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Modulation of Antioxidant Properties in Leaves of Betel Vine (*Piper betel* L.) Influenced by the Fungicide Blitox

Poulami Sil [a], Arunava Chakravarty [b], Shampa Purkaystha [a*], Punnam Chettri [a], Goutam Kumar Dash [c] and Shuvadeep Halder [d*]

[a] Centurion University of Technology and Management, Odisha 761211, India.
[b] Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia-741252, West Bengal, India.
[c] Department of Biochemistry and Crop Physiology, MS Swaminathan School of Agriculture, Centurion University of Technology and Management, Odisha 761211, India.
[d] Department of Horticulture and Food Science, School of Agriculture and Allied Sciences, The Neotia University, Diamond Harbour, S 24 Pargana-743368, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The experiment was conducted using a Randomized Block Design with three replications at the Plant Virus Research Station, BCKV, Kalyani, in Boroj. On betel vines, Blitox was sprayed at both double the recommended field dose (dRFD) and the recommended field dose (RFD) (50 WP/ha @ 15gm ai/6 lit). The vines were sprayed five more times at 15-day intervals beginning when they

**Corresponding author: E-mail: shuvadeep.halder@tnu.in, shampa.purkaystha@cutm.ac.in;*

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were 11.08.15 days old. Before every spray, leaf samples were taken and subjected to a number of criteria for analysis. Based on statistical analysis, the anti-oxidative characteristics improved most significantly when Blitox was administered at twice the recommended field dose (dRFD) (Blitox 50WP/ha @ 30gm ai/6 lit). Higher levels of free and total phenols, together with enhanced activity of enzymes such polyphenol peroxidase (PPO), superoxide dismutase (SOD), and peroxidase (POD), were indicators of this. Furthermore, testing for ferric reducing antioxidant power (FRAP) and diphenyl picryl hydrazyl (DPPH) revealed increased activity levels in the dRFD therapy in comparison to the RFD and control groups. Overall, these results imply that the larger Blitox dosage strengthens the antioxidant characteristics of betel vine leaves, hence enhancing their health and resistance to fungus-related infections.

Keywords: Betel Vine; blitox; antioxidant; fungicide; perennial crop; crop protection.

1. INTRODUCTION

Betel vine is renowned as the "Green Gold of India" [1]. West Bengal cultivates betel vine on 18,203 hectares of land, out of a national total of 55000ha [2]. West Bengal accounts for around 66% of total betel leaf output. In West Bengal, betel vines are mostly cultivated in conservatories called as Boraj and can be any length, rectangular or square form, with a height of 2m. It is a perennial crop with a height of 2-4 metres. Betelvine leaves are chewed or consumed as betelquidand widely used in Chinese and Indian folk medicine, as carminative, stimulant, astringent, against parasitic worms, conjunctivitis, rheumatism, wound, etc., and is also used for religious purposes (Biswas et al. 2022). The difficulty now is that a number of fungi are limiting its production, with losses ranging from 30 to 100% depending on the fungus species, plant variety, and climatic conditions (Biswas et al. 2022). Betel vines are commonly attacked by fungi such as *Phytopthera parasitica* and *Colletotrichum capsici*, which cause leaf root and stem foot rot. Blitox, a copper-based fungicide, works by disrupting the cell walls of fungal pathogens, preventing their growth and spread. Blitox can be applied as a foliar spray or soil drench [3-5]. When applied, the copper ions in Blitox are absorbed by fungal spores during germination, interfering with their enzymatic processes and inhibiting their growth. This dual-action approach ensures comprehensive disease control across various crops (Singh et al. 2000). Thus, the use of fungicides such as Blitox in betel production has been suggested by the AICRP on medicinal plants and betel vines [6-11].

