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Elucidation of biochemical induction of host defence by biomolecules/ bioagents against *Rhizoctonia solani* Kuhn in soybean (*Glycine max* Merr.)

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Abstract
An experiment was undertaken to understand the effect of elicitor compounds in imparting disease resistance to *Rhizoctonia solani* in Soybean. The results revealed that all the elicitor compounds were effective in managing the Rhizoctonia blight of soybean through the induction of host defence. Among the chemical inducers, isonicotinic acid was revealed to the most effective for inducing hydrogen peroxidase, ascorbate peroxidase, superoxide dismutase and polyphenol oxidase where as phenylalanine ammonia lyase and peroxidase was found to be highest in plants treated with salicylic acid and benzyl amino purine, respectively. All the elicitor biomolecules decreased the endogenous content of xylanase, laccase and cellulase to a greater extent, which have been reported as pathogenic determinants and assist pathogen attack. The bioagents were also effective in inducing the defence response to Rhizoctonia blight in soybean.

Keywords: Aerial blight, biogeants, elicitors, host defence, soybean

Introduction
Soybean (*Glycine max* (L.) Merrill) is one of the oldest crops grown. It is popularly known as "Miracle Crop or Golden Bean" owing to its multiple uses. Aerial blight of soybean (RAB) caused by *Rhizoctonia solani* Kuhn (teleomorph: *Thanetophorus cucumeris* (Frank) Donk) is a devastating disease especially in the hot and humid tropics. It infects the crop at all growth stages and cause severe defoliation and reduce plant vitality (Mathpal and Singh 2017) [1]. The disease has been accounted for approximately 35 per cent yield reduction in soybean which may increase to 70 per cent under favourable environmental conditions (Sinclair and Blackman 1989) [2]. Aerial blight alone caused a yield loss of around three lakh tones of soybean during 2011 to 2014 in U. S. states and Ontario (Allen *et al.*, 2017) [3]. Disease is wide spread in occurrence and has been reported from all the major soybean growing regions of the world like Mexico (Crispin and Gallegos 1963) [4], South American countries (Deslandes 1944; Ploper 1981) [5, 6], Japan, Sri Lanka and Kenya (Mukunya 1974; Weber 1939; Zaumeyer and Thomas 1957) [7, 8, 9].
In India, the disease is prevalent in states of Uttarakhand, Madhya Pradesh, Chhattisgarh and in north eastern states like Nagaland. The disease caused an average yield loss of 40-50 per cent in Uttarakhand (Joshi *et al.*, 2018) [10]. With the imminent climate change and increase in atmospheric temperature and humidity, the disease can occur in devastating proportions in the years to come. The soil borne nature of the pathogen and absence of a promising resistant variety further enhances the difficulty in managing the disease (Manian and Manibhushanrao 1990) [11]. At present, farmers are mostly dependent on the chemical fungicides for the management of the disease.

Materials and Methods
The experiments were undertaken in Department of Plant Pathology, G. B. Pant University of Agriculture and Technology Pantnagar. The effect of six chemical elicitors viz. oxalic acid, SA (salicylic acid), INA (Isonicotinic acid), Bap (Benzyl amino purine), GA3 (Gibberellic acid) and 2,4-D (2,4- dichloro, dimethyl trichloro ethane) and three bioagents PBA 1-3

(Pant Bioagent 1-3) on the induction of host defence in soybean (cv JS-335) against Rhizoctonia aerial blight in soybean leaves was studied. The elicitor/bioagent was sprayed in one month old soybean plants followed by the pathogen inoculation after 12 hrs.

A. Defence compound

a. Hydrogen peroxide content

The method given by Alexieva *et al.*, (2001) [12] was followed for measuring hydrogen per oxide content. 0.2 g of fresh leaf sample was crushed in 1.0 ml of 0.1 per cent (w/v) trichloroacetic acid (TCA) and centrifuged at 10000 rpm for 30 min at 4 ºC. The reaction mixture consisted of 0.5 ml supernatant, 0.5 ml of 0.1M potassium phosphate buffer and 2 ml of 1M potassium iodide (KI) reagent. The blank consisted of 0.1 per cent TCA in place of leaf extract. The reaction was allowed to develop for 1 hr in dark and absorbance was measured at 390 nm. The amount of H_2O_2 was calculated using standard curve prepared with different dilutions of a working standard of 100 µM of H_2O_2.

