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Endophytic *Bacillus velezensis* underscore induction of defense enzyme and confers resistance against *Xanthomonas axonopodis* PV. *Betlicola* in betel vine

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Abstract
Bacterial leaf spot is one of the major threats in betel vine production as it can cause economic loss. Endophytes are used in agriculture as a method to boost crop performance due to their capacity to colonize interior host tissues. In the present study, it was observed that accumulation of defense related enzyme and compounds (Peroxidise (PO), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL), total phenols, total sugars and proteins) has been increased in betel vine that were treated with endophyte *Bacillus velezensis* along with pathogen compared to untreated control. The endophyte treated plants had a significantly lower intensity of bacterial leaf spot than untreated control plants and also recorded a higher plant height and more of leaves.

Keywords: Peroxidase, polyphenoloxidase, Phenylalanine Ammonia Lyase, Xanthomonas axonopodis

Introduction
Betel vine (*Piper betle* L.) is an economically and medicinally important cash crop in the world. It belongs to the family Piperaceae and the most probable place of origin of betel is central Malaysia (Chattopadhyay and Maity, 1990) [2]. It is valued both as a mild stimulant and for its medicinal properties. Betel vine is extremely prone to diseases, pests and various types of natural disasters. The disease is a major barrier to the cultivation of betel vine, since the microclimate created in the orchards is highly conducive for the growth of pathogens. Major diseases of betel vine are foot rot or leaf rot caused by *Phytophthora parasitica* var. *piperina*, Anthracnose caused by *Colletotrichum piperis* and Bacterial leaf blight (BLB) caused by *Xanthomonas axonopodis* pv. *Betlicola*, among them bacterial leaf spot is the most destructive disease which decreases the production as well as the quality of betel vine to a great extent.
Chemical application is often required for disease control and considering the side effects of chemicals, an alternative method of protecting plants from disease is to activate their internal defense mechanisms *via* the use of specific biotic or abiotic elicitors. Moreover, endophytes have a profound impact on plant communities, as they can increase plant fitness by conferring abiotic and biotic stress tolerance, increasing biomass, plant growth and yield by increasing nutrient uptake and suppressing pathogens, as well safest for human health.
Therefore the present investigation deals with accessing the effect of endophytes isolated from betel vine in triggering defense signaling molecules and in management of bacterial spot of betel vine.

Materials and Methods
An assay for peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL), total phenols, total sugars and proteins was conducted to determine the influence of endophyte in triggering host-induced resistance. 3-month-old betel vine plants were used for the study. For this study, an effective bacterial endophyte (*B. velezensis*) was selected against *X. a.* PV. *Betlicola* and sprayed with endophytic suspension until the suspension run-off from the plant. Four different treatments were imposed under the pot condition. T_1- plants were challenge inoculated with the pathogen at one week after spraying of endophytic suspension, T_2- plants were only inoculated with endophytes, T_3- plants were only inoculated with the pathogen and T_4- plants were neither treated with endophytic suspension nor with the pathogen and it served as an untreated control.

Observations were recorded for 15 days with sampling performed at 2 days intervals starting from one day after endophytes application. During each sampling time, leaves were collected, washed and frozen overnight before enzymatic assay.

Assay of Phenylalanine Ammonia Lyase (PAL)

Leaf sample of 0.1 g was macerated with 25 mM Tris HCL buffer of pH 8.8 (w/v). The homogenate was centrifuged at 8000 rpm for 30 minutes at 4 °C and supernatant was used for enzyme assay. PAL activity was assayed by slight modification as described by Lisker *et al*. (1983) [10]. The reaction mixture contained 0.5 ml of crude sample, 2.50 ml of 50 mM L-phenylalanine as substrate and 0.2 ml of 25 mM Tris HCL (pH 8.8) and mixture was incubated for 2 hours at 40 °C, the activity was stopped by adding 0.03 ml of 5 N HCL and the absorbance was read at 290 nm against the same volume of reaction mixture without L-phenylalanine served as blank. The enzyme activity was expressed as µmol of trans-cinnamic acid (t-CA) /µg protein/h.

Assay of Peroxidase (PO)

One gram of **f**resh plant leaves homogenated in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) with mortar and pestle. The homogenate was centrifuged at 18, 000 rpm at 4°C for 15 minutes and supernatant served as an enzyme source. To a spectrophotometric sample cuvette, 3 ml of sodium phosphate buffer, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml H_2O_2 (30% w/v, 100 volumes stabilised) solution were added and mixed well. The absorbance was recorded at 420 nm using spectrophotometer. The enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} of fresh tissue (Hammerschmidt and Kuc, 1982) [5].

