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Monika Karn
Department of Plant Pathology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Satish K Sharma
Department of Plant Pathology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Joginder Pal
PG, Department of Agriculture, Khalsa College, Amritsar, Punjab, India

Sunita Devi
Department of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Anju Sharma
Department of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Naveen Sharma
Department of Fruit Sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Corresponding Author:
Monika Karn
Department of Plant Pathology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Efficacy of induced resistance chemicals in triggering defense mechanism of pomegranate plants against the pathogenic bacterium *Xanthomonas axonopodis* pv. *punicae*

Monika Karn, Satish K Sharma, Joginder Pal, Sunita Devi, Anju Sharma and Naveen Sharma

Abstract
Abiotic resistance inducers (ARI) are synthetic/natural compounds which induces innate resistance in plants against pathogens. Current study aimed to assess the foliar application of salicylic acid, potassium oxalate, β-amino butyric acid, sodium salicylate, and di-potassium phosphate @ 300ppm in managing bacterial blight of pomegranate in pot and field conditions. These inducers were assayed at 0, 2, 5, 10, and 14 days of pathogen inoculation. ARI treated plants recorded uttermost phenol and salicylic acid content accumulation at five days when challenged with pathogen. Similarly, activity of defense-related enzymes (phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase) was up regulated after challenge inoculation and it absolutely was most at fifth day. Interestingly, it was observed that regardless of sampling intervals, maximum content of biochemical parameters were perceived at fifth day followed by tenth day of pathogen inoculation. Our findings recommend that enhancing resistance in pomegranate plants before infection may be innovative, safe, and eco-friendly management strategy against bacterial blight of pomegranate.

Keywords: Abiotic resistance inducers, bacterial blight, pathogen inoculation, biochemical parameters

1. Introduction
Pomegranate is one, in all the foremost economically vital fruit crops of India. Maharashtra region is the largest producer of pomegranate within the country. It's cultivation and production suffers severely by known as oily spot disease which is predominant within the pomegranate cultivation in India. The aspect and productivity of pomegranate crop is hampered by 70 to 80% due to this disease (Chavan *et al*., 2017) [6]. Losses from diseases are ordinarily managed by the use of chemicals pesticides. The massive use of chemicals leads to the evolution of chemicals resistance within the infectious agent (Lanka *et al*. 2017; Preston and Malone, 2014) [25, 40]. Hence there is a necessity for developing novel management strategy which is much effective and also environmentally safe. Agricultural researchers these days recognized the benefits of natural plant hormones and their roles to pare off pests and disease symptoms (Mithofer and Boland, 2012) [35]. Once plants are attacked by pathogens, immune mechanisms are triggered by chemical elicitors. Salicylic acid is a natural phenolic compound present in several plants and is a crucial part within the signal transduction pathway and is concerned in native and innate resistance to pathogens (Delaney *et al*., 1995 and Maleck *et al*., 2000) [10, 32]. Phenolic compounds are a chemically distinct and biologically vital club of secondary metabolites. In apple trees, these compounds are involved in natural defence reactions against several diseases (Slatnar *et al*., 2010; Dao *et al*., 2011) [45, 8]. The three main plant signalling/warning molecules are: salicylic acid (SA), jasmonic acid (JA) and ethylene, which may well increase the amount of resistance against varied pathogens. Many reports have shown that SA and JA play very important roles in triggering the induced pathogenesis-related (PR) resistance proteins in plants (Stout *et al*., 2006b; Umemura *et al*., 2009; Sinha *et al*., 2014; Jiang *et al*., 2015) [48, 50, 44, 23]. External application of SA induces plant resistance to different types of pathogens that are related to aerobic burst, cell wall enforcement, up and down-regulation of gene expression (Oostendorp *et al*., 2001) [37]. SA may be a phenolic compound and growth regulator that enhances plant development and immunity (Lattanzio *et al*., 2006; Dempsey *et al*., 2011) [26, 11], also warning

to other plant chemical compounds that defend against infective microbes and insect pests (Sticher *et al*., 1997) [47]. It's essential for both local defense response and systemic acquired resistance (SAR). Salicylic acid and methyl salicylate (MeSA), upregulation usually occur at the same time in response to insect feeding (Frost *et al*., 2008; Arimura *et al*., 2011) [15, 2]. Volatile chemical communication in plants provides broader interplant communication among related and unrelated plant species across distances to upregulate their defenses (Song *et al*., 2010; Lopez *et al*., 2012) [46, 29]. Long distance signalling mechanisms usually use SAR immune responses (Shah and Zeier, 2013) [43]. Phenylalanine ammonia lyase (PAL) catalyzes the deaminization of L-phenylalanine to t-cinnamic acid, that is the first step in the phenylpropanoid pathway that provides the precursors for phenolics, lignin and furanocoumarin, phytoalexins and other downstream metabolites (Tsuge *et al*., 2004) [49]. Peroxidase (POD) oxidizes phenolics to additional toxic quinones and generates hydrogen peroxide (H_2O_2). The activities of PAL and POD might be increased under the influence of elicitors or infectious agent attack. An outsized family of class III plant peroxidases, POD, are pledged for several physiological and biochemical functions in plants, like cell wall growth (Senthil-Nathan, 2013; Passardi *et al*., 2004) [42, 38], elongation (Macadam *et al*., 1992) [30], lignification (Hammerschmidt and Kuc, 1980) [19], auxin catabolism (Gaspar *et al*., 1982) [16], expression of defense-related proteins (Van Loon *et al*., 2006; Duan *et al*., 2014) [51, 13], wound healing and defense mechanisms (Lagrimini, 1991; Hiraga *et al*., 2001) [24, 21].