B. Defence related antioxidant enzymes

a. Ascorbate peroxidise Activity (APX)

Ascorbate peroxidise (APX,EC 1.11.1.11) activity was determined using the method given by Nakano and Asada (1981). Thereaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2mM EDTA, 0.5mM ascorbic acid, 2 per cent H2O2 and 0.1 ml enzyme extract in a final volume of 3 ml. The decrease in absorbance at 290 nm up 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient (2.8mM-1 cm-1). The difference in absorbance (A290) (was divided bythe ascorbate molar extinction coefficient (2.8) (mM-1cm-1) and the enzyme activity expressed as µmol min-1mg-1 protein, taking into consideration that 1.0 mol of ascorbate was required for the reduction of 0.1 mol of H2O2. (McKersie and Leshem, 1994) [13].

b. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring the inhibition of photochemical reduction of nitro- blue tetrazolium (NBT) at 560 nm as described by Beauchamp and Fridovich (1971) [14]. The reaction mixture (3 ml) consisted of 50 mM Na- Phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM NBT, 10 µMEDTA, 2.0 µM riboflavin and 0.3 ml enzyme extract. The test tubes containing reaction mixture were kept under 4000 lux at 350C for 20 min. One unit SOD activity was defined as the amount of enzyme required to cause 50 per cent inhibition of the rate of NBT reduction measured at 560 nm.

c. Polyphenol oxidase (PPO) activity

Fresh leaf (0.2 g) were homogenized in 2 mlof 0.1M sodium phosphate buffer of pH 6.5 and centrifuged at 10,000 rpm for 15 min at 40C. The supernatant was used as source of enzyme. The reaction mixture comprised of 200 µl of the enzyme extract and 1.5 ml of the 0.1 M sodium phosphate buffer (pH 6.5). 200 µl of 0.01M catechol was added to start the reaction and the activity was expressed as the change in absorbance at 495 nm min-1 mg-1 protein (Mayer *et al.*, 1965) [15]

d. Peroxidase (POD) activity

Peroxidase (POD EC 1.11.1.17) activity was determined using the method as described by Tatiana *et al.*, (1999) [16] with minor modifications. The reaction mixture contained 0.05 M sodium phosphate buffer (pH 5.5), 2 per cent H_2O_2, 0.05 M guaiacol and 0.1 ml enzyme extract in a final volume of 5 ml. The reaction was started after the addition of enzyme extract. The formation of tetraguaiacol was measured at 470 nm. One unit of enzyme was defined as the amount of enzyme to decompose 1µmol of H2O2 min-1 at 25 ^{0}C.

e. Phenylalanine ammonia lyase (PAL) activity

Phenylalanine ammonia lyase activity was determined by the method given by Edward and Kesssmann (1992) [17]. Homogenization buffer consisted of 25 mM tris buffer (pH 8.8). reaction mixture was prepared by adding 0.1 ml of enzyme extract and 0.4 ml of 0.05 M tris buffer (pH 8.8) containing 0.2 mM phenylalanine and was incubated in water bath at 37 ^{0}C for 60 min. Reaction was stopped by adding 0.1 ml of0.5 N HCl. The trans-cinnamic acid was extracted by adding 2 ml of toluene. The absorbance was taken at 412 nm and the enzyme activity was expressed in µmol transcinnamic acid min-1g-1fresh weight.

C. Endogenously released defense related cell wall degrading enzymes

a. Xylanase

Xylanase activity was determined by DNS reducing sugar method. The reaction mixture consists of 500 µl enzyme extract, 1 ml of 1% xylan and 500 µl 100mM Sodium acetate buffer of pH 5. Enzyme blank consists of 500 µl enzyme extract and 1.5 ml, 100mM sodium acetate buffer pH 5. Substrate blank consists of 1 ml of 1% xylan solution and 1 ml of 100 mM sodium acetate buffer of pH 5. Prior to adding enzyme extract, the tubes were pre incubated for 5 min at reaction temperature. After 30 min incubation at 250 C, the reaction was stopped by adding 1 ml of DNS (40 g DNS, 8 g phenol, 2 g sodium sulphite, 800 g sodium potassium tartarate and 1% (w/v) NaOH in 4L distilled water and filtered before use) and the test tubes were boiled for 10 min and were allowed to cool to room temperature. The reducing sugar was measured using 6 mM D- glucose as standard at 550 nm. The enzyme activity is measured as 1 µmol of reducing sugar per minute.

b. Cellulase

Cellulase activity was determined by DNS reducing sugar method. The reaction mixture consists of 500 µl enzyme extract, 1 ml of 1% CMC and 500 µl 100mM Sodium acetate buffer of pH 5. Enzyme blank consists of 500 µl enzyme extract and 1.5 ml, 100mM sodium acetate buffer pH 5. Substrate blank consists of 1 ml of 1% xylan solution and 1 ml of 100 mM sodium acetate buffer of pH 5. Prior to adding enzyme extract, the tubes were pre incubated for 5 min at reaction temperature. After 30 min incubation at 250 C, the reaction was stopped by adding 1 ml of DNS (40 g DNS, 8 g phenol, 2 g sodium sulphite, 800 g sodium potassium tartarate and 1% (w/v) NaOH in 4 L distilled water and filtered before use) and the test tubes were boiled for 10 min and were allowed to cool to room temperature. The reducing sugar was measured using 6 mM D- glucose as standard at 550 nm. The enzyme activity is measured as 1 µmol of reducing sugar per minute.

