxidase gene from 144 hpi (3.54-folds) to 216 hpi (3.77). While, gene expression was low from 0 hpi (1.00-folds) to 72 hpi (1.27-folds) but continuous up and down regulation of ACC oxidase was observed. While, least expression was observed at 0 hpi (1.0-folds). While, PAL transcript abundance in roots of pomegranate shown higher expression at 24 and 48 hpi with fold change of 2.21 and 2.31 respectively. Thereafter the gene expression gradually decreased from 72 hpi (2.15-folds) to 216 hpi (0.46-folds). Lowest expression was observed at 216 hpi.

Overall the maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 24 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 3.77 fold change activity and least gene expression at 216 hpi was observed in T3 (2.56-folds) and T1 (2.64-folds). Lowest expression of ACC oxidase gene among all the time point intervals observed in T3 and T1 at 6 hpi with fold change of 0.29 and 0.38 respectively. Meanwhile, the PAL gene expression was maximum in T3 and T1 with fold change of 5.75 and 5.57 at 24 hpi respectively and lowest expression of PAL gene at 24 hpi was observed in T7 (2.21-folds). In contrast, among all the time points, lowest transcript levels of PAL was observed in T7 (0.46-folds) and T5 (0.48-folds) at 216 hpi.

4.4 Bio efficacy of ethylene against bacterial blight and phenological characters in field conditions

Present experiment was laid out to know the response of ethylene towards managing bacterial blight incidence under natural infection process and to know the effect of ethylene on phenological characters. Observations are recorded as follows.

4.4.1 Bio efficacy of ethylene against bacterial blight in field condition

4.4.1.1 Disease severity

4.4.1.2.1 Disease severity on leaves

Foliar spray of ethrel at different concentration found to increase bacterial blight disease severity significantly under field conditions. Ethrel sprayed at 200ppm (E3) showed higher disease severity of 6.65 per cent followed by, ethrel sprayed at.

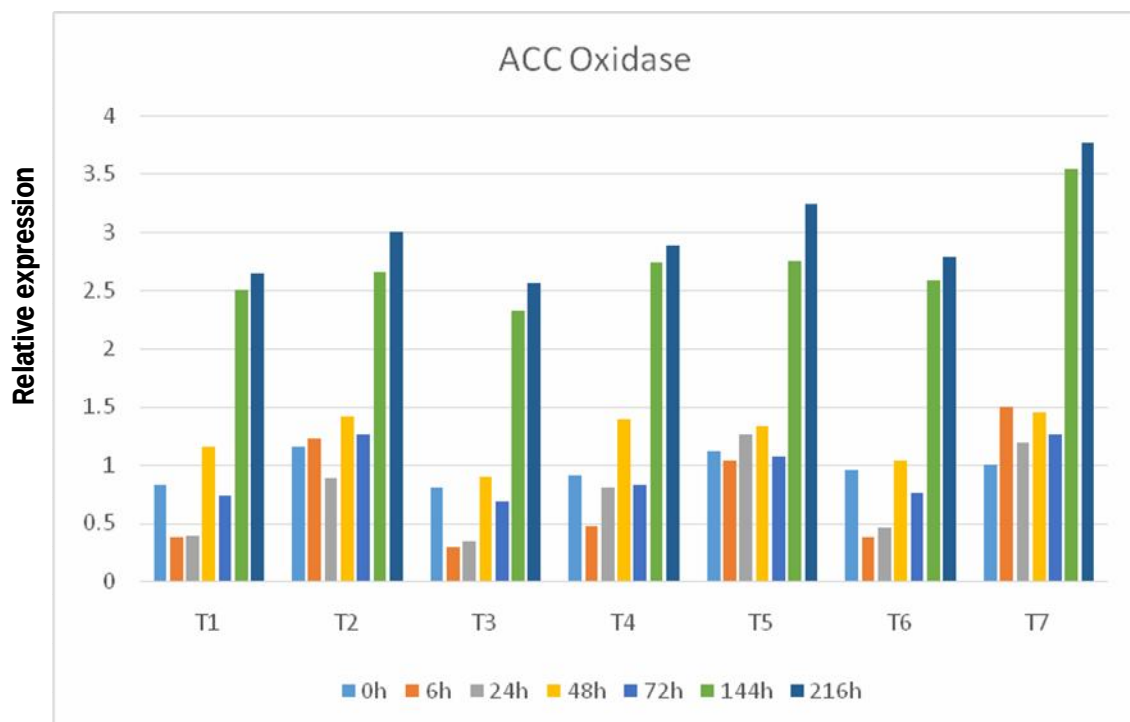


Fig. 7: Relative expression of ACC gene in roots of pomegranate upon bio agent application

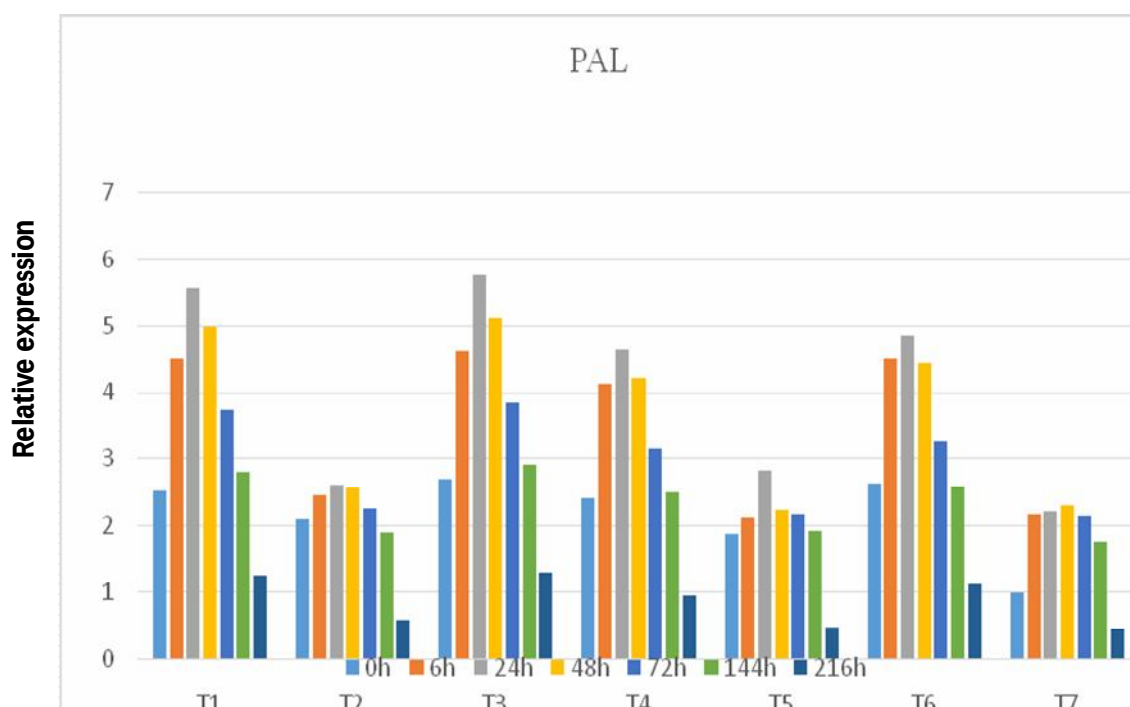


Fig. 8: Relative expression of ACC gene in roots of pomegranate upon bio agent application

Table 9: Bio efficacy of ethylene on severity of bacterial blight on leaves of pomegranate under field condition

Treatment	Disease Severity
Ethylene concentration	
E1-100 ppm	4.86 (12.32)
E2-150 ppm	5.60 (13.27)
E3-200 ppm	6.65 (14.67)
E4-Control	4.12 (11.40)
S. Em $\pm$	0.21
CD at 5%	0.60
Season (Bahar)	
S1-Ambia Bahar	4.40 (11.99)
S2-Mrig Bahar	8.51 (16.86)
S3-Hasta Bahar	3.01 (9.88)
S. Em $\pm$	0.18
CD at 5%	0.52
Interaction (Season x Concentration)	
S1E1	8.04 (16.40)
S1E2	9.14 (17.53)
S1E3	10.18 (18.58)
S1E4	6.69 (14.96)
S2E1	2.45 (8.99)
S2E2	2.77 (9.54)
S2E3	4.29 (11.93)
S2E4	2.51 (9.09)
S3E1	4.08 (11.58)
S3E2	4.9 (12.74)
S3E3	5.49 (13.51)
S3E4	3.15 (10.16)
S. Em $\pm$	0.37
CD at 5%	NS

Note: Figure in parenthesis indicate Arc sign transformed values

150ppm (E2) and 100ppm (E1) showed a disease severity of 5.6 per cent and 4.86 per cent respectively. Least disease severity was seen in E4 (control) treatment with 4.12 per cent (Table 9).

Among three different seasons, Mrig bahar (S2) shown higher disease severity of 8.51 per cent whereas Hasta bahar (S3) shown lower disease severity of 3.01 per cent.

The interaction between concentration levels and season was found non-significant differences on disease incidence.

4.4.1.2.2 Disease severity on fruits

Application of ethrel at different concentration shown significant influence on disease severity on fruits of pomegranate. Ethrel sprayed at 200ppm (E3) shown higher disease severity of 5.17 per cent. Followed by, ethrel at 150ppm (E2) (4.09%) and 100ppm (E1) (3.69%) shown on par results with each other. Whereas un-treated control plants (E4) shown lower disease severity of 3.07 per cent (Table 10).

Disease severity on fruit in different season differed significantly. Among three different seasons, Mrig bahar (S2) shown higher disease severity of 5.75 per cent whereas Hasta bahar (S3) shown lower disease severity of 2.69 per cent.

The interaction between concentration levels and season was found non-significant differences on disease severity.

4.4.1.2.3 Disease severity on twigs

Foliar spray of ethrel shown increase in the disease severity when compared with control plants without spray. Ethrel sprayed at 200ppm (E3) shown significantly higher disease severity of 0.57 per cent. Followed by, ethrel at 150ppm and 100ppm shown disease severity of 0.26 per cent and 0.18 per cent respectively and were on par with each other. Whereas un-treated control plants (E4) shown significantly lower disease severity of 0.15 per cent which was also on par with other treatment (Table 11).

Among three different seasons, disease severity did not show any significant difference in any of the seasons.