Abiotic resistance inducers (ARI) are artificial or natural chemical compounds, that don't show an instantaneous antimicrobial activity however are capable of protecting several kinds of plant species against a good range of phytopathogens by inducing systemic resistance. The common AIs used for plant protection includes salicylic acid, L-Glutamate, jasmonic acid, acibenzolar- S-methyl, b-aminobutyric acid, potassium salts, neem oil, isonicotinic acids, and benzothidiazoles (Morsy *et al*. 2011; Walters *et al*. 2013; Hartmann and Zeier 2019) [36, 52, 20]. These chemical elicitors are used on an individual basis or together to manage plant diseases of varied etiology. During this study, we have a tendency to evaluate the attainable utilization of salicylic acid, potassium oxalate, β-amino butyric acid, sodium salicylate, and di-potassium phosphate as inducers in host by activating defence mechanisms in host plant under pot and field conditions.

2. Materials and Method

An experiment was conducted at Model Farm of Dr YS Parmar university of Horticulture and Forestry Solan (H.P.), India during 2019 to evaluate the effect of IR chemicals, *viz*., salicylic acid, potassium oxalate, β-amino butyric acid, sodium salicylate and di-potassium phosphate against bacterial blight pathogen under pot and field conditions. The pots (size 45cm×30cm dia.) were filled with 10 kg of sterilized potting mixtures (soil mixed with farm yard manure and sand in the ration of 4:1:1). Subsequently, pomegranate seedlings raised through cuttings from cv. Kandhari were planted in each pot, each pot was planted with three seedlings and for each treatment 3 pots were used utilizing CRD experimental design and randomized complete design (RBD) for field conditions. In this study, five abiotic resistance inducers (AIR) chemicals with 300ppm concentrations were diluted with distilled sterilized water were evaluated for various biochemical parameters. Plants were sprayed twice before onset of monsoon in respective solution of above mentioned abiotic resistance inducing chemicals. Subsequently, after 20 days of application, treated plants were inoculated by spraying the culture suspension of *Xanthomonas axonopodis* pv. *punicae* grown on nutrient glucose broth (1×10^8 cfu/ml). The assay for plant defence enzyme was performed at 0, 2, 5, 10, and 14 days of pathogen inoculation.

2.1 Plant defence enzymes, total phenol and salicylic acid content

2.1.1 Preparation of enzyme extracts

From each treatment, 1 g of leaf samples (collected at 0, 2, 5, 10, and 14 days of pathogen inoculation) was homogenized in 1.5 ml of 50 mM Tris HCl buffer (pH 7.5) and centrifuged at 18,000 rpm for 20 min at 4ºC. The resulting supernatant was collected in sterilized 2 ml eppendorf tubes and stored in deep freezer (-20ºC) for further use as crude enzyme extract. This enzyme extract was used for assay of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD).

2.1.2 PAL, PPO, and POD assay

PAL activity was estimated based on the production of trans-cinnamic acid (Dickerson *et al*., 1984) [12]. Enzyme activity was expressed as µmol trans-cinnamic acid/min/g fresh plant weight. PPO activity was assayed by measuring the change in the colour intensity of catechol oxidation products (Mayer *et al*., 1965). PPO enzyme activity was expressed as a change in absorbance at 410 nm per min/mg of fresh weight of leaf. For POD assay, 0.5 ml crude enzyme extract was taken in a cuvette, and subsequently, 0.5 ml of 1% guaiacol solution and 1.5 ml 50 mM Tris buffer (pH 7.5) was added into it. Then, the reaction was started by adding 0.5 ml of 1% H_2O_2, and change in absorbance at 430 nm was recorded at an interval of 30 sec. for 3 min. One unit of peroxidase enzyme activity is expressed as the change in absorbance/min/mg of fresh weight (Hammerschmidt *et al*., 1982) [18].

2.1.3 Assay for total phenolic

The total phenole content in fresh leaves were analyzed using the Folin-Ciocalteu colorimetric method (Zieslin and Ben-Zaken, 1993) [54]. The OD of developed blue colour was measured at 650 nm using catechol as standard. The phenole content in plant tissue was expressed as µg/catechol/g fresh plant weight.

2.1.4 Assay for salicylic acid

The salicylic acid in fresh leaves was analyzed using 80% methanol, ascorbic acid, HCl (10N), and 1M $NaHCO_3$. The OD at 254nm was measured as per the procedure described by Dat *et al*. (2000) [9]. The content of salicylic acid was expressed as µg/g of fresh leaves.

2.2 Statistical analysis

The data was recorded in triplicate and analyzed, using the IBM SPSS software version 16.0. Analysis of variance was determined and the mean values were compared by Duncan's multiple range tests at $P< 0.05$.

3. Results and Discussion

Pomegranate plants pre-treated with abiotic induced resistance chemicals *viz*., salicylic acid, potassium oxalate, β-amino butyric acid, sodium salicylate and di-potassium phosphate were assayed at sampling intervals of 0 (day of pathogen inoculation), 2, 5, 10, and 14 days, after pathogen inoculation for various biochemical constitutions like PAL activity, peroxidase activity, polyphenol oxidase activity, total phenols, and salicylic acid activity. As per literature available so far, this is the first report carried out for the use of abiotic resistance inducer chemicals for estimating biochemical parameters of pomegranate against bacterial blight of pomegranate.