c. Laccase

Laccase activity was determined by the oxidation of 2,2'-azino-*bis* (3- ethylbenzthiazoline-6- sulfonic acid) (ABTS, Sigma) at 37 ºC (Buswell *et al.*, 1996) [18]. 1mL reaction

mixture was prepared by adding 600 µl enzyme extract, 300 µl sodium acetate buffer pH 4.5 (50 mM) and 100 µl ABTS solution (1 mM). The reaction was stopped by the addition of 100 µl of 20% (w/v) trichloroacetic acid. Oxidation of ABTS was monitored at 420 nm on a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 mmol of ABTS per minute. Standard graph for laccase was prepared by using laccase pure (Sigma). Stock of 1mg/ml was serially diluted. The concentration of laccase (mg/ml) is calculated with the help of standard graph. The obsevartion of Hydrgen peroxide, Polyphenol oxidase, ascorbate peroxidise, phenylalanine ammonia lyase, superoxide dismutase, peroxidise, cellulose, xylanase and laccase were estimated at 0, 2, 4 and 6 days after inoculation of *Rhizoctonia.*

Results and Discussion

The results revealed that all the tested chemical and biological elicitors were effective in inducing the defence related enzymes and reducing the the endogenous content of xylanase, laccase and cellulase to a greater extent, which have been reported as pathogenic determinants and assist pathogen attack. The effect of these elicitors on individual biochemical parameters are as discussed below.

A. Defence compound

a. Hydrogen per oxide H2O2 content

The biochemical properties of soybean cv. JS-335 plants inoculated with rhizoctonia aerial blight pathogen and treated with elicitor, hormones, bioagents were compared to the control plants. The level of H_2O_2 in inoculated leaves increased and became maximum, on second day after inoculation of pathogen and this decreasing trend continued up to 4-day after inoculation and thereafter, the level was nearly constant when compared with the control plants (Table 1). Among the biocontrol agents PBA-2 (407.00 µmol/g FW) showed maximum accumulation of H2O2 followed by PBA-1 (379.00 µmol/g FW) and PBA-3 (357.33 µmol/g FW) respectively. Among the other biochemical treated plants, INA was observed to show maximum accumulation of H2O2 (460.33 µmol/g FW) on second day after inoculation and then decreasing tend followed before attaining a nearly constant state and was significantly higher than GA3 (424.33 µmol/g FW) and 2,4-D (412.96 µmol/g FW). Hormones treated plants showed higher accumulation of H2O2 on second day as compared to other chemical elicitors viz. Oxalic acid (417.00 µmol/g FW) and salicylic acid (342.33 µmol/g FW) having highest accumulation at sixth day after inoculation. In general, the level of H2O2 remained high in all treatments compared to check till sixth day of observation. This result showed that oxidative burst was observed in all treatments is a result of induction of resistance. Bioagents were observed to show more responsiveness in terms of accumulation of H_2O_2 especially PBA-2 at earlier stage as compared to other chemicals.

Hydrogen peroxide is a toxic compound produced in response to various stress including biotic stress, is a result of dismutation of superoxide radicals. This triggers many downstream processes leading to a dynamic defence response characterized by inhibition of the growth of invaders through synthesis of secondary metabolites and pathogenesis related (PR) proteins, phytoalexin formation, callose deposition and strengthening of cell walls, (Shoresh *et al.*, 2010[19]; Xu *et al.*, 2008[20]; Vinale *et al.*, 2008[21]). Excess of H_2O_2 in the plant cells leads to the occurrence of oxidative stress. Its higher concentration, it is injurious to the plant as it may lead to lipid peroxidation and membrane injury (Nayar and Kaushal, 2002) [22]. Several studies have reported elevated H_2O_2 in response to stress. This increase in level of H_2O_2may be due to the slower activity of H_2O_2 scavenging enzymes as also been reported by Seloteel *et al.*, (2004) [23]. H_2O_2 appears to be the major reactive oxygen species (ROS) that accumulates during defense activation in most systems (Thakur and Sohal, 2013) [24]. According to Morsy (2005) [25] H_2O_2 caused significant reduction in pre and postemergence damping-off caused by *R. solani* of lentil plants. Deng *et al.*, (2012) [26] reported to have a positive influence on the physiological properties (root, shoot and production) of plant.