Table 10: Bio efficacy of ethylene on severity of bacterial blight on fruits of pomegranate under field condition

Treatment	Disease Severity
Ethylene concentration	
E1-100 ppm	3.69 (10.83)
E2-150 ppm	4.09 (11.43)
E3-200 ppm	5.17 (12.95)
E4-Control	3.07 (9.92)
S. Em $\pm$	0.25
CD at 5%	0.72
Season (Bahar)	
S1-Ambia Bahar	3.57 (10.78)
S2-Mrig Bahar	5.75 (13.71)
S3-Hasta Bahar	2.69 (9.35)
S. Em $\pm$	0.22
CD at 5%	0.62
Interaction (Season x Concentration)	
S1E1	5.3 (13.19)
S1E2	5.95 (13.99)
S1E3	7.41 (15.75)
S1E4	4.35 (11.94)
S2E1	2.35 (8.75)
S2E2	2.65 (9.35)
S2E3	3.71 (11.05)
S2E4	2.07 (8.25)
S3E1	3.42 (10.55)
S3E2	3.67 (10.94)
S3E3	4.4 (12.06)
S3E4	2.8 (9.57)
S. Em $\pm$	0.44
CD at 5%	NS

Note: Figure in parenthesis indicate Arc sign transformed values

Table 11: Bio efficacy of ethylene on severity of bacterial blight on twigs of pomegranate under field condition

Treatment	Disease Severity
Ethylene concentration	
E1-100 ppm	0.18 (1.50)
E2-150 ppm	0.26 (1.80)
E3-200 ppm	0.57 (3.20)
E4-Control	0.15 (1.20)
S. Em $\pm$	0.38
CD at 5%	1.07
Season (Bahar)	
S1-Ambia Bahar	0.28 (1.87)
S2-Mrig Bahar	0.35 (2.31)
S3-Hasta Bahar	0.24 (1.59)
S. Em $\pm$	0.33
CD at 5%	NS
Interaction (Season x Concentration)	
S1E1	0.20 (1.79)
S1E2	0.25 (2.16)
S1E3	0.74 (3.69)
S1E4	0.2 (1.59)
S2E1	0.12 (1.05)
S2E2	0.27 (1.51)
S2E3	0.48 (2.96)
S2E4	0.11 (0.85)
S3E1	0.21 (1.65)
S3E2	0.27 (1.73)
S3E3	0.50 (2.94)
S3E4	0.14 (1.15)
S. Em $\pm$	0.66
CD at 5%	NS

Note: Figure in parenthesis indicate Arc sign transformed values

The interaction between concentration levels and season was found non-significant differences on disease incidence.

4.4.2 Bio efficacy of ethylene on phonological characters under field condition.

4.4.2.1 Flower characters

4.4.2.1.1 Total number of flowers per plant

Table 12 depict the results on total number of flowers, hermoprodite flowers and male flower per plant. Data pertaining to total number of flowers per plant revealed that, foliar spray of ethrel at different concentration did not show any significant difference on total flowers per plant.

There was highly significant difference among the seasons with respect to total number of flowers per plant. Among different seasons, Ambe Bahar (S1) shown maximum number of flowers per plant (986.02). Followed by, Mrig bahar (S2) shown 374.35 flowers per plant and Hasta bahar (S3) shown significantly lower number of flowers per plant (264.50).

Interaction between concentration and season did not show any significant difference on total number of flowers.

4.4.2.1.2 Hermaphrodite flowers per plant

Foliar spray of ethrel had showed significant influence on production of hermaphrodite flowers per plant (Table 12). Among concentrations of foliar application of ethrel at 200 ppm (E3) showed significant influence towards enhancing maximum production of hermaphrodite flowers per plant (189.86), which was on par with ethrel at 150 ppm (179.43). In contrast, control (E4) plants without ethrel spray recorded minimum number of hermaphrodite flowers per plant (164.16) and also plant sprayed with ethrel at 100ppm (E1) shown lower number of flowers and were on par with control (Plate 12).

Among different seasons, Ambia Bahar (S1) shown maximum number of hermaphrodite flowers per plant (276.52). Followed by, Mrig bahar (S2) shown 134.775 hermaphrodite flowers per plant and Hasta bahar (S3) shown significantly lower number of hermaphrodite flowers per plant (118.05), were significantly superior over each other.

Table 12: Influence of ethylene on number of flowers per plant in pomegranate

Treatment	Total flowers per plant	Hermaphrodite flowers per plant	Male flowers per plant
Ethylene concentration			
E1-100 ppm	539.26	172.33	366.93
E2-150 ppm	542.43	179.43	363.00
E3-200 ppm	547.73	189.86	357.86
E4-Control	537.06	164.16	372.90
S. Em $\pm$	14.32	4.37	10.75
CD at 5%	NS	12.28	NS
Season (Bahar)			
S1-Ambia Bahar	986.02	276.52	709.50
S2-Mrig Bahar	374.35	134.77	239.57
S3-Hasta Bahar	264.50	118.05	146.45
S. Em $\pm$	12.40	3.78	9.31
CD at 5%	34.86	10.63	26.18
Interaction (Season x Concentration)			
S1E1	981.70	266.30	715.40
S1E2	987.20	282.30	704.90
S1E3	994.60	301.00	693.60
S1E4	980.60	256.50	724.10
S2E1	373.20	134.50	238.70
S2E2	375.70	138.50	237.20
S2E3	379.70	145.20	234.50
S2E4	368.80	120.90	247.90
S3E1	262.90	116.20	146.70
S3E2	264.40	117.50	146.90
S3E3	268.90	123.40	145.50
S3E4	261.80	115.10	146.70
S. Em $\pm$	24.81	7.56	18.63
CD at 5%	NS	NS	NS

Interaction between concentration and season did not show any significant difference on hermaphrodite flowers per plant.

4.4.2.1.3 Male flowers per plant

Production of male flowers per plant has not varied significantly among different concentrations of ethrel sprayed (Table 12). In spite of these, foliar application of ethrel at 200 ppm showed comparatively less number of male flowers (357.86) whereas untreated plants showed maximum production of male flowers (372.90) compared to rest of the treatments.

Among different seasons, Ambia Bahar (S1) shown maximum number of male flowers per plant (709.50). Followed by, Mrig bahar (S2) shown 239.57 male flowers per plant and Hasta bahar (S3) shown significantly lower number of male flowers per plant (146.45).

Interaction between concentration and season did not show any significant difference on male flowers per plant.

4.4.2.1.4 Percentage of male flowers

Experimental results in Table 13 reveals percentage of male flowers, percentage of hermaphrodite flowers and sex ratio. Percentage of male flowers differed significantly among the treatments. Among these, foliar spray of ethrel at 200 ppm concentration (T₅) showed less percentage of male flowers (61.83%), followed by, ethrel sprayed at 150 ppm (E2) and 100ppm (E1) shown lower number of male flowers with 63.25 per cent and 64.06 per cent respectively were on par with each other. Whereas control plants (E3) shown higher percentage of male flowers per plant (65.66%).

Among different seasons, Ambia Bahar (S1) shown significantly higher number of male flower percentage (71.94%). Followed by, Mrig bahar (S2) shown 63.92 per cent male flower percentage and Hasta bahar (S3) shown significantly lower number of Percentage of male flowers (55.24%).

Interaction between concentration and season did not show any significant difference on Percentage of male flowers.

Table 13: Effect of ethylene on flower characters in pomegranate

Treatment	Percentage of male flowers	Percentage of hermaphrodite flowers	Sex ratio
Ethylene concentration			
E1-100 ppm	64.06 (53.27)	35.94 (36.72)	1.912
E2-150 ppm	63.25 (52.76)	36.75 (37.23)	1.822
E3-200 ppm	61.83 (51.91)	38.17 (38.09)	1.705
E4-Control	65.66 (54.24)	34.34 (35.75)	2.056
S. Em $\pm$	0.23	0.23	0.03
CD at 5%	0.65	0.65	0.09
Season (Bahar)			
S1-Ambia Bahar	71.94 (58.03)	28.06 (31.96)	2.58
S2-Mrig Bahar	63.92 (53.09)	36.08 (36.90)	1.79
S3-Hasta Bahar	55.24 (48.01)	44.76 (41.99)	1.24
S. Em $\pm$	0.20	0.20	0.02
CD at 5%	0.56	0.56	0.08
Interaction (Season x Concentration)			
S1E1	72.79 (58.57)	27.21 (31.42)	2.69
S1E2	71.38 (57.67)	28.62 (32.33)	2.51
S1E3	69.75 (56.64)	30.25 (33.36)	2.31
S1E4	73.86 (59.25)	26.14 (30.74)	2.84
S2E1	63.8 (53.02)	36.2 (36.97)	1.78
S2E2	62.89 (52.48)	37.11 (37.52)	1.71
S2E3	61.76 (51.80)	38.24 (38.19)	1.62
S2E4	67.22 (55.07)	32.78 (34.92)	2.05
S3E1	55.6 (48.21)	44.4 (41.78)	1.26
S3E2	55.47 (48.14)	44.53 (41.85)	1.25
S3E3	53.98 (47.28)	46.02 (42.71)	1.18
S3E4	55.91 (48.39)	44.09 (41.60)	1.28
S. Em $\pm$	0.40	0.40	0.05
CD at 5%	NS	NS	NS

Note: Figure in parenthesis indicate Arc sign transformed values



100 ppm



150 ppm



200 ppm



Control

Plate 12: Effect of ethylene on flowering in pomegranate

4.4.2.1.5 Percentage of hermaphrodite flowers

Maximum percentage of hermaphrodite flowers were observed in treatment E3 (ethrel treatment at 200ppm) with 38.17 per cent. Followed by, ethrel at 150ppm and 100ppm showed significantly higher Percentage of hermaphrodite flowers with 36.75 per cent and 35.94 per cent respectively when compared with control while control plant showed least Percentage of hermaphrodite flowers per plant (34.34%) (Table 13).

Maximum Percentage of hermaphrodite flowers per plant were observed in Hasta bahar (S3) showed (44.76%) significantly higher Percentage of hermaphrodite flowers. Followed by, Mrig bahar (S2) and Ambia bahar (S3) which showed significantly lower number i. e., 36.08 per cent and 28.06 per cent of hermaphrodite flowers respectively.

Interaction between concentration and season did not show any significant difference on Percentage of male flowers.

4.4.2.1.6 Sex ratio

Exogenous application of ethrel exhibited minimum sex ratio. Foliar spray of ethrel at 200ppm (E3) showed least sex ratio (1.70) followed by E2 (ethrel at 150ppm) (1.82) compared to rest of treatments. Higher production of male flowers by lowering hermaphrodite flower production resulted in obtaining higher sex ratio in control treatment (2.05) (Table 13).

Among different seasons, Hasta bahar (S3) shown lower sex ratio (1.24) followed by Mrig bahar (1.79) whereas Ambia bahar (S1) recorded higher sex ratio (2.58).

Interaction between concentration and season did not show any significant difference on Percentage of male flowers.

4.4.2.1.7 Number of days taken from flower bud initiation to flowering

Foliar application of ethrel played a significant role on modifying duration of flowering. Data presented in Table 14 showed that, foliar application of ethrel at 200 ppm (E3) has taken less number of days from flower bud initiation to flower opening stage when compared to rest of the treatments. Data showed that, treatment E3 has

Table 14: Effect of ethylene on flower parameters in pomegranate

Treatment	Number of days taken	
	first bud initiation to flower opening	flowering to harvest
Ethylene concentration		
E1-100 ppm	18.36	148.46
E2-150 ppm	17.90	147.66
E3-200 ppm	17.36	147.00
E4-Control	19.63	151.13
S. Em $\pm$	0.19	0.28
CD at 5%	0.53	0.79
Season (Bahar)		
S1-Ambia Bahar	18.10	148.27
S2-Mrig Bahar	18.87	149.02
S3-Hasta Bahar	17.97	148.40
S. Em $\pm$	0.16	0.24
CD at 5%	0.46	NS
Interaction (Season x Concentration)		
S1E1	18.30	148.40
S1E2	18.20	147.90
S1E3	16.90	146.60
S1E4	19.00	150.20
S2E1	18.50	148.60
S2E2	17.90	148.00
S2E3	18.10	147.40
S2E4	21.00	152.10
S3E1	18.30	148.40
S3E2	17.60	147.10
S3E3	17.10	147.00
S3E4	18.90	151.10
S. Em $\pm$	0.33	0.48
CD at 5%	NS	NS

taken just 17.36 days from flower bud initiation to flower opening stage followed by T₂ (ethrel at 150ppm) where it has taken 17.90 days compared to untreated plants (19.63 days).