3.1 Estimation under pot and field conditions

3.1.1 Phenylalanine ammonia lyase (PAL) activity

Pre-treatment of pomegranate plants with all the resistance inducers chemicals, PAL, activity has been magnified considerably following pathogen inoculation (0 day) and henceforth continued to extend up to 5th days. Utmost PAL activity (1.270 µmol trans-cinnamic acid min^{-1}g^{-1}) was determined in salicylic acid treated plants (Fig. 1) followed by β-amino butyric acid (1.207) under pot conditions. However, least (0.826) was recorded in potassium oxalate treated plants under pot and field conditions, respectively. Maximum PAL (Fig. 2) activity (1.009µmol trans-cinnamic acid min-1g-1) was noted in salicylic acid treated pomegranate plant leaves followed by treatment β-amino butyric acid (0.990). However, least (0.793) was recorded in potassium oxalate treated plants under field conditions. The results are comparable with those of Mandal *et al*. (2009) [34] who detected 3.5 times higher activity of PAL on salicylic acid treated tomato plants than control after 72 h of inoculation. They conjointly recorded the activity of enzyme that was 5.9 times higher than control plants on day 7 (i.e. 168h) of the salicylic acid feeding of the roots. PAL acts as a key enzyme within the phenyl propanoid metabolism in plants that is involved in the conversion of amino acid phenylalanine to trans-cinnamic acid, the precursors for the synthesis of lignin polymer, flavanoid, and phytoalexins (Hahlbrock and Sheel, 1989) [17].