Table 1: Effect of elicitors, hormones and bioagents on activity of Hydrogen Peroxide (µmol/g Fresh weight) in leaves of soybean before and after inoculation of *R. solani* at two day interval

Treatment	Hydrogen Peroxide (µmol/g Fresh weight)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	315.67	376.67	376.22	417.00
Salicylic acid	249.67	307.00	302.56	342.33
INA	295.33	460.33	378.22	358.67
GA_3	239.00	424.33	401.89	206.33
2,4-d	326.33	412.96	251.00	317.00
BAP	229.00	283.33	257.89	300.29
PBA-1	278.00	379.00	183.22	410.00
PBA-2	329.33	407.00	312.89	335.00
PBA-3	272.65	357.33	220.22	350.00
Control	271.67	264.67	261.22	261.57
CD (p=0.05)	3.82	4.93	14.92	13.89
CV	0.80	0.79	2.97	2.47

B. Defence related antioxidant enzymes

a. Ascorbate peroxidise (APX) activity

The results (Table 2) indicated that all the nine elicitor treatments were found significantly increased the ascorbate peroxidase specific activity (µmol/min/mg protein) over the control after pathogen inoculation in glasshouse condition. Significantly maximum ascorbate activity was observed in PBA-1 (64.40 µmol/min/mg protein) followed by PBA-2 (58.10 µmol/min/mg protein) and PBA-3 (46.67 µmol/min/mg protein) after two days of fungal inoculation, after which they displayed declining trend till the sixth day compared to untreated control. The APX activity slightly reduced until second day and started increasing only after fourth day of fungal inoculation after which it again started declining. Among the elicitor and hormones, salicylic acid (59.90 µmol/min/mg protein) and BAP (46.31 µmol/min/mg protein) were recorded to have nearly doubled the ascorbate activity in post pathogen inoculation at second day following which the activity declined on fourth day and there after slightly increased. Oxalic acid was observed to show slowest activity among all chemicals but the activity kept on increasing at a slower pace till the sixth day (41.79 µmol/min/mg protein) of observation. APX exists as isoenzymes and utilizes ascobate as its specific electron donor and reduces H2O2 to water with the concomitant generation of monodehydroascorbate which is a univalent oxidant of ascorbate. Recent studies regarding the response of APX expression under specific stress conditions and pathogen attack indicate the importance of APX activity in controlling the H2O2 concentration and in intracellular signaling (Shigeoka *et al.*, 2002) [27]. Similar results were fund by Junga *et al.*, (2011) [28] who observed increased APX activity at 72 h after bacteriocin treatment in soybean leaves.

Table 2: Effect of *R.solani* aerial blight on specific activity of Ascorbaste peroxidase (µmol/min/mg protein) in leaves of soybean at two day interval

Treatment	Ascorbate per oxidase activity (µmol/min/mg protein)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	30.48	31.79	39.64	41.79
Salicylic acid	28.81	59.90	17.86	28.57
INA	27.86	39.40	47.86	52.14
GA_3	31.79	49.29	19.63	33.93
2,4-d	26.43	51.43	12.14	14.64
BAP	27.26	46.31	13.81	21.07
PBA-1	33.21	64.40	62.50	8.57
PBA-2	36.43	58.10	51.79	8.50
PBA-3	37.98	46.67	57.14	16.31
Control	37.02	32.50	44.40	19.28
CD(p=0.05)	5.50	6.07	6.91	10.15
CV	10.18	7.42	11.06	24.35

b. Superoxide dismutase (SOD) activity

The effect of foliar spray of bio-agents and other elicitor biomolecules on the activities of SOD in leaves of soybean (Table 3) did not show any regular trend, but increasing level of SOD activity after being inoculated with *R. solani* till second day was observed in most cases while it declined during the fourth day of inoculation thereafter which increased till sixth day post pathogen inoculation. Among the bio-agents PBA-2 (0.0133 ΔA/min/mg protein) showed the maximum increase in SOD activity at the second day which was significantly higher than PBA-3 (0.0091ΔA/min/mg protein) followed by PBA-1 (0.0089 ΔA/min/mg protein), in which the SOD content further reduced till the fourth day and there after showed an increasing trend. Among the other elicitor biomolecules 2, 4-D was most responsive towards the pathogen inoculation with highest level of SOD (0.0093 ΔA/min/mg protein) on second day after inoculation followed by INA (0.0080 ΔA/min/mg protein) and GA3 (0.0075 ΔA/min/mg protein). The chemical elicitors salicylic acid and oxalic acid showed a delayed response with SOD activity and kept on decreasing till the fourth day (0.0057 ΔA/min/mg protein and 0.0067 ΔA/min/mg protein respectively) after which it started to increased till sixth day of fungal inoculation. Untreated control showed minimum concentration of SOD accumulation throughout the study as compared to other treatments. It is evident from the above data that all the treatments induced SOD activity to a greater extent as compared to control but the trend is variable among the elicitors which indicates there ability to induce host defense against *R. solani*. Biocontrol agents were more responsive in initiating faster host defense towards pathogen attack while chemical elicitors display a delayed response compared to hormones and antagonists. The elicitors thus need to be individually studied for their detailed mode of action. The enzyme superoxide dismutase (SOD) has been reported to play a prime role in protecting cells against oxidative stress since they dismutase O_2 to H_2O_2 and O_2 (Scandalias, 1990) [29]. SOD activity increased during the hypersensitive response (HR) as superoxide radicals formed at site of infection are scavenged. Studies showed increased activity of SOD after elicitor treatment which might have induced HR. Similar observations for increased SOD activity were reported by Malolepsza *et al.,* (1994) [30] on strawberry leaves after treatment with salicylic acid.