Assay of Polyphenol Oxidase (PPO)

The polyphenoloxidase activity was determined as per the procedure given by Mayer *et al*. (1965) [11]. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added. The absorbance was recorded at 495 nm using spectrophotometer and enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} of fresh tissue.

Assay of total phenols

Total phenols in leaf samples were estimated by using Folin-Ciacalteu method (Singleton and Rossi, 1965) [15]. Two hundred µL of diluted sample was mixed with 1mL of 1:20 diluted Folin-Ciacalteu (FC) reagent and content was kept at room temperature for 5 min. Then 800 µL of 7.5 per cent sodium bicarbonate solution was added and incubated for 30 min at 37°C. The absorbance was measured at 750 nm against water blank. Gallic acid was used as standard and results were expressed as µg /g of leaf sample.

Assay of total sugars

Determination of total sugars in betel vine leaves was done by employing anthrone method (Hedge and Horeiter, 1962) [6] following the procedure given below. One gram of sample was taken and ground by pestle and mortar, the volume was made upto 5 ml using 2.5 N HCl. Then the samples were kept in hot water bath at 95 °C for 2 hours. Later samples were kept for cooling to room temperature, then pinch of sodium carbonate was added to neutralize the remaining acid in the sample. The sample was filtered and the volume was made up to 100 ml using distilled water. From that, 0.5 ml was taken and 0.5 ml distilled water was added followed by addition of 4 ml anthrone reagent was boiled (50-60 ^{0}C) for five minutes. The sample was cooled down and the intensity of dark green colour was read using spectrophotometer at 630 nm absorbance. A graph was drawn by plotting concentration of the standard on the x- axis and absorbance on the y-axis. From the graph, the amount of total sugar present in the sample was calculated.

Assay of proteins

Protein estimation was done by Lowry's method, which is sensitive enough to give moderately constant value and hence largely followed. 500 mg of the sample was grinded with a pestle and mortar in 5-10 ml of the sodium phosphate buffer. Centrifuged and used the supernatant for protein estimation. Pipette out 0.2 ml sample extract into the test tube and made up the volume to 1 ml with water, added 5 ml of alkaline copper solution to the test tube. Mixed the content well and allowed for 10 minutes, then added 0.5 ml of Folin-Ciacalteau reagent to the test tube, mixed well and incubated at room temperature in dark for 30 minutes. Observed for blue colour development and measured the readings at 660 nm using spectrophotometer. The amount of protein was calculated based on standard graph and expressed as mg/g.

Results

The experiment was conducted to know the role of endophytes in inducing the defense enzyme *viz*., Peroxidase (PO), Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL) along with these molecules, total phenol, Protein, total sugar content were estimated quantitatively at different time intervals (1, 3, 5, 7, 9 and 11 days after inoculation) in betel vine plants.

Significantly, the maximum mean phenylalanine ammonia lyase (PAL) activity was observed in T_1 (*B. velezensis* + pathogen) treated plants (40.68 µmol) followed by T_2 (38.23 µmol) (Table 1). The PAL activity in all treatment was initially high then decreased on 3rd day and again increased from 5th to 11th day as compared to untreated control. The data on estimation of PO activity at 1, 3, 5, 7, 9 and 11 days is presented in Table 2. Among the four treatments, mean PO activity was higher in T_1 (*B. velezensis* + pathogen- 0.71 absorbance/min/g of leaf tissue), next best was T_2 (only pathogen- 0.55 absorbance/min/g of leaf tissue). In T_4 (Control), PO activity was maximum at first day and the enzyme activity decreased further (0.34 absorbance/min/g of leaf tissue). PPO activity was high in endophyte treated plants as compared to other treatments. T_1 (*B. velezensis* + pathogen) recorded significantly higher PPO activity (0.45 absorbance/ min/g of leaf tissue) (Table 3), next best treatment was T_2 (only pathogen) where in recorded (0.38 absorbance/ min/g of leaf tissue), followed by T_3 (0.35 absorbance/min/g of leaf tissue). Least PPO activity was observed in untreated control (0.27 absorbance/min/g of leaf tissue). The PPO activity was initially high at by 1st day, then decreased on 3rd day and increased from 5th to 11th day. Total phenol was estimated on 1, 3, 5, 7, 9 and 11th day after treatment imposition and results are presented in Table 4. Among the four treatments, *B. velezensis* + pathogen showed significantly higher phenol content as compared to all other treatments and this was

followed by only pathogen treatment. The mean total phenol content was more in T_1 (159.26 µg/100g of fresh tissue) treatment followed by T_3 (144.73 µg/100g of fresh tissue) treatment. The less mean phenol content was observed in only pathogen (131.85 µg/100g of fresh) as compared to untreated control.