3.1.2 Peroxidase activity

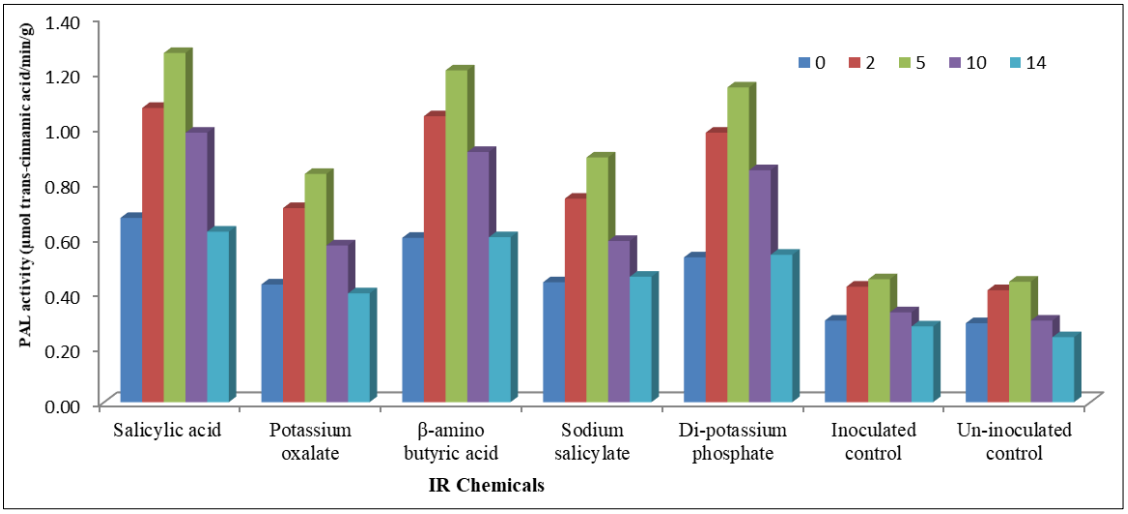

Fig 1: PAL activity in pomegranate seedling

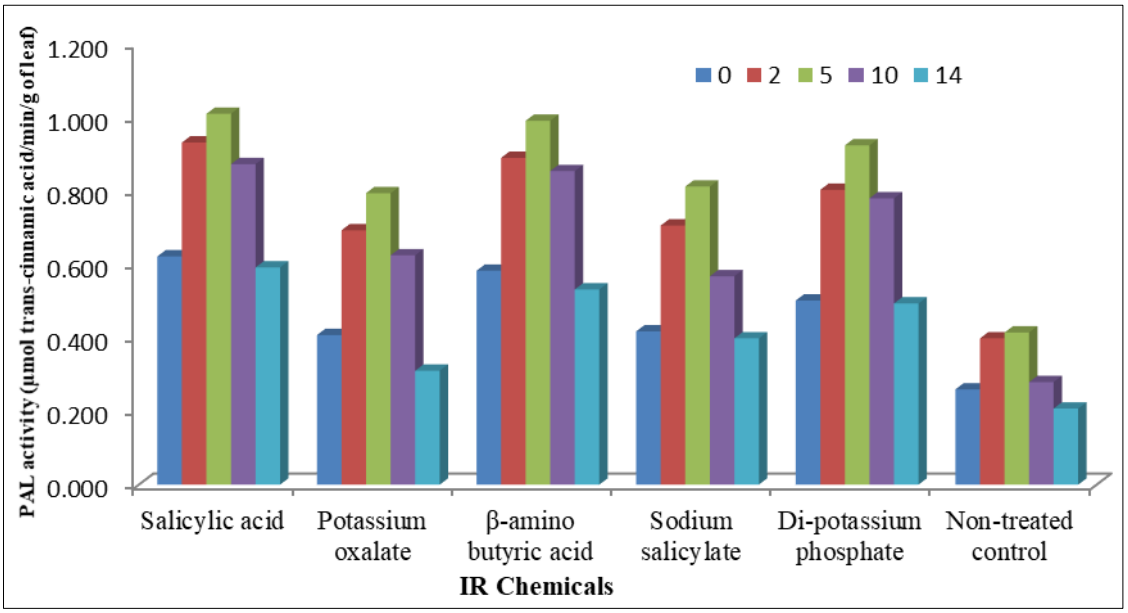

Fig 2: PAL activity in pomegranate plants

Peroxidase activity has exaggerated considerably following pathogen inoculation (0 day) and thenceforth continuing to extend up to five days in pot conditions and field conditions. Maximum peroxidase activity (0.914) in terms of change in absorbance/min/mg fresh weight of leaf tissue was determined in plant treated with salicylic acid (Fig. 3 and 4) followed by β-amino butyric acid (0.885). Similarly, Almoneafy *et al*. (2013) [1] recorded high induction of POD activity on 9th day once inoculation on salicylic acid treated tomato plants. Increase in POD activity was primarily because of increased respiratory rate induced by the pathogen activity, and its predominant participation in the wall-building processes like speedy oxidization of phenols, suberization, and lignification of host plant cells therefore displayed numerous role in defence reaction against pathogenic agents (Asha and Kannabiran, 2001) [3].