Among different seasons, Hasta bahar (S3) has taken less number of days (17.97) from flower bud initiation to flower opening stage when compared to other seasons. Mrig bahar (S2) has taken more number of days from flower bud initiation to flower opening stage (18.87) and followed by S1 has taken 18.10 days.

Interaction between concentration and season did not show any significant difference on number of days from flower bud initiation to flower opening stage.

4.4.2.1.8 Number of days taken from flowering to fruit harvest

Number of days taken from flowering to fruit harvest has varied significantly among the treatments (Table 14). Among these, foliar application of ethrel at 200 ppm (E3) significantly reduced the duration of cropping period by taking less number of days from flowering to harvest (147.00 days) and followed by ethrel treatment at 150ppm (E2) which was statistically on par with T3 (147.66 days), while untreated plants has taken maximum number of days (151.13 days) from flowering to its harvesting stage.

Among different seasons, number of days taken from flowering to fruit harvest has not shown any significant difference.

Interaction between concentration and season did not show any significant difference on number of days taken from flowering to fruit harvest.

4.4.2.2 Fruit parameters

Experimental results (Table 15) depict the fruit setting percentage, fruit retention percentage and fruit weight.

4.4.2.2.1 Fruit setting percentage

Application of plant ethylene shown significant difference among the treatments. Among these, foliar application of ethrel at 200 ppm (E3) showed maximum percentage of fruit setting (30.77%) statistically on par with E2 (ethrel at 150ppm) (30.49%) and significantly superior over E1 (ethrel at 100ppm) (29.29%),

Table 15: Effect of ethylene on fruit setting, fruit retention percentage and fruit weight of pomegranate

Treatment	Fruit setting percentage	Fruit retention percentage	Fruit Weight
Ethylene concentration			
E1-100 ppm	29.29 (32.56)	19.11 (25.4)	262.55
E2-150 ppm	30.49 (33.35)	19.88 (26.05)	263.08
E3-200 ppm	30.77 (33.51)	20.2 (26.26)	264.23
E4-Control	28.3 (31.90)	18.44 (24.85)	261.90
S. Em $\pm$	0.35	0.38	2.07
CD at 5%	0.98	1.09	NS
Season (Bahar)			
S1-Ambia Bahar	21.45 (27.54)	10.04 (18.4)	267.64
S2-Mrig Bahar	28.39 (32.14)	19.89 (26.41)	259.24
S3-Hasta Bahar	39.3 (38.81)	28.3 (32.09)	261.95
S. Em $\pm$	0.303	0.33	1.794
CD at 5%	0.85	0.94	5.042
Interaction (Season x Concentration)			
S1E1	20.46 (26.87)	9.31 (17.74)	267.28
S1E2	21.93 (27.90)	10.49 (18.89)	267.96
S1E3	23.29 (28.83)	11.2 (19.52)	268.15
S1E4	20.13 (26.56)	9.15 (17.46)	267.16
S2E1	28.26 (32.08)	20.25 (26.69)	258.91
S2E2	30.26 (33.35)	20.8 (27.12)	259.75
S2E3	28.3 (32.07)	20.09 (26.51)	260.82
S2E4	26.74 (31.07)	18.42 (25.36)	257.47
S3E1	39.16 (38.73)	27.78 (31.77)	261.47
S3E2	39.29 (38.80)	28.34 (32.13)	261.55
S3E3	40.72 (39.64)	29.32 (32.74)	263.71
S3E4	38.03 (38.06)	27.75 (31.73)	261.06
S. Em $\pm$	0.60	0.67	3.588
CD at 5%	NS	NS	NS

Note: Figure in parenthesis indicate Arc sign transformed values

while untreated plants showed least fruit setting percentage (28.3%) (Table 15) (Plate 13).

Among different seasons, Hasta bahar (S3) has shown maximum percentage of fruit setting (39.3%) and least Fruit setting percentage was observed in Ambia Bahar (S1) with 21.45 per cent, followed by Mrig bahar (S2) (28.39%).

Interaction between concentration and season did not show any significant difference on fruit setting percentage.

4.4.2.2 Fruit retention percentage

Application of ethrel showed significant effect on maximizing fruit retention percentage among the treatments (Table 15). Among these, foliar application of ethrel at 200 ppm (E3) showed maximum fruit retention percentage (20.2%). Plants sprayed with ethrel at 150ppm (E2) and 100ppm (10ppm) showed on par results with E3 by showing 19.88 per cent and 19.11 per cent fruit retention respectively. In contrast, control plants showed lower fruit retention percentage (18.44%).

Among different seasons, Hasta Bahar (S3) showed maximum fruit retention percentage (28.3%). Lowest fruit retention percentage was observed in Ambia Bahar (S1) with fruit retention percentage of 10.04 per cent.

Interaction between concentration and season did not show any significant difference on fruit setting percentage.

4.4.2.3 Fruit Weight

Foliar application of ethrel at different concentrations did not show significant role towards enhancement of fruit weight (Table 15).

Among different seasons, fruit weight was maximum in Ambia Bahar (S1) with fruit weight of 267.64g. In contrast, low fruit weight was observed in Mrig bahar (S2) (259.24g) and Hasta Bahar (S3) shown results on par with S2 (261.95g).

Interaction between concentration and season did not show any significant difference on Fruit Weight.

4.4.2.2.4 Color values (L^* a^* b^*)

Fruit color is one of the most important factors in marketing satisfaction. The colour of fruit was measured using colorimeter. The data on changes in L^* , a^* , b^* colour value (colorimeter reading values) of pomegranate fruit as influenced by different concentrations of ethrel are displayed in table 16.

L^* scale: Light vs. dark where a low number (0-50) indicates dark and a high number (51-100) indicates light. In pomegranate plants sprayed with ethrel at 200ppm (E3) shown maximum L^* value (45.34). Followed by, E2 (ethrel at 150ppm) shown higher L^* value (44.30) whereas control (E4) shown least L^* value (43.71). E1 (ethrel at 100ppm) shown on par results with E4 (43.79). a^* scale: Red vs. green where a positive number indicates red and a negative number indicates green. Plants sprayed with ethrel shown maximum enhancement in the redness of the pomegranate fruit skin, when compared with control (Plate 14). E3 (ethrel at 200ppm) shown maximum a^* value (40.55) when compared with other treatments. Whereas, E2 (ethrel at 150ppm) shown on par results with E3 (39.83) and in contrast, control (E4) shown least a^* value (38.75). E1 (ethrel at 100ppm) shown on par results with E4 (39.51). b^* scale: Yellow vs. blue where a positive number indicates yellow and a negative number indicates blue. Application of ethrel shown increase in b^* value (blue to yellowness) when compared with un-treated plants. E3 has shown maximum b^* value when compared with control (30.70). Whereas, E2 (ethrel at 150ppm) shown on par results with E3 (30.22) while, control (E4) shown least b^* value (29.63). E1 (ethrel at 100ppm) shown on par results with E4 (29.76).

Among different crop seasons, season has not shown any significant difference on Color values (L^* a^* b^*) of pomegranate.

Interaction between concentration and season did not show any significant difference on Color values (L^* a^* b^*) of pomegranate.

4.4.2.3 Yield parameters

Results in table 17 displays the data pertaining to total number of fruit per plant, fruit yield per plant and fruit yield per hectare.



100 ppm



150 ppm



200 ppm



Control

Plate 13: Effect of ethylene on fruit set in pomegranate

Table 16: Effect of ethylene on colour parameter of pomegranate

Treatment	<i>L</i>* value	<i>a</i>* value	<i>b</i>* value
Ethylene concentration			
E1-100 ppm	43.79	39.51	29.76
E2-150 ppm	44.30	39.83	30.22
E3-200 ppm	45.34	40.55	30.70
E4-Control	43.71	38.75	29.63
S. Em $\pm$	0.29	0.38	0.26
CD at 5%	0.82	1.08	0.73
Season (Bahar)			
S1-Ambia Bahar	44.30	39.88	30.47
S2-Mrig Bahar	44.17	39.14	29.74
S3-Hasta Bahar	44.39	39.95	30.02
S. Em $\pm$	0.25	0.33	0.22
CD at 5%	NS	NS	NS
Interaction (Season x Concentration)			
S1E1	43.62	39.70	30.06
S1E2	44.14	40.12	30.62
S1E3	45.92	41.13	31.16
S1E4	43.54	38.55	30.03
S2E1	43.72	39.02	29.46
S2E2	44.14	39.23	29.72
S2E3	45.12	40.25	30.46
S2E4	43.69	38.06	29.33
S3E1	44.04	39.80	29.76
S3E2	44.62	40.12	30.32
S3E3	44.99	40.25	30.46
S3E4	43.92	39.63	29.53
S. Em $\pm$	0.51	0.66	0.45
CD at 5%	NS	NS	NS

Table 17: Effect of ethylene on number of fruits, fruit yield per plant and per hectare in pomegranate

Treatment	Total number of fruits per plant	Fruit yield per plant (kg)	Fruit yield per hectare (t)
Ethylene concentration			
E1-100 ppm	79.56	15.91	13.26
E2-150 ppm	85.50	17.10	14.25
E3-200 ppm	91.46	18.29	15.24
E4-Control	74.33	14.86	12.39
S. Em $\pm$	2.49	0.49	0.41
CD at 5%	7.00	1.40	1.16
Season (Bahar)			
S1-Ambia Bahar	98.60	19.72	16.43
S2-Mrig Bahar	75.20	15.04	12.53
S3-Hasta Bahar	74.35	14.87	12.39
S. Em $\pm$	2.15	0.43	0.36
CD at 5%	6.06	1.21	1.01
Interaction (Season x Concentration)			
S1E1	91.60	18.32	15.26
S1E2	103.50	20.70	17.25
S1E3	111.80	22.36	18.63
S1E4	87.50	17.50	14.58
S2E1	74.60	14.92	12.43
S2E2	78.30	15.66	13.05
S2E3	84.00	16.80	14.00
S2E4	63.90	12.78	10.65
S3E1	72.50	14.50	12.08
S3E2	74.70	14.94	12.45
S3E3	78.60	15.72	13.10
S3E4	71.60	14.32	11.93
S. Em $\pm$	4.31	0.86	0.71
CD at 5%	NS	NS	NS

4.4.2.3.1 Total number of fruits per plant

Foliar application of ethrel played prominent role towards enhancing total number of fruits per plant. Data presented in Table 17 revealed that, foliar spray of ethrel at 200 ppm (E3) concentration showed significant results towards producing maximum number of fruits per plant (91.46). Application of ethrel at 150ppm (E2) showed on par result with E3 (85.50). Whereas, control plants (E4) shown least number of fruits per plant (74.33). E1 (ethrel at 100ppm) also showed on par results with E4 (79.56).

Among different seasons, Ambia Bahar (S1) shown maximum number of fruits per plant (98.60). In contrast, lowest number of fruits per plant was observed in Hasta bahar (S3) (74.35). Whereas, Mrig bahar (S2) results recorded are on par with S3 (75.20).

Interaction between concentration and season did not show any significant difference on number of fruits per plant.