The protein content found increased in endophyte treated plants from day one to 11th day after inoculation, decreased production of protein was observed in pathogen inoculated plants. The mean protein was significantly higher in T_1 (*B. velezensis* + pathogen) (2.97 µg/g of fresh) followed by T_3 (*B. velezensis* alone) (2.11 µg/g of fresh tissue). Lesser mean protein content was observed in pathogen (1.47 µg/g of fresh tissue) treated plants as compared to untreated control (1.96 µg/g of fresh tissue) (Table 5). Effect of different treatments on total sugar production in betel vine plants in response to endophyte and pathogen inoculation is presented in the Table 6. The total sugar was maximum in endophyte treated plants compared to treated, pathogen treated and untreated control. At the end of 11th day after inoculation, among the treatments, T_1 (2.12 µg/100g of fresh tissue) recorded highest total sugar production followed by T_3 (1.94 µg/100g of fresh tissue) and least sugar (1.06 µg/100g of fresh tissue) was produced in T_2 (only pathogen inoculation). In untreated control sugar production was more compared to pathogen inoculation alone.

Table 1: The activity of phenylalanine ammonia-lyase enzyme following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	Phenylalanine ammonia-lyase enzyme (µmol of transcinnamic acid/µg protein/h)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	40.54	36.47	40.80	41.59	43.51	40.83	40.68
2.	T_2 (only pathogen)	35.39	34.39	38.62	39.32	41.52	39.65	38.23
3.	T_3 (only endophytes)	27.20	21.37	23.62	25.54	26.41	22.79	24.65
4.	T_4 (neither endophyte nor pathogen)	21.11	20.82	20.42	20.69	20.12	20.07	20.54
Mean B		31.06	28.26	30.86	31.78	32.89	30.83	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.15		0.18		0.36		
C.D. at 1%		0.43		0.53		1.05		

Table 2: The activity of peroxidase enzyme following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	Peroxidase activity (change in absorbance per minute/g of leaf tissue)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	0.58	0.48	0.64	0.79	0.84	0.91	0.71
2.	T_2 (only pathogen)	0.46	0.45	0.56	0.45	0.61	0.76	0.55
3.	T_3 (only endophytes)	0.41	0.38	0.45	0.50	0.63	0.64	0.50
4.	T_4 (neither endophyte nor pathogen)	0.38	0.35	0.28	0.22	0.19	0.19	0.34
Mean B		0.45	0.41	0.50	0.52	0.60	0.65	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.01		0.01		0.33		
C.D. at 1%		0.03		0.04		0.09		

Table 3: The activity of polyphenol oxidase enzyme following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	polyphenol oxidase (change in absorbance per minute/g of leaf tissue)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	0.41	0.32	0.38	0.44	0.56	0.63	0.45
2.	T_2 (only pathogen)	0.36	0.25	0.31	0.34	0.44	0.59	0.38
3.	T_3 (only endophytes)	0.40	0.24	0.30	0.35	0.38	0.45	0.35
4.	T_4 (neither endophyte nor pathogen)	0.25	0.19	0.23	0.31	0.32	0.28	0.27
Mean B		0.36	0.25	0.30	0.36	0.43	0.49	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.01		0.01		0.03		
C.D. at 1%		0.03		0.04		0.08		

Table 4: Production of total phenol following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	Total phenols (µg /100g of fresh tissue)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	108.17	121.17	146.41	162.18	192.52	225.15	159.26
2.	T_2 (only pathogen)	118.51	123.86	128.38	131.44	143.36	145.57	131.85
3.	T_3 (only endophytes)	105.36	112.39	131.51	148.48	165.10	205.55	144.73
4.	T_4 (neither endophyte nor pathogen)	112.83	126.58	133.40	139.39	144.50	147.40	134.02
Mean B		111.22	121.00	134.93	145.37	161.37	180.92	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.16		0.20		0.41		
C.D. at 1%		0.47		0.58		1.16		