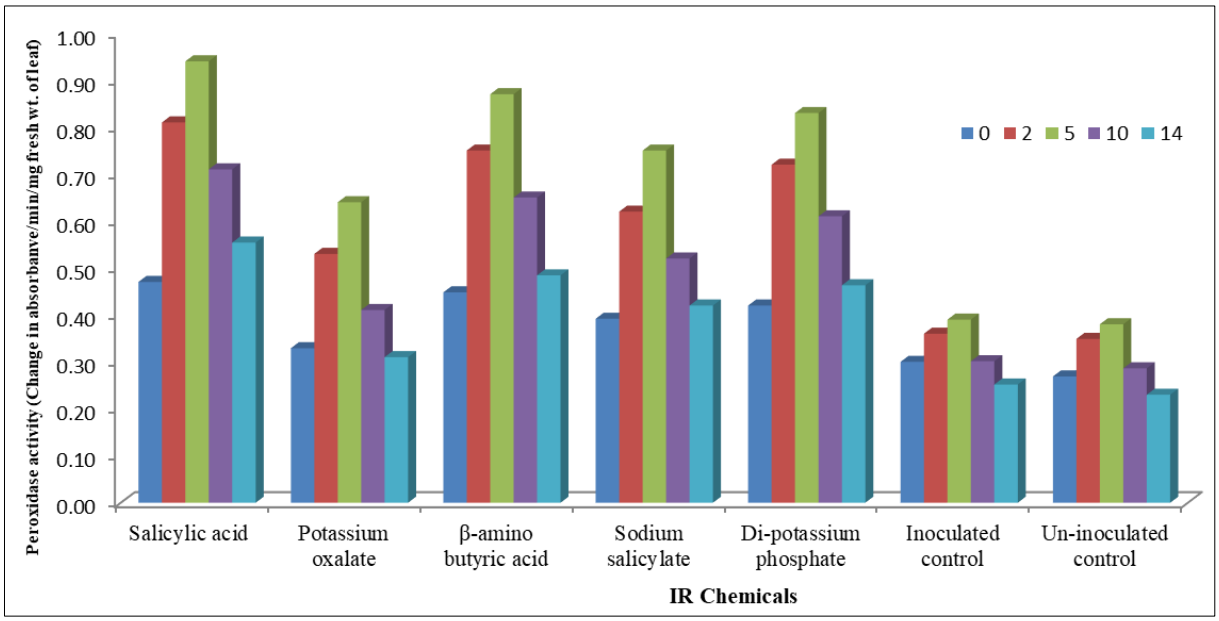

Fig 3: Peroxidase activity in pomegranate seedling

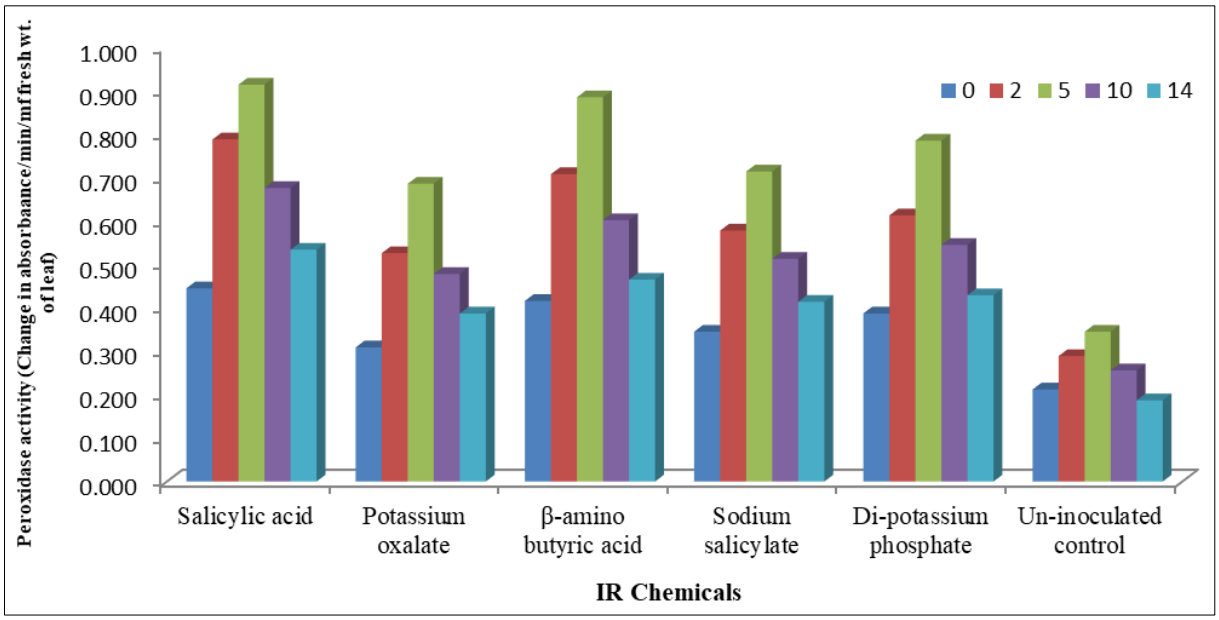

Fig 4: Peroxidase activity in pomegranate plants

3.1.3 Polyphenol oxidase activity

The maximal (0.840 and 0.794) level of PPO activity (Fig. 5 and 6) in terms of change in absorbance/min/mg fresh wt. of leaf tissue resolved in salicylic acid treated pomegranate seedlings followed by β-amino butyric acid (0.767 and 0.701) under pot and field conditions, respectively. However, the least (0.527 and 0.518) record of PPO activity was determined in potassium oxalate. However, PPO activity in control plants was systematically below that in BABA treated plants throughout the whole sampling period. Similarly, higher level of PPO was determined in roots, and shoots of susceptible cultivars of chickpea on treatment with salicylic acid (Raju *et al*., 2008) [41]. Additionally, Chandra *et al*. (2007) [5] confirmed a decline in infection by *Rhizoctonia solani* with a rise in activity of PPO. The magnified PPO activity increased the rate of oxidization of phenolics to more toxic compound quinines that have fungitoxic properties, and reduced inaccessibility of

nutrients or cellular proteins to the pathogens (Wuytz *et al*., 2006) [53]. Additionally, cross linking of quinines with phenolic compounds design a physical barrier to pathogens within the semi-permeable membrane, and generation of H_2O_2 and other reactive species (Li and Steffens, 2002) [27]. Thus, indicating the substantial role of IR chemicals in imparting resistance through the improved activity of PPO enzyme.