Table 3: Effect of treatments on activity of Superoxide dismutase (ΔA/min/mg protein) in leaves of soybean before and after inoculation of *R. solani* at two day interval

Treatment	Superoxide dismutase (ΔA/min/mg protein)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	0.0103	0.0077	0.0067	0.0070
Salicylic acid	0.0090	0.0087	0.0057	0.0072
INA	0.0069	0.0080	0.0059	0.0107
GA3	0.0070	0.0075	0.0048	0.0068
2,4-D	0.0080	0.0093	0.0059	0.0091
BAP	0.0080	0.0081	0.0053	0.0080
PBA-1	0.0049	0.0089	0.0057	0.0054
PBA-2	0.0090	0.0133	0.0090	0.0147
PBA-3	0.0069	0.0091	0.0063	0.0075
Control	0.0055	0.0074	0.0043	0.0050
CD(p=0.005)	0.0057	0.0130	0.0029	0.0027
CV	28.65	19.43	28.38	19.62

c. Polyphenol oxidase (PPO) activity

The leaves of soybean plants treated with bio-agents and other elicitor biomolecules showed an increase in the level of polyphenol oxidase activities after inoculation with *R. solani* (Table 4). The polyphenol oxidase activity increased from second day after pathogen inoculation and reached a maximum at second day and then started declining. Among the bio-agents, PBA-2 showed the maximum PPO content (0.0194 ΔA/min/mg protein) at second day which was significantly higher than PBA-1 (0.0183 ΔA/min/mg protein) followed by PBA-3 0.0178 ΔA/min/mg protein). The PPO activity decreased till the sixth day with values of 0.0034 ΔA/min/mgprotein and 0.0049 ΔA/min/mg proteins in PBA-1 and PBA-3 respectively. Our study showed that the salicylic was most responsive towards the pathogen inoculation with highest level of PPO on second day (0.0188 ΔA/min/mg protein) after pathogen inoculation followed by GA3 (0.0168 ΔA/min/mg protein) and INA (0.0167 ΔA/min/mg protein) which were insignificantly different. It is evident from data that all the treatments induced polyphenol oxidase activity to a greater extent as compared to control indicating their ability to induced host defense against *R. solani*. Biocontrol agents were more responsive in initiating the host defense towards pathogen attack when compared to other biomolecules. Polyphenol oxidase is an oxygen transferring enzyme. It uses O2 to catalyse through dehydrogenation of catechols to orthoquinones and the orthohydrogenation of phenols to catechols. The present findings clearly showed increased activity of polyphenol oxiadse in treated plants as compared to control plants are supported by Mayer (2006) [31] who observed induction of polyphenol oxidase in plants, particularly under conditions of stress and pathogen attack. Similar results were observed in *Alternaria* leaf blight infection in Brassica species where the activity of polyphenol oxidase increased at a much faster rate in the susceptible plants (Gupta *et al.,* 1995) [32]. The increased activity of the polyphenol oxidase has been reported due to either solubilisation of polyphenolases from cellular compartments or activation of latent polyphenol oxidase (Robb *et al.,* 1964) [33] and also the possibility of its being released by the pathogen (Farkas and Kirarly 1962) [34]. Foliar spray with salicylic acid to control *Fusarium oxysporum f. sp. lycopersici* in tomato also showed an increased PAL and POD activities and the increase was 3.7 and 3.3 times respectively higher compared to control treatment (Mandal *et al.,* 2009) [35].