Table 5: Production of total protein following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	Protein (µg /g of fresh tissue)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	1.95	2.45	2.69	3.24	3.56	3.91	2.97
2.	T_2 (only pathogen)	1.76	1.74	1.66	1.45	1.27	0.92	1.47
3.	T_3 (only endophytes)	1.67	1.77	1.81	1.97	2.22	3.20	2.11
4.	T_4 (neither endophyte nor pathogen)	1.96	1.92	1.94	1.95	1.98	2.01	1.96
Mean B		1.83	1.97	2.02	2.15	2.25	2.51	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.04		0.05		0.10		
C.D. at 1%		0.11		0.14		0.29		

Table 6: Production of total sugar following colonization of *B. velezensis* in betel vine

SL. No.	Treatment details	Protein (µg /g of fresh tissue)						Mean A
		1st day	3rd day	5th day	7th day	9th day	11th day	
1.	T_1 (pathogen inoculation after one week of endophyte application)	0.72	0.98	1.12	1.44	1.55	2.12	1.32
2.	T_2 (only pathogen)	0.80	1.11	1.34	1.28	1.17	1.06	1.12
3.	T_3 (only endophytes)	0.65	0.73	0.98	1.21	1.35	1.94	1.14
4.	T_4 (neither endophyte nor pathogen)	0.78	0.80	0.83	0.81	0.85	0.88	0.82
Mean B		0.73	0.9	1.06	1.18	1.23	1.5	
		Between the treatments		Between the time		Interaction		
SE(m) ±		0.01		0.01		0.02		
C.D. at 1%		0.03		0.03		0.06		

Effect of endophyte *B. velezensis* on PDI and plant growth parameters

The data on the effect of different treatments on Per cent Disease Index and growth parameters at 15th and 30th day after treatment imposition were presented in the table 7. Overall results showed significantly increased plant growth parameters and decreased the Per cent Disease Index when compared with control. Among the different treatments, T_3 (*B. velezensis* only) showed reduced per cent disease index on 30th day (10.42) followed by T_1 (pathogen inoculation after one week of endophyte application) showed 11.53 PDI as compared to treated and treated control. The effect of endophyte *B. velezensis* on plant growth parameters revealed that there was significant difference among treatments with respect to plant height, number of leaves. Among the different treatments, T_3 (only endophyte) showed maximum plant height (110 cm) and number of leaves (52) on 30th day followed by T_1 (pathogen inoculation after one week of endophyte application) recorded the plant height (100 cm) and number of leaves (45) as compared to treated and untreated control.

Table 7: Effect of endophyte *B. velezensis* on PDI and growth in betel vine

SL. No.	Treatment details	PDI		Plant height (cm)			Number of leaves		
		15st day	30th day	Before spray	15st day	30th day	Before spray	15st day	30th day
1.	T_1 (pathogen inoculation after one week of endophyte application)	12.31	11.53	35.00	65.00	100.00	20.00	30.00	45.00
2.	T_2 (only pathogen)	19.58	25.34	45.00	72.00	94.00	15.00	24.00	33.00
3.	T_3 (only endophytes)	11.15	10.42	38.00	69.00	110.00	21.00	33.00	52.00
4.	T_4 (neither endophyte nor pathogen)	15.19	22.62	30.00	60.00	90.00	17.00	27.00	39.00
	SE(m) ±	0.47	0.38	0.88	0.71	0.97	0.70	0.89	0.66
	C.D. at 5%	1.43	1.18	2.66	2.16	2.95	2.13	2.72	2.03

Discussion

Defense enzymes, produced as a natural response to presence or infection by biotic or abiotic stimulators, have conventionally been thought to be advantages as it forms mechanisms to disease resistance. These enzymes are typically studied for their roles in the production of antimicrobial compounds (quinone molecules) and the reinforcement of cell walls (lignification) to prevent spread of pathogens (Naveen *et al*. 2013). In general, the activity of the enzymes PAL, PO and PPO was higher in endophyte pretreated and pathogen inoculated plants followed by only endophyte treated plants as compared to the corresponding treated and untreated control.

Phenylalanine Ammonia Lyase is the key enzyme in the plant phenyl propanoid pathway catalyzing synthesis of secondary metabolites from L-phenylalanine including lignin, flavanoid and phytoalexins. PAL has been extensively studied because of its role in plant development and its response to wide environmental stimuli. The importance of this enzyme in plant metabolism is demonstrated by the huge diversity and large quantities of products found in plant materials. Among four treatments, *B. velezensis* + pathogen showed more PAL activity against *X. a.* PV. *Betlicola* followed by only endophyte treatment as compared to treated and untreated control. The PAL activity in all treatment was initially high then decreased on 3rdday and reached maximum at 11th day as compared to untreated control. These results are in conformity with the previous work of Li *et al*. (2012) [9] as they revealed that plants inoculated with *Bacillus amyloliquefaciens* and *Xanthomonas axonopodis* PV. *Dieffenbachiae* induced the plants to synthesize Phenylalanine Ammonia Lyase (PAL) and the activity reached the maximum level on eighth day after inoculation.