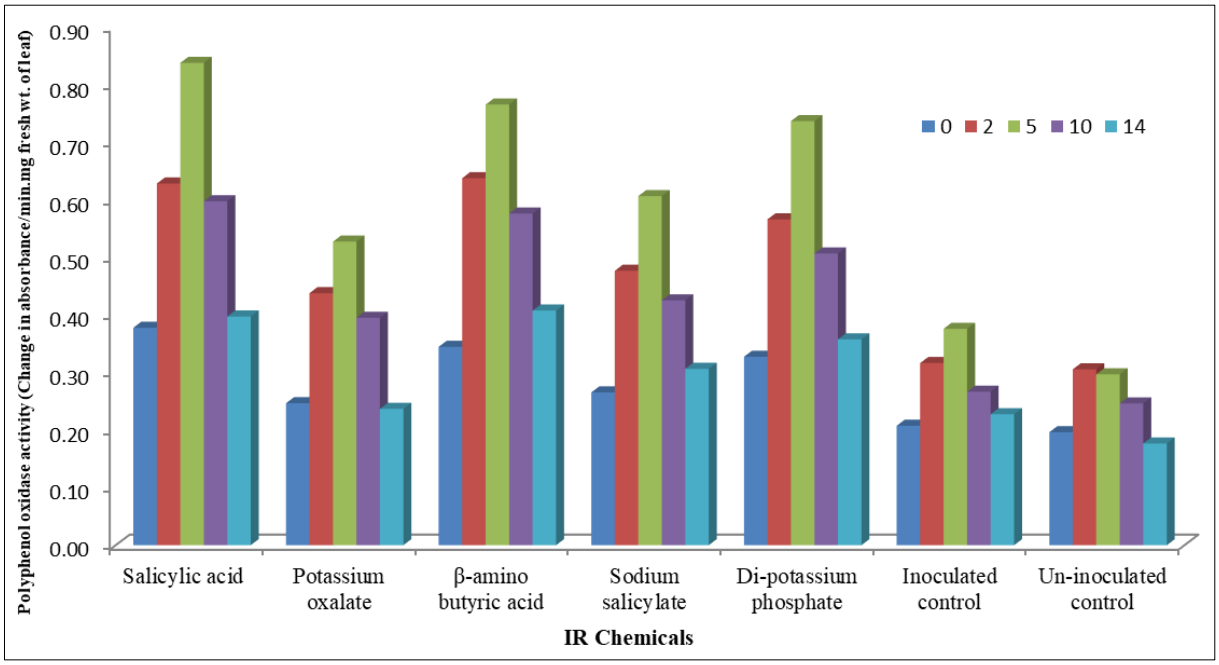

Fig 5: Polyphenol oxidase activity in pomegranate seedling

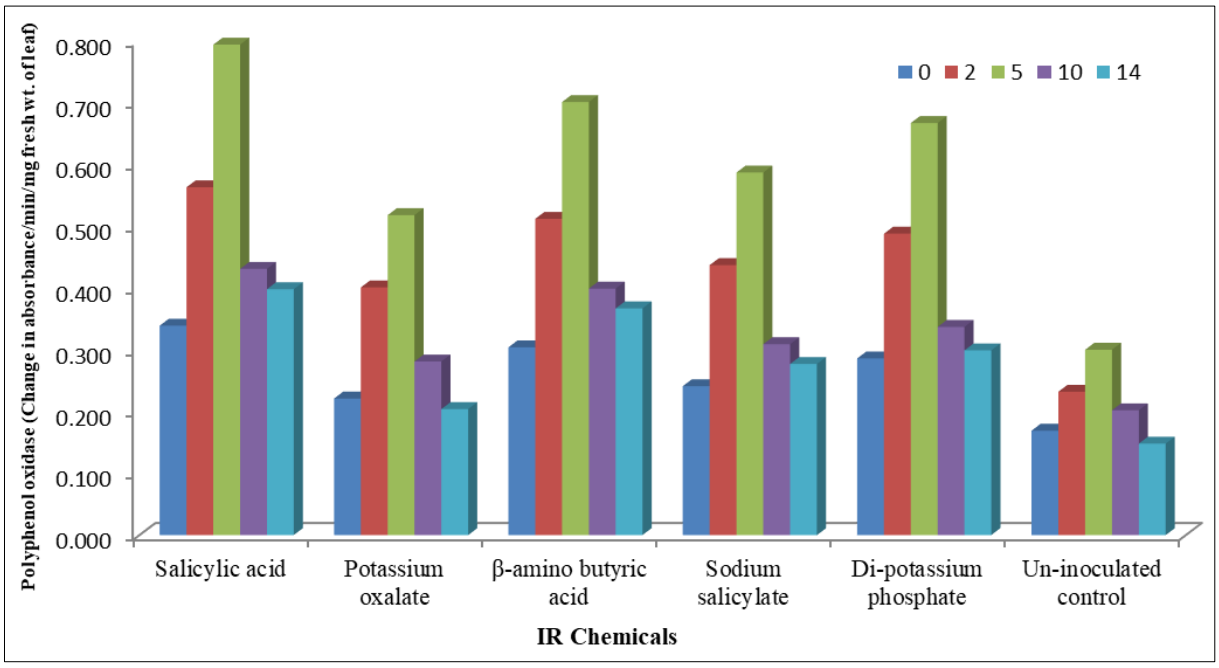

Fig 6: Polyphenol oxidase activity in pomegranate plants

3.1.4 Total phenols

The maximum quantity of phenol content (Fig. 7 and 8) was found in pomegranate leaves treated with salicylic acid (641.10 and 543.21µg/g leaf tissue) as foliar spray followed by β-amino butyric acid (605.67 and 502.67µg/g leaf tissue) and di-potassium phosphate (568.66 and 470.73µg/g leaf tissue) under pot and field conditions, respectively. However, sodium salicylate (523.99 and 450.71µg/g leaf tissue) followed by potassium oxalate (475.00 and 400.71µg/g leaf tissue) treated seedlings registered least increase in quantity of phenol content. Magnified total phenol content was additionally observed in pathogen (350.67) and water treated control (281.67) over a period of time, however the amount was lower as compared to other treatments. The interaction of nitrogen and salicylic acid had been found positive for the phenol concentration of leaves however it had been negative for the proline concentration once applied exogenously (Maity *et al*., 2017) [31]. These findings are in accordance with those of El-Hendawy *et al*. (2010) [14] who found that faba bean plants treated with salicylic

acid either foliar spray or seed soaking showed the utmost accumulation of total phenols in inducing resistance against chocolate spot disease as compared with the untreated ones. Similarly, Biswas *et al*. (2012) [4] showed that total phenol contents for all the treatments multiplied from 5-10 days period but thenceforth, again decreased from 10-15 days. The multiplied phenol contents in treated plants can be liable for defence mechanisms in plants. Jaypal and Mahadevan (1968) [22] reported that sharp increase in phenol contents in incompatible host microbial interaction promote resistance through hypersensitive reaction. Phenolic compounds being fungitoxic in nature, their accumulation either provided mechanical strength to host cell membrane or created a barrier to limit the entry of pathogens.