4.4.2.3.2 Fruit yield per plant (kg/plant)

Variation in treatment with respect to fruit yield per plant differed significantly among the treatments (Table 17) (Plate 15). Data revealed that, foliar application of ethrel at 200 ppm has recorded maximum fruit yield (18.29 kg/plant) and application of ethrel at 150ppm (E2) shown on par results with E3 (17.10 kg/plant). While untreated plants showed minimum yield (14.86 kg/plant) compared to other treatments and T1 (15.91 kg/plant) shown on par results with T4.

Among different seasons, Ambia Bahar (S1) shown maximum fruit yield per plant (19.72 kg/plant). In contrast, lowest fruit yield per plant was observed in Hasta bahar (S3) (14.87 kg/plant). In season, Mrig bahar (S2) results recorded are on par with S3 (15.04 kg/plant).

Interaction between concentration and season did not show any significant difference on fruit yield per plant.

4.4.2.3.3 Fruit yield per hectare (t/ha)

Data in Table 17 showed that, foliar application of ethrel at 200 ppm (E3) showed highest yield per hectare (15.24 t/ha) and followed by T2 (ethrel at 150ppm) shown on par results with E3 (14.25 t/ha). While, Lowest yield per plant was recorded in un-treated plants (E4) with 12.39 t/ha. T1 (13.26 t/ha) shown on par results with E4.

Among different seasons, Ambia Bahar (S1) shown maximum fruit yield per hectare (16.43 t/ha). In contrast, lowest fruit yield per hectare was observed in Hasta bahar (S3) (12.39 t/ha). In season, Mrig bahar (S2) results recorded are on par with S3 (12.53 t/ha).

Interaction between concentration and season did not show any significant difference on fruit yield per hectare.

4.5 Elucidation of resistance with other hormones

Experimental analysis revealed that, among different hormones sprayed, ethrel sprayed at 300ppm (T1) shown maximum disease severity (33.2%) when compared with water control (T4). Followed by, methyl jasmonate sprayed at 300ppm (T2) shown higher disease severity (29.97 %) but it is on par with water control (27.32%) and ethanol control (26.81%). Lowest disease severity was observed in T3 (Salicylic acid at 300ppm) (15.08%) (Table 18).



100 ppm



150 ppm



200 ppm



Control

Plate 14: Effect of ethylene on fruit colour of pomegranate



100 ppm



150 ppm



200 ppm



Control

Plate 15: Effect of ethylene on fruit yield in pomegranate

Table 18: Effect of different hormones on severity of bacterial blight of pomegranate under greenhouse condition

Treatment	Disease Severity
T1-Ethrel-300 ppm	33.20 (35.17)
T2-Methyl jasmonate-300 ppm	29.97 (33.18)
T3-Salicylic acid-300 ppm	15.08 (22.83)
T4-Water Control	27.32 (31.50)
T5-Ethanol Control	26.81 (31.18)
S. Em $\pm$	0.36
CD at 5%	2.62

Note: Figure in parenthesis indicate Arc sign transformed values

5. DISCUSSION

Pomegranate is a fruit which grows with a very high yield in many states of India and one of the most profits gaining fruit in the market. But due to various conditions, the plants are infected by various diseases which destroy the entire crop leaving very less fruit yield. As an outcome of intensive cultivation led to more attack of pest and diseases mainly bacterial blight disease which was regarded as minor disease till 1990's. Sudden outbreak of bacterial blight disease from 2002 onwards resulted in severe yield loss both in terms of quality and quantity of pomegranate.

Severity of bacterial blight disease led to use of antibiotics towards its management incurred more cost of cultivation. Use of hormones also helps in improving quality (colour, texture *etc.*) and yield parameters (total number of flowers, fruits *etc.*) of the fruits. Thus, finding alternative approaches for managing disease by understanding the mechanism of action of hormones like ethylene in disease resistance and implementing the outcomes in developing management strategies as well as helps in obtaining higher production with quality fruits. With regard to the above relevance of study was to attempt to identify and evaluate role of ethylene in disease resistance. The experimental results obtained by present investigations are discussed here under.

5.1 Symptomatology

Symptoms of bacterial blight observed on above ground parts of the plant. On leaves, one to several, small, regular to irregular grayish water soaked lesion appeared on abaxial surface that looked transparent yellow when observed against light. The lesions further enlarged and turned dark brown to black, eventually becoming visible on both leaf surfaces. Lesions coalesced to form larger lesions which resulted in blighted appearance followed by defoliation. Nearly identical symptoms observed on spray inoculated plantlets. On midrib of a leaf lamina, the bacterial inoculation led to extensive chlorosis of leaf lamina, browning of the blade, blackening of the veins and eventual defoliation. On twigs and branches, the symptoms began as brown to black water soaked lesions around the nodes or injuries. The lesions under high humid conditions were sticky, enlarged and led to girdling and cracking of the nodes on

slight pressure. It was further observed that when lesions appeared on young new twigs, all the leaves above the lesion turned intense yellow and fell off. On floral parts the bacterium could neither cause any visible symptoms nor was it ever observed in naturally infected plants. On fruits, water soaked lesions initially appeared on fruit surface that later turned dark brown. These lesions enlarged and coalesced to cover large areas. Thin shining white encrustation of dried bacterial ooze or droplets of glue like bacterial ooze was often observed on lesions. Under high humidity, blight lesions were sticky or slimy. Formation of L or Y cracks on necrotic fruit spots and splitting of fruits was invariably observed. Interestingly, infection was restricted only to the rind. Symptoms described in the present investigation corroborate the findings of earlier workers (Hingorani and Mehta, 1952; Kanwar, 1976; Rangaswamy, 1962; Sohi *et al.*, 1964; Sharma *et al.*, 2010).

5.2 Isolation of pathogen

Isolations from blight lesions yielded glossy yellow colonies of *X. axonopodis* pv. *punicae* with characteristic brown fuscan pigments in NGA. Colonies appeared after 48 hours of incubation at 28°C were selected and streaked on NGA plates for getting pure culture. The pathogen was isolated by following the standard protocol of practical manual by IARI, New Delhi (Mondal and Kumar, 2011). After confirmation and proving pathogenicity, the virulent isolates were used for further studies. The observations made pertaining to the isolation in the present investigation were in conformity with the work of Sharma *et al.* (2017), who obtained pure cultures by isolating from infected parts on NGA medium.

Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity. Infection was readily seen by them on tender leaves of plants which on artificially inoculated with pathogens in seven to ten days of incubation. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976), he observed the symptoms within four to seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts.

5.3 Phenotype based identification of *Xanthomonas axonopodis* pv. *punicae*

The morphological characters like its fuscan Development, Unique dark brown fuscan pigmentation was observed in *Xap* inoculated NGA medium. Its intensity increases after seven days of incubation. Production of pigmentation is a unique character of *Xap*, helps in differentiating with other non-pathogenic bacteria. Colonies on NGA plates are circular, convex, mucoid, shiny and yellow were observed. Results of both phenotype tests were in conformity with earlier report by Mondal and Kumar, 2011 and sharma *et al.*, 2017.

5.4 Molecular identification of *Xanthomonas axonopodis* pv. *punicae* using specific primers

To identify pathogen a primer set, KKM-5 Forward and KKM-6 Reverse designed by Mondal *et al.* (2012) were used for molecular detection of pathogen. The primer set gave product size of 491 bp, which is specific to only *X. axonopodis* pv. *punicae* (*Xap*). These results are in accordance with Mondal *et al.* (2012).

5.5 Pathogenicity test

The symptoms observed during pathogenicity and their confirmation with phenotypic expression is matching with earlier studies as reported by many workers. Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity. Infection was readily seen by them on tender leaves of the artificially inoculated plants in seven to ten days with congenial greenhouse conditions. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976). He has observed the symptoms within four to seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts.

5.6 Understanding the role of ethylene concentration on pathogen/disease development in plant

5.6.1 Disease severity

Phytohormones play an important role in mediating defense responses against biotic and abiotic stresses in plant system (Verma *et al.*, 2016). Foliar application of ethrel showed higher disease severity whereas control plants showed lower disease

severity in all the varieties. Among the different treatments, ethrel at 300ppm showed significantly higher disease severity of 28.64 per cent followed by 200ppm (26.47%). Among different varieties, Bhagwa showed higher disease severity of 31.57 per cent followed by Wonderful and Ruby with disease severity of 28.64 per cent and 25 per cent whereas least disease severity was noted in Nana with 15 per cent respectively. Similar results were observed by Rodrigues *et al.* (2020) where foliar spray of Ethrel (0.0001 M) 24 h before inoculation with *Pyricularia oryzae*. Blast severity was higher on ethylene-sprayed plants regardless of the cultivar. High foliar ethylene concentration for inoculated plants from cultivars BRS Guamirim and BRS 220 was associated with an increase in leaf tissues susceptibility to blast. Ethylene biosynthesis was observed on wheat leaves infected with *P. oryzae* infection. In the same way, The ethylene-insensitive *Never ripe (Nr)* mutant displays increased tolerance to the virulent strain, while maintaining resistance to the avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* in tomato. Transgenic plants overexpressing a wild-type NR cDNA were infected with virulent *X. campestris* pv. *vesicatoria* (Ciardi *et al.*, 2000).

The different results regarding the role of ethylene in plant defense could reflect its involvement in multiple physiological processes in the plant. Chlorosis, senescence and abscission are well-known responses of plants to ethylene and thus ethylene can promote predisposition of plant tissue to disease (Weingart *et al.*, 2001). Ethylene can accelerate senescence in leaves and ripening in fruits (Abeles *et al.*, 1992). This might predispose the tissue for development of disease caused by necrotrophic pathogens. On the other hand, ethylene stimulates the development of necrosis (Lund *et al.*, 1998) and in many cases the hypersensitive response (HR) (Ciardi *et al.*, 2001). HR is a defense phenomenon involving a rapid localized necrosis of plant cells at the infection site, followed by a local and systemic activation of defense-related genes (Pontier *et al.*, 1998). It can be envisaged that cell death during HR is able to restrict the proliferation of biotrophs because it deprives the pathogen of access to nutrient sources present in living cells (Cohn *et al.*, 2001). A necrotroph, however, might benefit from HR because it feeds on dead plant cells. It has been reported that HR facilitates infection of *Arabidopsis* by necrotrophs such as *Botrytis cinerea* or *Sclerotinia sclerotiorum* (Govrin and Levine, 2000). It may be expected that necrotrophic pathogens are well adapted to deal with HR-based defense

mechanisms that are active against biotrophs (Mayer *et al.*, 2001). In another study, *Xanthomonas campestris* pv. *vesicatoria*, the causal organism of bacterial scab in pepper (*Capsicum annuum*) produced small amounts of ethylene when grown under low oxygen tensions in liquid culture. Spraying with methionine increased ethylene production and disease severity. It is suggested that the ethylene produced during bacterial scab infection contributes to the development of disease symptoms including leaf abscission (Ben-David *et al.*, 1986). *Xanthomonas citri* ssp. *citri* (Xcc) infection is directly related to ethylene production and subsequent defoliation, which occurs more rapidly with higher inoculum concentration and with infections closer to the stipules (Dutta and Biggs, 1991; Goto *et al.*, 1980) and also Pathogens like *R. solanacearum* and *P. syringae* possess the ability to produce ET, independent of a plant host (Freebairn and Buddenhagen, 1964; Weingart *et al.*, 2001). Ethylene reduced SA-mediated tolerance against *P. syringae* pv. *tomato* (Broekaert *et al.*, 2006).