Peroxidase is an important enzyme system found in plants and plays a measurable role in the synthesis of lignin. It is known to catalyze the oxidation of mono and di-phenols and aromatic amines to the highly toxic quinones in the presence of hydrogen oxides (Bonner, 1950) [1]. The enzyme has been reported to be toxic to several microorganisms. In the present findings the increase in peroxidase activity was more in endophyte + pathogen inoculated plants. Among four treatments, *B. velezensis* + pathogen showed more PO activity as compared to control. However, increased peroxidase activity was observed at first day then decreased on 3rdday and reached maximum at 11th day as compared to untreated control. Similarly Dalal *et al*. (2015) [3] reported that the endophytes and pathogen inoculated treatment significantly increased the levels of defense compounds and enzymes in soybean plants namely phenols, chitinase, peroxidase (PO), 3-glucanase, polyphenoloxidase (PPO) and phenylalanine ammonia-lyase (PAL).

PPO is a copper containing enzyme and functions by transferring electrons from the phenols to a cytochrome rather than directly to atmospheric oxygen. PPO oxidizes phenolics to highly toxic quinones. The oxidation may be responsible for the color changes following infection or injury of plant tissues (Goodman *et al.,* 1967) [4]. In the present findings the increase in PPO activity was more in endophyte + pathogen inoculated plants. Among four treatments, *B. velezensis* + pathogen showed more PPO activity as compared to control. However, increased peroxidase activity was observed at first day then decreased on 3rdday and reached maximum at 11th day as compared to untreated control. Similar changes in PO, PPO and PAL activity as a consequence of endophyte + pathogen infection was reported by other investigators Karthikeyan *et al*. (2005) [8] and Ting *et al*. (2009) [16].

It has long been recognized that responses are characterized against pathogen invasion by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death. Among four treatments, *B. velezensis* + pathogen showed more phenol content as compared to untreated control followed by only pathogen application (positive control. Phenol content increased day by day, but maximum phenol content was observed on 11th day. These results are in conformity with work done by Mishra *et al*. (2018) [12] showed that the level of total phenolics was higher in the plants treated with the two endophytes (109.65 mmol gallic acid g^{-1} fresh weight) at 48 hapi under pathogenic (*A. alternata*) stress conditions in *Withania somnifera* (L.).

Plant proteins play various enzymatic, structural and functional roles (Photosynthesis, biosynthesis, Transport, immunity). Among four treatments, mean protein was significantly higher in T_1 (*B. velezensis* + pathogen) followed by T_2 (*B. velezensis* alone). Lesser mean protein content was observed in pathogen treated plants as compared to untreated control. High level of sugars in plant tissues enhances the plant immune response against pathogens. Among the treatments, the plants treated with *B. velezensis* + pathogen showed highest total sugar content followed by *B. velezensis* alone treated plants. Total sugar content was maximum in the T_1 on 11th day followed by T_2 compared to treated and untreated control. Jaroszuk *et al*., (2020) [7] when the bioagents were mixed together or combined with antioxidants, resulting in substantial increases chemical components (protein, sugar, and chlorophyll) compared to control treatments.

Effect of endophyte *B. velezensis* on PDI and plant growth parameters

Among the different treatments, T_3 (only endophyte) showed reduced per cent disease index (84.39%) and increased plant height (110 cm), number of leaves (52) on 30th day (10.42) as compared to treated and untreated control. Effect of mixing the bioagents with each other or in combination with antioxidants led to significant decrease in percentages of disease incidence with considerable increase to the fruit yield compared with control treatment expressed by Jaroszuk *et al*., (2020) [7] while working with strawberry.

Conclusion

The induction of host defense enzymes and accumulation of molecular compounds in plants treated with endophytes under biotic stress confers resistance. The result thus highlights the pivotal role of endophyte *Bacillus velezensis* to alleviate stress condition caused by phytopathogens in the tested crops. Overall, this study enlightens novel insights into the molecular mechanisms involved in the endophytes induced resistance against *Xanthomonas axonopodis* PV. *Betlicola* infection in host.

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