3.1.5 Salicylic acid content

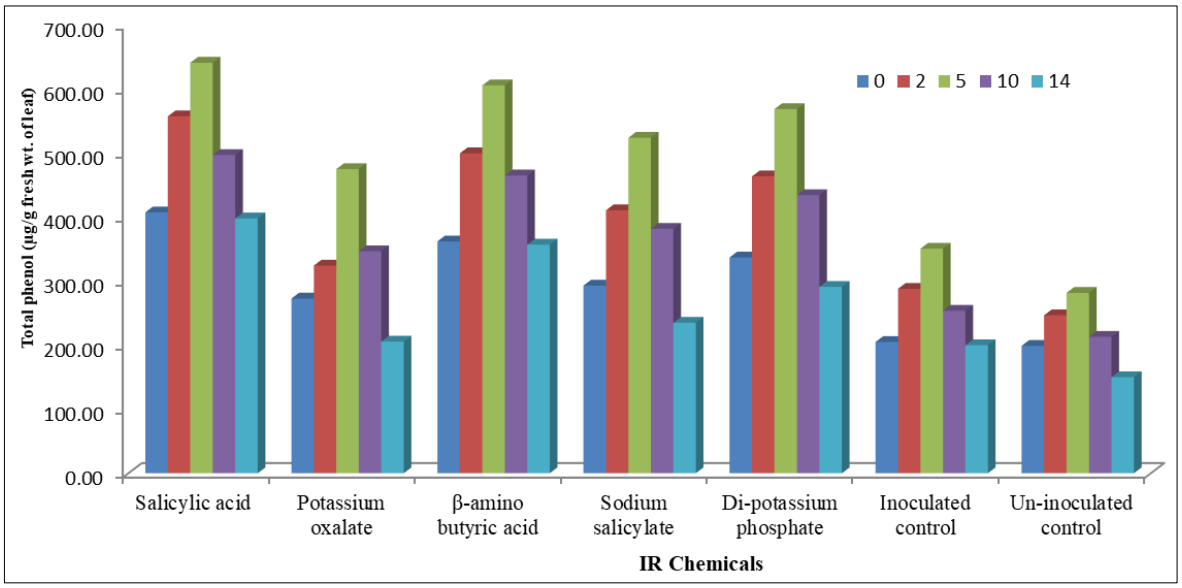

Fig 7: Total phenol content in pomegranate seedling

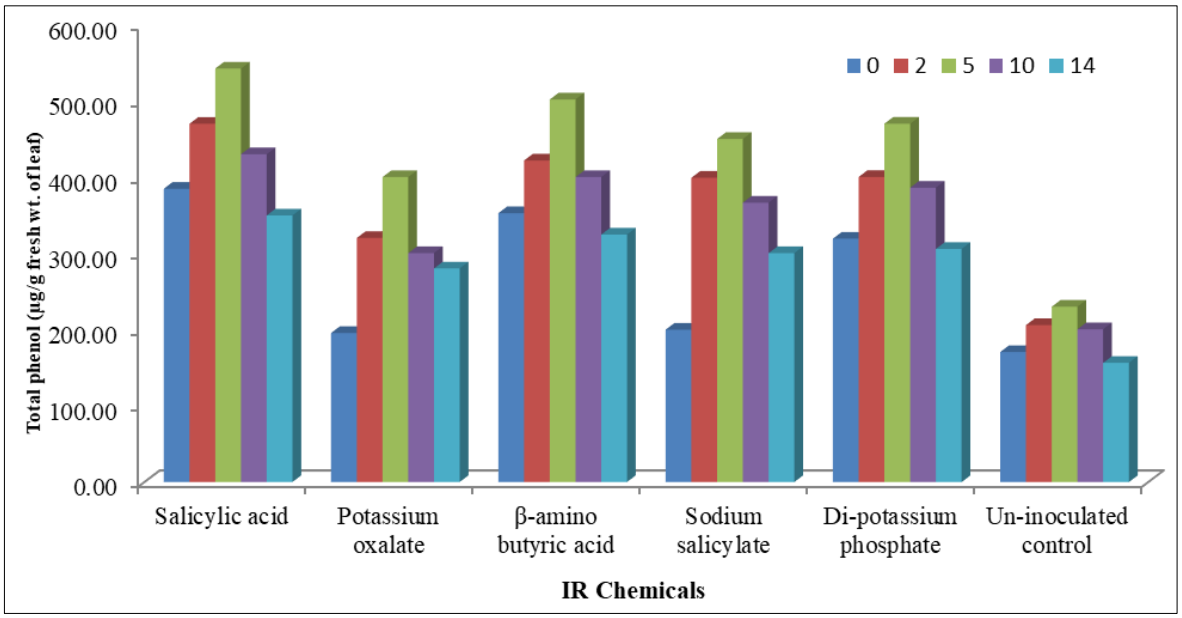

Fig 8: Total phenol content in pomegranate plant

This increment in SA content was determined mainly up to 5th days of pathogen inoculation and thenceforth, it declined steadily in all the treatments (Fig. 9 and 10) under pot and field conditions. The maximal (87.34µg/g recent wt. of leaf tissue) SA level was determined in salicylic acid followed by β-amino butyric acid (81.67µg/g fresh wt. of leaf tissue). However, least SA content (51.34µg/g recent wt. of leaf tissue) was recorded in pomegranate seedlings treated with potassium oxalate. Increased SA content was also determined in pathogen (33.00) and water treated control (29.67) over a period of time, however the amount was lower as compared to other treatments. Salicylic acid content increased (Fig. 10) rapidly after pathogen inoculation (0 day). This increment in SA content was determined primarily up to 5th days of challenge inoculation and thenceforth, it diminished slowly in all the treatments. The highest SA level (55.49µg/g recent wt. of leaf tissue) was determined in salicylic acid followed by β-amino butyric acid (54.02). However, least SA content (36.65) was recorded in pomegranate plants treated with potassium oxalate.