Table 4: Effect of foliar spray of bio agent, elicitors and other biomolecules on Polyphenol oxidase (ΔA/min/mg protein) activity in soybean leaves

Treatments	Polyphenol oxidase (ΔA/min/mg protein)			
	Days after inoculation			
	0	2	4	6
oxalic acid	0.0079	0.0161	0.0057	0.0045
salicylic acid	0.0066	0.0188	0.0051	0.0042
INA	0.0057	0.0167	0.0060	0.0047
GA_3	0.0068	0.0168	0.0055	0.0046
2,4-D	0.0091	0.0156	0.0048	0.0018
BAP	0.0058	0.0148	0.0042	0.0043
PBA-1	0.0077	0.0183	0.0060	0.0034
PBA-2	0.0044	0.0194	0.0041	0.0038
PBA-3	0.0051	0.0178	0.0061	0.0049
Control	0.0062	0.0106	0.0024	0.0030
CD(p=0.005)	0.0054	0.0055	0.0069	0.0080
CV	4.90	1.93	8.08	12.08

d. Peroxoidase (PO) activity

Effect of biomolecules and bioagents spray on the peroxidase activity in leaves of soybean plants in (Table 5) showed the activity of PO was immediate increased in the leaves of plants over the control after being inoculated with *R. solani.* The highest peroxide level was recorded after the second day of pathogen inoculation. Among the biocontrol agents maximum increase was observed in PBA-3 (0.340 ΔA/min/mg protein) and PBA-1(0.337 ΔA/min/mg protein) which were insignificantly different followed by PBA-2 (0.324 ΔA/min/mg protein) after which the level drastically reduced till fourth day and the slightly increased till sixth day of pathogen innoculation. Among other biomolecules, cytokinin BAP (0.308 ΔA/min/mg protein) exhibited higher increase followed by 2,4-D (0.283 ΔA/min/mg protein) and INA (0.236 ΔA/min/mg protein) in peroxidase level after two days of pathogen inoculation and least being in chemical elicitors viz. oxalic acid and salicylic acid. Results of the study reveal that biocontrol were again most responsive to peroxidase followed by phytohormones and chemical elicitors. Similar study was conducted by Pathak (2003) [36], who observed induction of ISR by PGPR fluorescent Pseudomonas strain PRP3 against *R.solani* and found 9.95 per cent increase in PO level. Shalata and Tal (1998) [37] also reported increased PO activity under stress condition in tomato.

Table 5: Activity of peroxidase (ΔA/min/mg protein) in soybean leaves before and after inoculation of *R. solani* at two day interval

Treatment	Peroxidase (ΔA/min/mg protein)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	0.070	0.114	0.032	0.048
Salicylic acid	0.097	0.197	0.007	0.021
INA	0.116	0.236	0.002	0.058
GA_3	0.069	0.227	0.016	0.051
2,4-D	0.089	0.283	0.040	0.066
BAP	0.073	0.308	0.029	0.102
PBA-1	0.109	0.337	0.004	0.027
PBA-2	0.078	0.324	0.011	0.029
PBA-3	0.086	0.340	0.019	0.031
Control	0.065	0.105	0.020	0.020
CD(p=0.05)	0.016	0.015	0.005	0.01
CV	10.92	3.64	17.27	13.96

e. Phenylalanine ammonia lyase (PAL) Activity

Effect of bioagents and other biomolecules on PAL activity in leaves of soybean inoculated with *R. solani* at two days interval (Table 6) showed that the level of PAL (expressed as µg- t- cinnamic acid produced/ hr/ gFW) was significantly increased in all the treatments as compared to control, but increase was not always significant. The highest Phenyl ammonia lyase level was recorded at the fourth day of pathogen inoculation. Among the biocontrol agents maximum increase was observed in PBA-3 (0.340 ΔA/min/mg protein) and PBA-1(0.337 ΔA/min/mg protein) which were insignificantly different followed by PBA-2 (0.324 ΔA/min/mg protein) after which the level drastically reduced till fourth day and the slightly increased till sixth day of pathogen innoculation. Among other biomolecules, cytokinin BAP (0.308 ΔA/min/mg protein) followed by 2,4-D (0.283 ΔA/min/mg protein) and INA (0.236 ΔA/min/mg protein) recorded highest increase in peroxidase level after two days of pathogen inoculation and least being in chemical elicitors viz. oxalic acid and salicylic acid. Results of the study revealed that plant or animal origin elicitors were most responsive to peroxidase compared to chemical elicitors. PAL is reported as the key enzyme in the synthesis of phenyl propanoid derivatives involved in disease resistance mechanism. Activity of PAL increased in soybean plant inoculated with *R. solani*, treated with biomolecules was higher than in the control plant. PAL is induced in many resistance reactions and after treatment withvarious elicitors of defence reactions (Davies and Ausubel, 1989) [38]. Zhi Huai *et al.,* (2009) [39] reported increased activities of POD, PPO and PAL enzymes reducing the *R.solani* infection causing sheath blight in rice by 82.9 per cent with *Trichoderma harzianum* indicating induction of resistance. Chen *et al.,* (2000) [40] also reported high levels of PAL, POD and PPO induced in cucumber roots inoculated with *Pythium aphanidermatum* compared with plant growth-promoting rhizobacteria (PGPR).