5.6.2 Pathogen quantification

The quantification of bacterial growth is indispensable for analyzing the interaction between *X. axonopodis* pv. *punicae* and pomegranate. Although the currently employed plate counting method is highly reliable, this procedure has several disadvantages. Most detrimental is the need of direct processing of the harvested samples excluding the possibility of analyzing short interval time points. Furthermore, it is not well suited for the quantification at early time points. The samples are taken by punching out leaf discs, which only define a surface area but not the volume of the excised leaf disc. When comparing different mutants or plant ecotypes the leaf morphology can significantly differ. Finally, the method is rather labor intensive and vulnerable for repetitive technical mistakes for instance during pipetting of the dilution series and during counting of the single independent colonies. DNA-based quantification of *Xap* by qRT-PCR seems to be an obvious alternative approach that has meanwhile been adapted to several other plant microbe interaction studies (Gachon and Saindrenan, 2004).

Among all the genotypes higher pathogen load was observed on Baghawa variety sprayed with ethrel at 300ppm (9522.67-folds) and followed by Wonderful

variety sprayed with ethrel at 300ppm with fold change of 9371.62. In contrast, Nana variety shown least load of pathogen when sprayed with ethrel at 300ppm with fold change of 4301.75. Followed by Ganesh variety shown lower pathogen load at 300ppm by exhibiting fold change of 5323.49. Among different concentrations, 300ppm shown higher pathogen load on all the genotypes and Control plants in all genotypes shown the least pathogen load among different treatments.

Similary, Doddaraju *et al.* (2019) quantified the pathogen load in pomegranate cultivars upon pathogen inoculation under greenhouse condition using *XopQ* effector gene primer. They have noted higher pathogen load in Bhagwa (Δ CT-241.9) and least pathogen load in Daru-IC318735 (Δ CT-6.8).

To study the interaction of *Pseudomonas syringae* with Arabidopsis, present an alternative robust approach, which is based on the quantification of bacterial DNA by real-time PCR. They have directly compared this assay with the routinely used plate counting method to access bacterial titers in a number of well described Arabidopsis mutants using *OprF* (outer membrane porin) gene primers specific to *Pseudomonas syringae*. Study showed that the DNA-based technique is highly reliable and comparable. Moreover, the technique is easily applicable, robust and ideal for routine experiments or for larger scale analyses (Ross and Somssich, 2016).

To detect and quantify YDVs using primers specific for Barley yellow dwarf virus-PAV (BYDVPAV) and Cereal yellow dwarf virus-RPV (CYDV-RPV) coat protein genes because of the higher sensitivity of RT-PCR. BYDV-PAV virus load was more on 8th day on susceptible oat (15,181-folds) and CYDV-RPV virus load was maximum on 12th day (10,685-folds). BYDV-PAV RNA accumulated more rapidly and to a higher level than CYDV-RPV RNA in both oat and wheat, which may account for PAV being more prevalent and causing more severe viral disease than CYDV (Balaji *et al.*, 2003).

The amount of DNA is measured by a calibrated PCR machine instead of by a somewhat subjective counting of independent single bacterial colonies within a small area. Additionally, the actual plant biomass instead of a leaf disc diameter is used for the calculation of bacterial growth. The plant biomass can be defined by the PCR-based method because the analyzed plant specific gene is a single copy gene. Each plant cell harbors one DNA copy of this gene to which the primer can bind during the

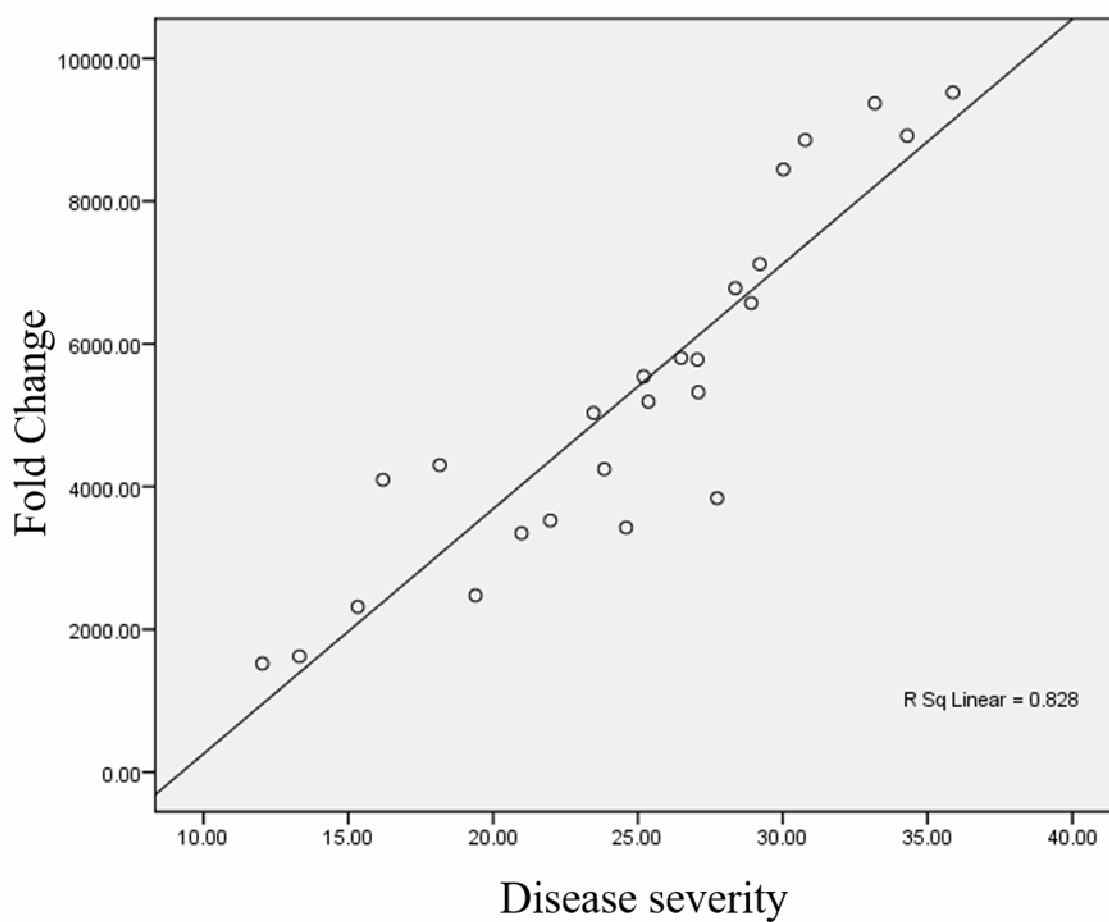


Fig. 9: Comparison of pomegranate bacterial blight severity by visual symptoms and qPCR. Correlation between visual symptoms based disease severity (%) and qPCR (Fold change)

first round of PCR. The same holds true for the bacteria specific gene *XapQ*. Each copy of *XapQ* DNA bound by the primer at the beginning of the reaction is representative of one bacterial cell. By subtracting the Ct value of the *XapQ* gene from the Ct value of the plant specific gene GAPDH (Δ CT), the relative abundance of bacterial cells in comparison to the amount of plant cells can be monitored (Ross and Somssich, 2016).

Disease identification by visual symptoms in the field is a simple and inexpensive approach. However, symptom-based diagnostics are subject to observer bias and errors and are unreliable at asymptomatic stage of infection. Most importantly, the symptoms of one disease can also be confused with those of other diseases (Mahlein *et al.*, 2012). The disease severity based on the visual symptoms was compared with pathogen detection by quantification by fold change method using qPCR in different varieties of pomegranate genotypes; Bhagwa, Wonderful, Ruby, Ganesh, Nana. Fold change values of *XopQ* correlated with the disease severity based on visual symptoms ($R^2 = 0.82$, Fig. 9). Among the Varieties, Nana shown low disease severity and low fold change values of *XopQ* and Bhagwa shown higher disease severity and higher fold change values of *XopQ*. Ethrel sprayed at 300ppm shown more disease severity and higher fold change values irrespective of the varieties and in contrast control plants shown lower fold change values as well as lower disease severity. This data clearly shows correlation of disease severity and fold change values of *XopQ* and R is close to 1, it tells us that the linear relationship is very strong.

5.7 Microbe induced ethylene regulation in pomegranate and its effect on disease development

5.7.1 Primer design and PCR amplification of ACC deaminase producing gene in bio-control agents used in the experiment

Ethylene is an essential plant hormone produced by all plants and mediates a wide range of responses and developmental processes. It is also a stress hormone since its synthesis is accelerated by induction of a variety of stress signals, such as mechanical wounding, salinity, drought, water logging, extreme temperatures, pathogenic infection and pollutants (Saleem *et al.*, 2007). However, excessive ethylene produced in germinating seeds or by different environmental stresses can hinder plant growth especially root growth retardation. The synthesis of ethylene in

plants is directly proportionate to the concentration of its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Shaharoona *et al.*, 2006). Many plant growth promoting bacteria produce ACC deaminase and these bacteria can decrease the level of ethylene produced in stressed plants by cleaving ACC to α -ketobutyrate and ammonium ion (Glick, 2005; Hontzeas *et al.*, 2004).

ACC deaminase primers, PacdF/R (accession: CP007620.1) and TacdF/R (accession: FJ751936.1) were designed for *Pseudomonas putida* and *Trichoderma asperellum* respectively. PCR amplification using designed primers shown predicted product size of 130 bp for *Pseudomonas putida* and 141bp for *Trichoderma asperellum* and amplification shows the presence of ACC deaminase gene in both the biocontrol agents. Similarly, the presence of ACC deaminase gene in *Pseudomonas putida* was reported by earlier workers (Nadeem *et al.*, 2010; Hontzeas *et al.*, 2004). The presence of ACC deaminase gene was confirmed with 140-bp fragment using the primers QAF and QAR in *Trichoderma asperellum* (Viterbo *et al.*, 2010).

5.7.2 Disease severity

Conventional applications of copper bactericides are not only ineffective but also pose severe ecological hazards (Byrne *et al.*, 2005). An environmentally safe strategy to control plant diseases is the use of biocontrol agents (Ji *et al.*, 2006). The widely recognized mechanisms for biocontrol includes competition for an ecological niche or a substrate, production of inhibitory allelochemicals and induction of systemic resistance in host plants to a broad spectrum of pathogens (Bloemberg and Lugtenberg, 2001; Compant *et al.*, 2005; Wang *et al.*, 2001).

Application of bioagents shown greater reduction in disease severity when compared to untreated control plants. Treatment T3 (combination of both soil and foliar spray of *Pseudomonas putida*) shown lower disease severity (4.24%) whereas control plant shown higher disease severity of 28.2 per cent.

Similarly, Saikia and Bora (2021) studied the the effectiveness of the cell suspension (10^7 CFU/ml) of *Pseudomonas putida*, *Metarhizium anisopliae*, *Streptomyces fimicarius* and *Streptomyces laurentii* in suppressing bacterial blight of rice (*Xanthomonas oryzae* pv. *oryzae*) was tested *in planta* by applying as seed treatment, root treatment, soil treatment and spray application. Lowest disease

incidence was observed in plants treated with the combination of *P. putida* + *S. fimicarius* + *S. laurentii* (10.29%) as compared to other treatments. Plants were also coinoculated with *A. tumefaciens* C58 and *P. putida* UW4 or *P. putida* UW4-accS (an ACC deaminase minus mutant strain). In both types of experiments, it was observed that the presence of ACC deaminase generally inhibited tumour development on both tomato and castor bean plants (Hao *et al.*, 2007). Application of *P. Putida* A1 and streptomycin could delay wilt symptom development and reduce wilt severity. The severity index of *P. putida* A1 was less than 40 per cent, more effective than the application of streptomycin. Inhibition was most effective on *Xanthomonas oryzae* pv. *oryzae*, for which the inhibition zone diameter was as high as 5.5 cm (Sun *et al.*, 2017).