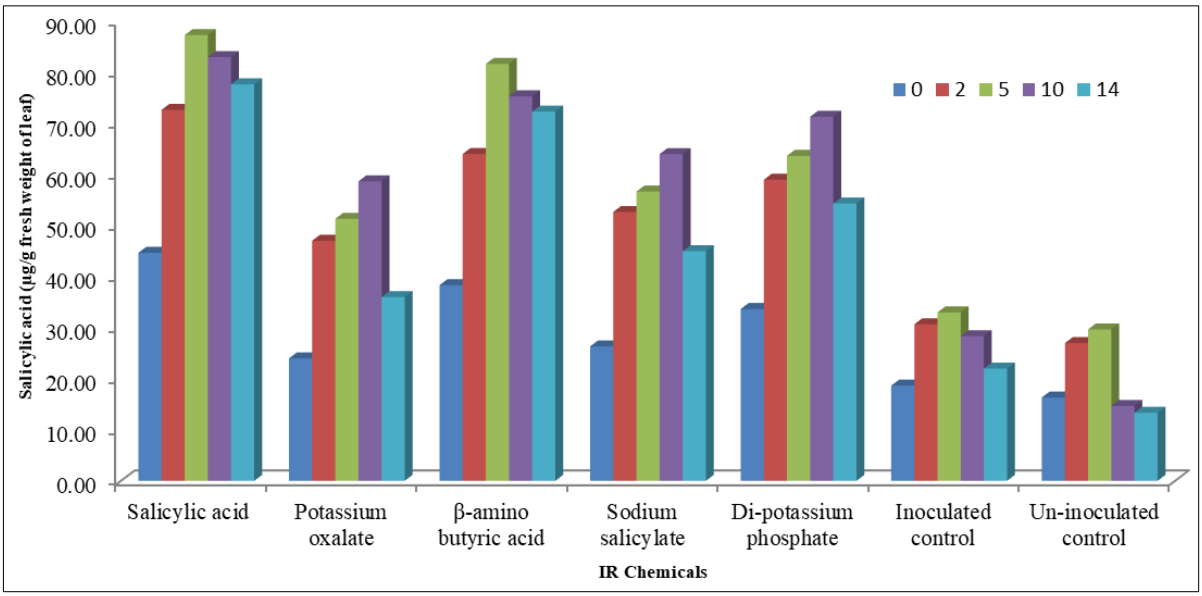

Fig 9: Salicylic acid content in pomegranate seedling

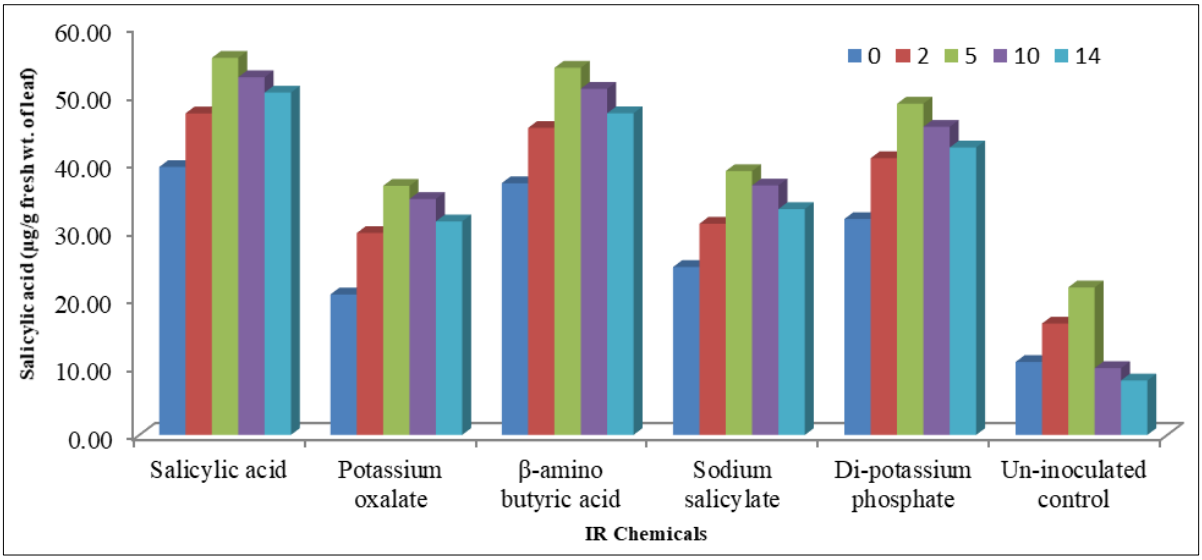

Fig 10: Salicylic acid content in pomegranate plant

Increased SA content was also determined in checked plant (21.71) over a period of time, however, the extent was lower as compared to other treatments. Prakongkha *et al.* (2013) [39] studied grapevine plants treated with chitosan, and BTH, SA level has found to exaggerated considerably after 7 days of treatment, and even higher once challenged inoculation. In contrast, SA accumulation in pathogen-inoculated grapevine was significantly low. Apparently, in the earlier studies it was noticed that exogenous application of SA did not spread quickly within plants that did not considerably increase the endogenous levels of SA. The increment of SA concentration in the leaf tissues might have contributed for increased resistance to the pathogen. SA was found to be an important signal molecule concerned in triggering defense responses, and/or in sensitizing plant cells for a faster, and stronger response to wide range of pathogen attack. Moreover, SA inhibits catalase production that leads to an increase in the concentration of H_2O_2 or active oxygen species throughout the hypersensitive response against pathogens thereby, acts as intermediate within the signaling cascade for the expression of genes associated with defence (Chen *et al.*, 1993) [7].

4. Conclusions

This biochemical study indicated that there was pronounced increase in all the biochemical parameters in pomegranate plants once pre-treated with resistance inducer chemicals. Foliar treatment of plants with SA and BABA @ 300ppm could offer a safety against *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight and may be used as a possible substitute for chemical pesticides. Within the present study, safe concentration of SA and BABA for pomegranate application determined, that opens new horizons and might be commercially used as an eco-friendly, safe low-cost and readily applied methodology to manage bacterial blight pathogen.

5. Statements and Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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