Table 6: Changes in phenylalanine ammonia lyase (µg cinnamic acid/mg/min) content in leaves of soybean before and after inoculation of *R. solani* at two day interval

Treatment	Phenylalanine ammonia lyase (µg cinnamic acid/mg/min)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	2.31	2.00	3.03	1.59
Salicylic acid	1.32	3.13	4.56	3.05
INA	0.94	2.38	3.24	1.29
GA_3	1.50	1.21	3.33	2.67
2,4-d	0.95	1.08	3.13	1.66
BAP	0.91	1.08	4.50	1.22
PBA-1	1.03	1.45	3.38	1.06
PBA-2	0.84	1.08	2.94	0.93
PBA-3	1.41	2.01	4.54	1.18
Control	1.55	0.97	1.73	1.30
CD(p=0.05%)	0.64	0.13	0.06	0.22
CV	27.00	4.73	1.17	7.61

C. Endogenously released defense related cell wall degrading enzymes

a. Xylanase

From Table 7 it is clear that the level of endogenous xylanase content decreased considerably in the plants treated with bioagents and other elicitor biomolecules after being innoculated with *R. solani* under study. However, the lowest level of xylanase was observed up to fourth day after the inoculation thereby it started increasing. Among the bio-agents, PBA-3 showed the maximum reduction of xylanase content (452 µmol glucose/mg protein) at the second day which was significantly lower than PBA-2 and PBA-1

respectively. The xylanase content was reducing till the fourth day with values of 563 µmol glucose/mg protein and 949 µmol glucose/mg protein respectively, there after showed an increasing trend in bioagent treated plants. Among the other elicitor biomolecules, INA was most responsive towards the pathogen inoculation with lowest level on second day (556 µmol glucose/mg protein) followed by BAP (571 µmol glucose/mg protein) and 2, 4-D (580 µmol glucose/mg protein) which were insignificantly different. Among other chemical elicitors salicylic acid was more responsive than oxalic acid with maximum reduction up to fouth day (784 µmol glucose/mg protein) after inoculation of the pathogen thereby increasing progressively. All the treatments showed similar pattern as more reduction of the endogenous xylanase level as compared to control where there was a slight reduction on second day (1284 µmol glucose/mg protein) but thereafter it started increasing. Critical perusal of Table 7 revealed that all the treatments induce xylanase activity to a greater extent as compared to control which indicates there ability to induce host defense against *R. solani*. Biocontrol agents were more responsive and initiated faster defense towards infection by pathogen when compared to other biomolecules. Being the natural compounds thus are more ecofriendly and non toxic to the plant health.

Table 7: Xylanase (µmol glucose/mg protein) activity in leaves of soybean infected by *R. solani*

Treatment	Xylanase (µmol glucose/mg protein) Days after inoculation			
	0	2	4	6
Oxalic acid	1715	1188	1152	1356
Salicylic acid	1875	869	784	939
INA	1562	556	591	629
GA_3	1526	904	827	1156
2,4-D	1383	782	580	1341
BAP	1324	665	571	1165
PBA-1	1496	1076	949	950
PBA-2	1634	951	563	1193
PBA-3	1525	452	719	1143
Control	1477	1284	1304	1336
CD (p=0.05)	19.97	10.14	7.02	11.43
CV	0.76	0.68	0.51	0.60

b. Laccase

Table 8 revealed that the response of soybean plants treated with bioagents and other biomolecules showed a decreasing trend in the total endogenous laccase level after being inoculated with *R. solani*. The highest rate of reduction was observed on the fourth day after which the laccase content started reducing at decreasing rate till the sixth day of inoculation. Among the biocontrol agents maximum reduction was observed in case of PBA-3 (10.5 µg/ml of crude extract), which was significantly lower than PBA-2 (10.5 µg/ml of crude extract) and PBA-1 (16 µg/ml of crude extract). However the most drastic reduction was observed in case of PBA-2 between second day (399 µg/ml of crude extract) and fourth day (104.5 µg/ml of crude extract) of inoculation, thereby revealing more responsiveness. Among other biomolecules, cytokinin, BAP showed the steepest reduction after two days of inoculation (598.5 µg/ml of crude extract) till the fourth day (57.75 µg/ml of crude extract) thereafter attaining a nearly constant value. In other growth regulators, the reduction in GA3 was maximum on sixth day (11.25 µg/ml of crude extract) after inoculation which was significantly higher than 2, 4-D 115.25 µg/ml of crude extract) and INA (146 µg/ml of crude extract) as on sixth day of inoculation. The effect of exogenous foliar application of salicylic acid (239.75 µg/ml of crude extract) and oxalic acid (304.5 µg/ml of crude extract) exhibited gradual reduction in total laccase content up to the fourth day after which it was fairly constant. *R.solani* is a soil borne pathogen which infects roots and degrades the tissue. Laccases play a very crucial role in maceration of lignified host tissues leading to loss of host cell integrity and maceration. The enzymes produced by different organisms are not always identical and can vary even within a single species where different isozymes may be present (Collmer and Keen, 1986) [41]. The enzymes here produced in the plant system are of broadly two types. First that belong to the plant system and second that belong to the infecting pathogen. With the help of the above mentioned results it can be concluded that the plant tries to reduce the endogenous level of the total enzyme content post infection. This may be due to the antagonistic activity of the host macerating enzymes against the pathogen macerating enzymes thereby showing initial reduction of total enzymes content initial few days after which the endogenous level of host enzymes gradually increases to its prior level. Laccases role in cell wall breakdown and are essential for there important role in pathogenicity of *R. solani* was also observed by Bora (2003) [42].