Pseudomonas isolates were assessed for their potential to produce chitinase, cellulase, β -1,3-glucanase, protease, reduce nitrate and produce mycolytic enzymes, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, phenazine antibiotics and N-acyl homoserine lactones (AHLs) (Jayamohan *et al.*, 2020).

Similarly, the application of *Trichoderma asperellum* isolates delayed wilt (*Ralstonia solanacearum*) development, effectively decreased the disease incidence, increased fruit yield and improved plant growth promotion under field conditions. *Trichoderma asperellum* treatment decreased the disease incidence of bacterial wilt by upto 52.75 per cent (Konappa *et al.*, 2018). Similar outcomes were previously approved by Saksirirat *et al.* (2009) the result showed that *T. harzianum* (T9) induced resistance with the highest reduction in number of spots (44.77%) against *Xanthomonas campestris* pv. *vesicatoria* causing bacterial leaf spot in tomato. Singh *et al.* (2019) proved that the nitrate-fed tobacco plants displayed an increased HR when *Xanthomonas oryzae* pv. *oryzae* infected, which was enhanced in the *Trichoderma*-treated plants. This mechanism was enhanced by the involvement of *Trichoderma*, which elicited NO production and enhanced the expression pattern of NO-modulating genes (NR, NOA and ARC). Similar observation made by Chien and Huang (2020) they observed that both the combined use and individual application of *Bacillus amyloliquefaciens* and *T. asperellum* via foliar spray or growth medium application or both have the potential to control bacterial spot on tomato.

The plant-Trichoderma interaction, as an indirect and complex process, would result in a molecular dialog, where in both the plant and Trichoderma will produce molecules to mediate the interaction (Pozo *et al.*, 2005). These signaling molecules (effectors/elicitors), including enzymes, proteins and secondary metabolites secreted by *Trichoderma* spp., can activate the expression of resistance related genes in plants against pathogenic microorganisms and promote the growth of the plant and root system, as well as nutrient availability (Vinale *et al.*, 2008), as well as can induce plant systemic resistance to pathogens (Yedidia *et al.*, 2000). *T. asperellum* induces series of defense responses such as accumulation of phenols and induction of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase enzymes involved in phenylpropanoid metabolism and of PR protein (β -1,3-glucanase).

5.7.3 Gene expression

The gaseous plant hormone ethylene is known to be enhanced in plants due to pathogenic stress (Ma *et al.*, 2003) and accumulation of ACC, the immediate precursor of ethylene biosynthesis and an important mediator of stress response in plants (Abeles *et al.*, 1992). In present investigation application of ethylene increased the disease severity in pomegranate. Biosynthesis of ethylene begins with the compound S-adenosylmethionine (SAM) that is also required as the precursor in many other pathways. The committed step in the biosynthesis of ethylene from SAM is the conversion of ACC by the enzyme ACC synthase (ACS). ACC is converted to ethylene by ACO (Stearns and Glick, 2003). Earlier, our experimental studies shown that application of ethylene increased the disease severity in pomegranate plant. Hence, the study aimed to know the effect of reduced ethylene levels in plant to pathogen infection. In this study ACO accumulation were significantly reduced in the pomegranate leaves and root due to application of ACC deaminase producing bioagents (*T. asperellum* and *Pseudomonas putida*) compared to pathogen inoculated control. It was expected a higher rate of ethylene production with a corresponding increase in ACO activity in pathogen-stressed pomegranate. Wang *et al.* (2000) contradicted the role of ACC deaminase activity in *Pseudomonas fluorescens* CHA0 having introduced ACC deaminase gene, capable of increasing resistance against *Pythium* damping-off and susceptibility to *Fusarium* crown and root rot in tomato.

In leaves, maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 6 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 6.16 fold change activity and least gene expression at 216 hpi was observed in both spray and soil application of *Pseudomonas putida* (T3) (2.13-folds) and spray application of *Pseudomonas putida* (T2) (2.53-folds). Lowest expression of ACC oxidase gene among different time points was observed in T3 and T6 at 0 hpi with fold change of 0.41 and 0.47 respectively. In roots, overall the maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 24 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 3.77 fold change activity and least gene expression at 216 hpi was observed in T3 (2.56-folds) and T1 (2.64-folds). Lowest expression of ACC oxidase gene among different time point intervals observed in T3 and T1 at 6 hpi with fold change of 0.29 and 0.38 respectively.

These results are in accordance with findings of Madhaiyan *et al.* (2006), reported that the ACC deaminase producing *Methylobacterium* decreased ACC oxidase activity in the inoculated canola plants. Among the *Methylobacterium* spp., CBMB12 resulted lowest ACO related gene expression (1.46 Normalized Fold Expression), whereas CBMB20 had high gene expression (3.42 Normalized Fold Expression) in pathogen challenged tomato (Yim *et al.*, 2014). An ACC deaminase gene, together with its regulatory region, was transferred into *P. fluorescens* strains CHA0 and CHA96. Introduction of ACC deaminase genes into strain CHA0 improved its ability to protect cucumber against *Pythium* damping-off and potato tubers against *Erwinia* soft rot in small hermetically sealed containers. In the presence of ACC deaminase-expressing *P. putida* UW4, the lower level of ACC was expected to cause a reduction in ACC oxidase activity in plant tissues. In the absence of ACC deaminase, resulted in the increased accumulation of ACC in plant tissues (Madhaiyan *et al.* 2006). Similarly, Plants were also coinoculated with *Agrobacterium tumefaciens* C58 and *P. putida* UW4 or *P. putida* UW4-acdS (an ACC deaminase minus mutant strain). It was observed that the presence of ACC deaminase generally inhibited tumour development on both tomato and castor bean plants and also ethylene levels are low on these plants (Hao *et al.*, 2007). Application of 1-

aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing fungal endophyte *Trichoderma asperellum* (strain MAP1) significantly reduced ethylene biosynthesis/signaling gene expression (1-fold reduction ACC oxidase) and reduced ethylene-induced leaf senescence (Rauf *et al.*, 2021).

Phenylalanine ammonia-lyase (PAL) has a crucial role in secondary phenylpropanoid metabolism and is one of the most extensively studied enzymes with respect to plant responses to biotic and abiotic stress (Kim and Hwang, 2014). In this study, analysed the expression profile of PAL gene upon inoculation of bio-agents at different time points. The PAL gene expression was maximum at 24 hpi in all the treatments. Expression profile of PAL in leaves and roots of pomegranate studied. In both leaves and roots PAL expression was higher in bio agent applied treatment at the initial time points. In leaves, maximum up-regulation of PAL gene was observed in T3 (both spray and soil application of *Pseudomonas putida*) and T2 (spray application of *Pseudomonas putida*) with fold change of 7.14 and 6.58 at 24 hpi respectively and at 24 hpi lowest gene expression of PAL was observed in T7 (control) (2.54-folds) and T4 (soil application of *Trichoderma asperellum*) (4.13-folds). In contrast, among all the time points, lowest transcript levels of PAL was observed in T7 at 6 hpi (0.90-folds) and followed by T4 at 0 hpi (1.46-folds). Meanwhile in roots, the PAL gene expression was maximum in T3 and T1 with fold change of 5.75 and 5.57 at 24 hpi respectively and lowest expression of PAL gene at 24 hpi was observed in T7 (2.21-folds). In contrast, among all the time points, lowest transcript levels of PAL was observed in T7 (0.46-folds) and T5 (Spray application of *Trichoderma asperellum*) (0.48-folds) at 216 hpi.

Similarly, Karthikeyan *et al.* (2006) observed activity of PAL and it reached maximum levels within three days after application of a mixture of *P. fluorescens*, *T. viride*. Konappa *et al.* (2018) who reported that the inoculation of *Trichoderma asperellum* against tomato bacterial wilt caused by *Ralstonia solanacearum*, shown the increase in the level of phenylalanine ammonium lyase (PAL), polyphenol oxidase (PPO), β -1,3-glucanase, peroxidase (POX). PAL related gene expression was 0.11 fold expression in control tomato plants and it was doubled with a fold expression of 0.22 in bacterial leaf spot pathogen (*Xanthomonas campestris* pv. *vesicatoria*) treated plants. Respective high gene expression of PAL was recorded with 0.67 and 0.30 Fold Expression in *Methylobacterium* spp. CBMB12 and CBMB15 (strains having ACC

deaminase gene) inoculated plants and challenged with bacterial pathogen (Yim *et al.*, 2014). *Brassica napus* seeds treatment with *P. putida* caused enhanced activities of peroxidase, PAL, catalase, proline and hydrogen peroxide compared to control (Yali and Hajmalek, 2021).

In view of these observations, it is not surprising that inhibitors of ethylene synthesis, such as the enzyme ACC deaminase, can suppress disease severity in plants. The results of the work described here confirm and extend the previous observation of Hao *et al.* (2007) that bacterial ACCD being expressed in a pathogenic *A. tumefaciens* strain or being produced by a non-pathogenic rhizosphere bacterium (e.g. *P. putida* UW4) used for co-inoculation of the plant together with the pathogen can decrease crown gall formation. The results support the idea that ACC deaminase provided by bioagents (*Trichoderma asperellum* and *Pseudomonas putida*) prevents the over production of ethylene (ACC oxidase) in the pathogen infected tissues and in turn reduce the disease severity in plants infected by pathogenic *Xanthomonas axonopodis* pv. *punicae*.

5.7.1 Bio efficacy of ethylene against bacterial blight in field condition.

5.7.1.1 Disease severity

Several bacteria and fungi have been shown to produce ethylene by pathways that are different from the biosynthetic pathway in higher plants (Fukuda and Ogawa, 1991; Weingart *et al.*, 2001). The physiological significance of ethylene production by microorganisms is far from clear. In plant-pathogenic microorganisms, ethylene can act as a phytotoxin and, therefore, may be considered as a virulence factor. However, ethylene is not always involved in virulence. The bacterial leaf pathogens *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola* both produce ethylene in plant (Weingart *et al.*, 2001). Whereas ethylene biosynthesis mutants of *P. syringae* pv. *glycinea* were impaired in their ability to multiply in leaves of soybean and bean. Population sizes of ethylene biosynthesis mutant that did not produce the phototoxin coronatine were 10- and 15-fold lower than those of the wild-type strain on soybean and on bean and 16/83-E1 produced very weak symptoms compared with the wild-type strain (Weingart *et al.*, 2001).

Foliar spray of ethrel showed significant results towards increasing bacterial blight disease severity on leaves, fruits and twigs where, foliar spray of ethrel at different concentration found to increase higher bacterial blight disease severity under field conditions. Ethrel sprayed at 200ppm shown higher disease severity on leaves (6.65%), fruits (5.17%) and twigs (0.57%). Least disease severity was seen in (control) treatment on leaves (4.12%), fruits (3.07%) and twigs (0.15%).

Among three different seasons, Mrig bahar shown higher disease severity on leaves (8.51%) and fruits (5.75%) whereas Hasta bahar shown lower disease severity on leaves (3.01%) and fruits (2.69%). In contrast, twig blight severity did not show any difference with respect to different seasons. Similar kind of results with respect to seasons observed by previous workers (Gowda *et al.*, 2020; Manjula and Khan, 2002; Maity *et al.*, 2020).

The present findings are also in conformity with the work of Manjula and Khan (2002), who reported the devastating nature of bacterial blight of pomegranate on all the varieties, irrespective of age of the plant during late summer and kharif season. Gowda *et al.* (2020) observed similar results, among the seasons, Mrig bahar was found more vulnerable than Ambe bahar and Hasta bahar because of environmental factors like rainfall and temperature which are less in Ambe bahar (January-February) and Hasta bahar (September-October) season. Maity *et al.* (2020) reported crop loss due to bacterial blight upto 80-100 per cent in Mrig bahar.

5.8 Bio efficacy of ethylene on phonological characters in field condition.

5.8.1 Flower characters

Production of perfect flowers results in obtaining fruit yield per plant. In present investigation, use of ethylene hormone played a prominent role towards enhancing hermaphrodite flowers per plant. Among different concentrations of ethrel, foliar application of ethrel at sub-optimal dose played a significant role towards enhancing productive flowers in all seasons. Moreover, its application at flower bud initiation helped in inducing early flowering in pomegranate. These statements are in line with findings of Subhadrabandhu and Koo-Duang (1987) where application of

ethephon at 100 ppm before flowering resulted in production of highest per cent of flowering in litchi.

Ethylene suppressed production of staminate flower and helped in development of maximum pistillate flowers in cucumber (Jadav *et al.*, 2010). Similar results were noticed in the present findings where foliar application of ethrel at 200 ppm resulted in obtaining maximum number of hermaphrodite flowers per plant (189.86). Obtained results are in agreement with findings of Chaudhari and Desai (1993) where, foliar application of ethrel induced maximum flowering in pomegranate. Synthesis of endogenous ethylene by external application of ethephon stimulated early flowering in mango (Davenport and Nunez-Elisea, 1990). Similar results were observed by Cunha (2005) where endogenous synthesis of ethylene by its exogenous application induced early flowering in pineapple.

Increased rate of hermaphrodite flowers (38.17%) and lesser per cent of male flowers (61.83%) resulted in minimizing sex ratio (1.70). These results are in accordance with findings of Girek *et al.* (2013) where application of ethephon at 100 ppm before flowering resulted in obtaining minimum sex ratio in melon. Similar results were noticed by Hilli *et al.* (2010) where its application at 500 ppm resulted in lowering male to female sex ratio in ridge gourd. Contradictory results to findings of Peacock (2010) where foliar application of ethrel maximized male to female sex ratio in grapes while findings of Subhadrabandhu and Koo-Duang (1987) showed significant results towards minimizing sex ratio in longan. This statement is in accordance with findings of Jadav *et al.* (2010) where foliar application of ethrel at 200 ppm resulted in minimizing sex ratio in cucumber. Moreover, ethylene and nutrient may be the upstream regulators that regulated some genes related to ovule development in pomegranate, ethylene application promoted the female flower production and there is noted increase in gene expression of ETR (ethylene-resistant) and ERF1/2 (ethylene-responsive factor) upon ethrel treatment in flower tissues (Chen *et al.*, 2017).

Results revealed that, plants which received foliar spray of ethrel at 200 ppm has taken 17.36 days for completion of flowering from bud initiation to its opening stage when compared to untreated plants where it has taken 19.63 days for completion of flowering. Obtained results are in close conformity with findings of Jadav *et al.*

(2010) where foliar application of ethrel enhanced early flowering in cucumber. The results are in accordance with findings of Ahire *et al.* (1994) where, foliar application of ethrel at sub-optimal condition helped in enhancing early bud initiation, as well as in minimizing duration of flowering. Similar results were observed by Lakshmipathi *et al.* (2014) and Subhadrabandhu and Koo-Duang (1987) where foliar application of ethrel induced early flowering in cashew and litchi respectively.

Foliar application of ethrel at 200 ppm also helped in inducing early maturity in pomegranate by taking 147.00 days from flowering to harvest compared to other treatments. Similar results were observed by Jadav *et al.* (2010) where foliar application of ethrel at 200 ppm induced early picking in cucumber. Endogenously synthesized ethylene enhanced early maturity in most of the fruits crops (Crane, 1969). This statement is in close conformity with the findings of Yadava (2012) where its exogenous application induced early ripening in Cape gooseberry fruits.

Among different seasons, Ambia Bahar shown maximum number of hermaphrodite flowers per plant (276.52). More flower production in Ambe bahar compared to other seasons (Anonymous, 2011). Hasta bahar shown lower sex ratio (1.24) and Maximum Percentage of hermaphrodite flowers (44.76%). Hasta bahar (S3) has taken less number of days (17.97) from flower bud initiation to flower opening stage.

5.8.2 Fruit parameters

Ethylene played a significant role on enhancing fruit setting and fruit retention percentage in pomegranate. Data in Table revealed that, foliar application of ethrel resulted in obtaining higher fruit setting percentage (30.77%) in pomegranate. Moreover maximum production of hermaphrodite flowers followed by lesser flower dropping in treatment plants resulted in obtaining higher fruit setting percentage in pomegranate. These statements are in line with findings given by Lakshmipathi *et al.* (2014) where maximum production of hermaphrodite flowers by ethrel treatment resulted in obtaining maximum fruit setting percentage in cashew. Similar results were obtained by Jadav *et al.* (2010) and Girek *et al.* (2013) where higher production of perfect flowers resulted in obtaining maximum fruit yield per vine both in cucumber and as well as in melon accordingly. The results are in accordance with findings of Uniyal and Misra (2015) where foliar application of ethrel at 100ppm

helped in maximizing fruit setting percentage in bael. The fruit set in pomegranate depends on number of hermaphrodite flowers (Anonymous, 2011).

Application of ethrel showed significant effect on maximizing fruit retention percentage among the treatments. Among these, foliar application of ethrel at 200 ppm (E3) showed maximum fruit retention percentage (20.2%). Similar observation in pomegranate reported by Kumar *et al.* (2020) when ethrel applied at 3 ml/l + DAP 5 g/l application for defoliation. Similarly, Bhatt *et al.* (2017) proved application of ethrel at 150ppm increased the fruit retention percentage in plum when compared to control. Chandola *et al.* (2020) observed foliar application of ethrel at 400ppm improved fruit retention in litchi.

Fruit color is one of the most important consumer preference in marketing. Pomegranate (*Punica granatum* L.) cultivars generally have fruit with colors ranging from green to red during ripening. Total chlorophylls and carotenoids decreased during fruit maturation, while a continually increase of total anthocyanins occurred (Zhao *et al.*, 2015). In pomegranate plants sprayed with ethrel at 200ppm (E3) shown good improvement in external colour of the fruit when measured using colorimeter. Colorimeter provides reading in terms of L^* , a^* and b^* . Where L^* indicates whiteness and darkness. Chromatic portion of the solids is defined by: a (red), $-a$ (green), b (yellow) and $-b$ (blue) (Shahir and Rangaraju, 2014). Plants sprayed with ethrel at 200ppm shown (E3) Maximum L^* value (45.34), a^* value (40.55) and b^* value (30.70) which indicates increase in redness of fruit colour. Similarly, Zhou *et al.* (2010) reported increase in L^* value increased when fruits started turning from green to orange colour (decrease in chlorophyll and increase in carotenoids) when treated with ethrel in Ponkan (*Citrus reticulata*) fruit. Shahir and Rangaraju (2014) confirmed increase in colour values (L^* a^* b^*) when bananas are treated with ethrel. Singh and Shafiq (2008) observed pre-harvest ethrel spray significantly increased the concentrations of total anthocyanin and cyanidin 3-galactoside in the fruit skin and improved red blush and chromaticity values a^* of apple fruit. Ethrel spray at 600 ppm induced uniform ripening with attractive yellow colour within four days while untreated control fruits failed to ripen uniformly and remain light green even after eight days of storage. This indicates that degradation of chlorophyll pigments in fruit peel in ethrel treated fruits was more rapid than in untreated fruits, which is due to accelerated rate of diffusion of exogenous ethylene into peel of ethrel treated fruits.

Also total carotenoid content in ethrel treated fruits increased (Sebastian *et al.*, 2019). El-Wafa *et al.* (2020) noted that spraying ethrel at 125 ppm increased juice and fruit peel anthocyanin content in pomegranate fruit.

Among different seasons, Hasta bahar has shown maximum percentage of fruit setting (39.3%) and fruit retention percentage (28.3%). fruit weight was maximum in Ambia Bahar with fruit weight of 267.64g.

5.8.3 Yield parameters

Maximum production of hermaphrodite flowers and increase in fruit setting percentage by ethrel treatment helped in maximum production of fruit yield per plant. Moreover, maximum production of fruits per plant resulted in obtaining highest yield per plant (17.10 kg/plant) as well as yield per hectare respectively (15.24 t/ha). Synthesis of endogenous ethylene by exogenous ethylene application stimulated development of floral primordia and enhanced maximum production of hermaphrodite flowers per plant. Similar results were observed by Jadav *et al.* (2010) and Lakshmipathi *et al.* (2014) in cashew where application of ethrel helped in production of maximum yield per plant.

Among different seasons, Ambia Bahar shown maximum fruit yield per hectare (16.43 t/ha) and fruit yield per plant (19.72 kg/plant). Gowda *et al.* (2020) noticed more yield of pomegranate in Ambia bahar and low yield in Hasta bahar. More flower production in Ambe bahar leads to more fruit yield compared to other seasons (Anonymous, 2011).

5.9 Elucidation of resistance with other hormones

Plant growth and response to environmental cues are largely governed by phytohormones. The plant hormones ethylene, jasmonic acid and salicylic acid (SA) play a central role in the regulation of plant immune responses (Denancé *et al.*, 2013). Among different hormones sprayed, ethrel sprayed at 300ppm shown maximum disease severity (33.2%) when compared with water control (T4). Similar observations are made by Rodrigues *et al.* (2020) where he observed Blast (*Pyricularia oryzae*) severity was higher on ethylene-sprayed plants regardless of the cultivar in wheat crop. Increase in ethylene concentration on plants induces their susceptibility

against pathogens infection due to an acceleration on senescence, ripening and abscission of organs (Zhang *et al.*, 2018).

Methyl jasmonate sprayed at 300ppm shown higher disease severity (29.97 %) but it is on par with water control (27.32%) and ethanol control (26.81%). It has been well known that JA, as an activating signal molecule, triggers immunity to confer broad-spectrum resistance for plants (Okada *et al.*, 2015). Pathogen infection or other forms of biotic attack stimulate rapid biosynthesis of JA and its derivatives, which would promote the expression of defense-related proteins and secondary metabolites such as alkaloids, terpenoids and PR proteins (Campos *et al.*, 2014). For instance, some pathogens can activate the JA-signaling pathway to disable SA-mediated immune responses and, thus, facilitate pathogen proliferation (Brooks *et al.*, 2005; Yang *et al.*, 2017). Jasmonic acid appeared to contribute to citrus disease susceptibility by antagonizing SA-mediated effective defences against *Xanthomonas citri* subsp. *citri* causing citrus canker in citrus (Long *et al.*, 2019). Susceptibility to *Hyaloperonospora arabidopsidi* increases upon salicylic acid application in Arabidopsis. Coronatine (mimic molecule of jasmonic acid) involved in the elicitation of susceptibility to *P. syringae* by down-regulating SA-inducible defense response pathways via a SA/JA antagonism. Application of jasmonic acid (methyl jasmonate) increased the disease susceptibility (Cui *et al.*, 2005). JA and Coronatine activate the expression of the NAC (transcription factor) tomato homolog, JASMONIC ACID2-LIKE. This transcription factor binds to and activates the expression of SAMT1 and SAMT2, which encode enzymes that deactivate SA by methylation, thereby suppressing the accumulation of SA and promoting stomatal opening (Du *et al.*, 2014).