Table 8: Activity of laccase (µg/ml of crude extract) in leaves of soybean inoculated with *R. solani*

Treatment	Laccase (µg/ml of crude extract of soybean leaves) Days after inoculation			
	0	2	4	6
Oxalic acid	470.5	388.25	304.5	182.25
Salicylic acid	428.5	349	239.75	191
INA	504.75	509.25	351	146
GA3	488.25	575.75	271.75	11.25
2,4-D	600.5	442.75	273.75	115.25
BAP	588.5	598.25	57.75	20
PBA-1	586.5	413	228.75	16
PBA-2	575	399	104.5	58.75
PBA-3	451	245	95.75	10.5
Control	631.5	488.75	192	28.5
CD (p=0.05)	18.12	103.29	6.61	3.47
CV	2.00	12.75	1.83	2.62

c. Cellulase

The results on effect of bioagents and other biomolecules on *R. solani* inoculated leaves of soybean revealed a decreasing trend in endogenous cellulase level (Table 9). The highest rate of reduction was observed till the second day after which the cellulase content started increasing in treated plants. Maximum reduction was recorded in GA3 (60 µmol glucose/mg protein) followed by salicylic acid (61 µmol glucose/mg protein), oxalic acid (70 µmol glucose/mg protein) and INA (71 µmol glucose/mg protein) was recorded up to second day of pathogen inoculation, while the level started to increase afterwards. Among the biocontrol agents maximum reduction was observed in case of PBA-2 (125 µmol glucose/mg protein) followed by PBA-1+2 (129 µmol glucose/mg protein) and PBA-1(130 µmol glucose/mg protein). However reduction observed was drastically low as compared to other biomolecules. The reduction was significant in all the treatments as compared to check. However the level of cellulase kept on increasing in the succeeding days.

Table 9: Effects of elicitors, hormones and bioagents on cellulase activity in leaves of soybean inoculated with *R. solani*.

Treatment	Cellulase (µmol glucose/mg protein)			
	Days after inoculation			
	0	2	4	6
Oxalic acid	168	70	204	189
Salicylic acid	152	61	114	166
INA	172	71	112	193
GA_3	163	60	189	210
2,4-D	161	85	162	242
BAP	156	126	177	229
PBA-1	163	130	217	221
PBA-2	207	125	155	135
PBA-3	142	129	121	126
Control	156	131	189	164
CD (p=0.05)	18.92	10.88	4.17	6.27
CV	7.03	6.47	1.49	1.08

It has been reported that extracellular enzymes including pectinases, cellulases and proteases, that can degrade components of plant cell wall. Patil and Pathak (1994) [43] have also implicated these enzymes in disease development in plants studied by them. These enzymes produced by microorganisms during host invasion cause cell wall modifications that can initiate plant defense responses. Thus, heterologous expression of CWMEs can lead to a broad range of effects that could potentially mimic the plant's responses to endogenous or external CWMEs (Pogorelko *et. al*, 2013) [44]. So the probable reason of reduction in the level of cellulose in our data may be to reduce these kind pathogenic determinants and initiate timely host defense. Induction of resistance against diseases utilizing bio-agents and chemicals which may be synthetically or naturally produced either by microorganisms or host plants has been well demonstrated by Many scientists. The biochemical barriers in plant tissues to restrict pathogen growth and development under the influence of systemic resistance inducers can in future pave way for more effective and environment friendly alternative to chemical control (Akram and Anjum, 2011) [45].

Conclusion

Elicitor compounds including chemicals, hormones and bioagents increased the accumulation of defense compounds viz. H2O2, APX, POD, PPO, SOD and PAL, initiating a whole array of defense mechanisms thereby inducing induced systemic resistance (ISR). All the elicitor biomolecules decreased the endogenous content of xylanase, laccase and cellulase to a greater extent, which have been reported as pathogenic determinants and assist pathogen attack. So reduction in their concentration strengthens the plant defense.

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