SA is a multifaceted hormone and is a key regulator in plant-pathogen signalling pathways thereby protecting a broad spectrum of pathogens (bacterial, viral, fungal *etc.*) (Vlot *et al.*, 2009). It is observed that application of exogenous SA or increase in the level of endogenous SA is accompanied by a burst of reactive oxygen species such as H₂O₂, O₂—etc (Shasmita *et al.*, 2019). Results shown, lowest disease severity was observed in T3 (Salicylic acid at 300ppm) (15.08%). Treatment of plants with salicylic acid has provided best protection to plants from bacterial blight disease (*Xanthomonas oryzae*pv. *oryzae* (Xoo)) thereby inducing resistance against the pathogen. Findings of Shasmita *et al.* (2019) suggested a rapid increase in defence

enzyme (superoxide dismutase, catalase, peroxidase, polyphenol oxidase) activity and TP content of SA (2 mM) treated plants as a result of a defence mechanism against *Xoo*. Similarly, Mondal and Shanmugam (2013) observed SA induced effective defense responses in tomato plants against *R. solanacearum*. The reduced disease incidence in tomato by SA may be a result of cell wall strengthening through deposition of lignin and induction of defense enzymes.

6. SUMMARY AND CONCLUSIONS

Pomegranate is a high-profitable crop that thrives in a variety of agro-climatic conditions. It is well-known for its high nutritional value and medicinal properties and as a result, it is in high demand on both the domestic and international markets. The majority of pomegranate farmers are in dire straits as a result of the bacterial blight disease, which has resulted in significant economic losses in terms of both quality and quantity. The ineffectiveness of most chemicals in controlling this disease has shifted growers' attention to the quest for a new molecule and hence the use of plant growth regulators and signal molecules may be a novel approach to controlling this disease as well as improving pomegranate quality and quantity. As a result, the aim of this study was to determine the impact of plant hormone ethylene on patho-physiological functions related to bacterial blight of pomegranate.

The important phytohormone ethylene was taken for present study to know impact towards phenological characters and disease resistance against bacterial blight. Ethylene reducing bioagents like *Pseudomonas putida*, *Trichoderma asperellum* were taken for the present study to know its response towards controlling bacterial blight disease and regulation of ethylene pathway genes under greenhouse conditions. Obtained results are summarised below accordingly.

The causal organism of bacterial blight disease was studied after isolation from infected leaves showing typical symptoms of bacterial blight. Typical yellow, mucoid bacterial colonies of *X. a. pv. punicae* isolates were authenticated on phenotype and genotype based identification followed by pathogenicity test. Further it was confirmed with amplification of *gyrase B* (GyrB) specific to *Xap*. Isolated pathogen was tested for pathogenicity test and it confirmed the Koch's postulates.

Foliar application of ethrel showed higher disease severity whereas control plants showed lower disease severity in all the varieties. Among the different treatments, ethrel at 300ppm (E4) showed significantly higher disease severity of 28.64 per cent and pathogen load whereas control plant showed lower disease severity (34.93 %). Among different varieties, Bhagwa (V1) showed higher disease severity of 31.57 per cent whereas least disease severity was noted in Nana with 15 per cent respectively. Among all the genotypes higher pathogen load was observed on Bhagwa

variety (V1) sprayed with ethrel at 300ppm (9522.67-folds). In contrast, Nana (V5) variety shown least load of pathogen when sprayed with ethrel at 300ppm with fold change of 4301.75. Among different concentrations, 300ppm (T3) shown higher pathogen load on all the genotypes and Control (T4) plants in all genotypes shown the least pathogen load among different treatments.

PCR amplification of ACC deaminase using designed primers shown predicted product size of 130 bp for *Pseudomonas putida* and 141bp for *Trichoderma asperellum* and amplification shows the presence of ACC deaminase gene in both the biocontrol agents. Application of bioagents shown greater reduction in disease severity when compared to untreated control plants. Treatment T3 (combination of both soil and foliar spray of *Pseudomonas putida*) shown lower disease severity (4.24%) followed by T2 (foliar spray of *Pseudomonas putida*) and T6 (combination of both soil and foliar spray of *Trichoderma asperellum*) with the disease severity of 5.79 per cent and 8.78 per cent respectively whereas control plant shown higher disease severity of 28.2 per cent.

In leaves, maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 6 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 6.16 fold change activity and least gene expression at 216 hpi was observed in both spray and soil application of *Pseudomonas putida* (T3) (2.13-folds) and spray application of *Pseudomonas putida* (T2) (2.53-folds). Lowest expression of ACC oxidase gene among different time points was observed in T3 and T6 at 0 hpi with fold change of 0.41 and 0.47 respectively. In roots, overall the maximum up-regulation of ACC oxidase gene was observed at 216 hpi in all the treatments and lowest observed at the initial time points from 0 to 24 hpi. Among all the treatments maximum gene expression of ACC oxidase was observed in control (T7) with 3.77 fold change activity and least gene expression at 216 hpi was observed in T3 (2.56-folds) and T2 (3.0-folds). Lowest expression of ACC oxidase gene among different time point intervals observed in T3 and T1 at 6 hpi with fold change of 0.29 and 0.38 respectively.

Foliar spray of ethrel showed significant results towards increasing bacterial blight disease severity on leaves, fruits and twigs where, foliar spray of ethrel at

different concentration found to increase higher bacterial blight disease severity under field conditions. Ethrel sprayed at 200ppm (E3) shown higher disease severity on leaves (6.65%), fruits (5.17%) and twigs (0.57%). Least disease severity was seen in E4 (control) treatment on leaves (4.12%), fruits (3.07%) and twigs (0.15%). Among three different seasons, Mrig bahar (S2) shown higher disease severity on leaves (8.51%) and fruits (5.75%) whereas Hasta bahar (S3) shown lower disease severity on leaves (3.01%) and fruits (2.69%). In contrast, twig blight severity did not show any difference with respect to different seasons.

Foliar application of ethrel at 200 ppm resulted in obtaining maximum number of hermaphrodite flowers per plant (189.86). Increased rate of hermaphrodite flowers (38.17%) and lesser per cent of male flowers (61.83%) resulted in minimizing sex ratio (1.70). Plants which received foliar spray of ethrel at 200 ppm has taken 17.36 days for completion of flowering from bud initiation to its opening stage when compared to untreated plants where it has taken 19.63 days for completion of flowering. Foliar application of ethrel at 200 ppm also helped in inducing early maturity in pomegranate by taking 147.00 days from flowering to harvest compared to other treatments. Among different seasons, Ambia Bahar (S1) shown maximum number of hermaphrodite flowers per plant (276.52). Hasta bahar (S3) shown lower sex ratio (1.24) and Maximum Percentage of hermaphrodite flowers (44.76%). Hasta bahar (S3) has taken less number of days (17.97) from flower bud initiation to flower opening stage.

Application of ethrel showed significant effect on maximizing fruit retention percentage among the treatments. Among these, foliar application of ethrel at 200 ppm (E3) showed maximum fruit retention percentage (20.2%). Plants sprayed with ethrel at 200ppm shown (E3) Maximum L^* value (45.34), a^* value (40.55) and b^* value (30.70) which indicates increase in redness of fruit colour. Among different seasons, Hasta bahar (S3) has shown maximum percentage of fruit setting (39.3%) and fruit retention percentage (28.3%). fruit weight was maximum in Ambia Bahar (S1) with fruit weight of 267.64g.

Maximum production of fruits per plant resulted in obtaining highest yield per plant (17.10 kg/plant) as well as yield per hectare respectively (15.24 t/ha). Among

different seasons, Ambia Bahar (S1) shown maximum fruit yield per hectare (16.43 t/ha) and fruit yield per plant (19.72 kg/plant).

Among different hormones sprayed, ethrel sprayed at 300ppm (T1) shown maximum disease severity (33.2%). Methyl jasmonate sprayed at 300ppm (T2) shown higher disease severity (29.97%) but it is on par with water control (27.32%) and ethanol control (26.81%). Lowest disease severity was observed in T3 (Salicylic acid at 300ppm) (15.08%).

It is evident from the present study that, foliar application of ethrel in optimal dose at different growth and developmental stages enhanced production of productive flowers, yield and quality attributes of pomegranate but meanwhile it also increased disease severity of bacterial blight disease. Application of bioagents having ACC deaminase gene (reducing ethylene levels in plant) resulted in reduction of disease severity as well as ethylene levels (ACC oxidase). Also among different hormones applied ethrel application showed maximum disease severity and salicylic acid showed lowest disease severity.

Future line of work

1. Expression studies of ethylene pathway genes in different varieties to identify resistant varieties
2. Mechanism behind susceptibility of pomegranate to bacterial blight upon ethylene treatment
3. Effect of different ethylene inhibitors on diseases resistance of pomegranate against bacterial blight
4. Holistic module development by using effective molecules along with other integrated approaches to develop inbuilt resistance in plant system towards management of bacterial blight disease.

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ETHYLENE MEDIATED RESISTANCE AGAINST BACTERIAL BLIGHT IN POMEGRANATE AND ITS INFLUENCE ON PHENOLOGICAL CHARACTERS

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2021

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ABSTRACT

Bacterial blight in pomegranate is a major disease caused by *Xanthomonas axonopodis* pv. *punicae*, which has resulted in significant economic losses in terms of both quality and quantity. The ineffectiveness of most chemicals in controlling this disease has shifted grower attention to the quest for a new molecule and hence the use of plant growth regulators and signaling molecules is a novel approach to control the disease as well as improving quality and quantity attributes of pomegranate. Hence, the aim of present study was to determine the impact of plant hormone ethylene on bacterial blight of pomegranate and also its effect on phenological characters. Result shown that, in present study, among all cultivars, foliar application of ethrel at 300ppm shown higher pathogen load (9371.62-folds) and disease severity (28.64%) in Bhagwa cultivar. Application of ACC deaminase producing bioagents like *Pseudomonas putida* and *Trichoderma asperellum* resulted in reduction of disease severity as well as ethylene levels (ACC oxidase) in plants under greenhouse condition. Field study revealed that foliar application of ethrel (200ppm) at different growth and developmental stages enhanced production of productive flowers (38.17%), yield (15.24 t/ha), colour of pomegranate but meanwhile it also shown higher disease severity on leaves (6.65%), fruits (5.17%) and twigs (0.57%). Among different hormones applied, ethrel application shown maximum disease severity (33.2%) and salicylic acid shown lowest disease severity (15.08%) under greenhouse condition. Hence, it is evident from present study that ethylene is a negative regulator of disease resistance and application of ethylene deaminase producing bioagents like *Pseudomonas putida* and *Trichoderma asperellum* decreased the bacterial blight disease severity and also ethylene levels (ACC Oxidase) in plants, meanwhile ethylene application increased number of productive flowers, yield and quality attributes of pomegranate.

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