Blitox, a member of the copper oxycloride family, interferes with photosynthesis by destroying biosynthesis machinery and modifying the pigment and protein composition of the photosynthetic membrane [12]. Maksymice et al. [13] attribute the reduction in chlorophyll content to a copper-induced iron deficiency. On the other hand, the survival of this chemical in plants is mostly due to its metabolism via enzyme-mediated reactions that include oxidation, reduction, and conjugation with other plant elements [14-17]. Fungicides, when employed as a crop protection tool, leave hazardous residues in the harvested product, affecting consumer health. As a result, the use of these fungiicides is governed by severe national international legislation, which generate residue data that is used to determine the maximum residue limit of a given chemical on a certain crop [18-22]. Fungicides, in addition to their known effect, have been demonstrated to modify or impede physiological or metabolic activity in plants. For example, triforins (saprol) substantially impede chloroplast electron transport reactions (Robinson et al. 1977). In certain situations, the reason of the fungicide's phytotoxicity was shown to be a specific product coming from the fungicide's breakdown following penetration of the host cell and integration into cell metabolism [23,24]. Thus, dybutyl urea, a result of benonyl breakdown, may be particularly responsible for the phytotoxicity. Thus, dybutyl urea, a byproduct of benonyl breakdown, may be primarily responsible for the phytotoxicity of the fungicide benlate (Kara et al. 2020). Furthermore, fungicides, due to their substantial impact on phenol and carbohydrate metabolism enzymes, can modify the concentration of these phytochemicals. Fungicides, on the other hand, impose oxidative stress, which can modulate the activity of many enzymes [25-27].

With these background information, the current study attempted to determine the effect of fungicide Blitox, applied at recommended and double recommended doses, on the change in

levels of Chlorophyll, Ascorbic acid, Phenol (free and total phenol), and antioxidative enzyme activities such as Polyphenol oxidase (PPO), Phenol oxidase (POD), and Super oxide dismutase (SOD), as well as lipid peroxidation. Furthermore, the antioxidant activity of total phenol extract in betel leaves was investigated using two distinct assay systems: DPPH and FRAP, as well as the impact of fungicide. Finally, statistical analysis was performed on the chemical data collected during this investigation.

2. MATERIALS AND METHODS

In order to study the influence of fungicide viz. blitox when applied at their recommended field dose (RFD) and double the recommended field dose (dRFD) on the change of nonenzymatic antioxidant levels namely chlorophyll, ascorbic acid, copper content, free phenol, total phenol and activities of antioxidative enzymes like polyphenol peroxidase (PPO), phenol oxidase (POD), and superoxide dismutase (SOD), along with lipid peroxidation as well as radical scavenging ac In this experiment, three types of samples were gathered, such as: Control (T0)- No fungicide treatment (T1):- Blitox 50 WP/ha at 15gm ai/6 lit; Treatment (T2): 50 WP/ha at 30gm ai/6 lit.

2.1 Sample Preparation

First, 100g of betel leaf samples were obtained from each replicated plot before to fungicide application, and then 100g of betel leaf samples were collected from each treatment replication at intervals of 15, 30, 45, 60, and 75 days before spraying. On 11.08.15, 25.08.15, 10.09.15, 25.09.15, 10.10.15, and 25.10.15, various doses of Blitox (RFD and dRFD) were sprayed onto betel leaves. A portion of the leaf sample was immediately used without drying for biochemical analysis (like polyphenol peroxidase (PPO), phenol oxidase (POD), and superoxide dismutase (SOD) along with lipid peroxidation) to see the effect of natural conditions. The rest was oven dried at 40ºC until constant weight and ground using an electric grinder and kept in the poly pack for the biochemical analysis (total phenol, free phenol content). Leaf samples were collected and crushed in a mortar and pestle with appropriate reagents for the analysis. In the case of dry samples, the leaves were oven dried and pulverised in a mortar and pestle with the appropriate reagents for the assay.

2.2 Physiological Analysis

2.2.1 Estimation of total moisture content

The moisture content of the leaf sample was determined using the gravimetric technique. The leaves (10 g) were dried at 40ºC in a hot air oven. The sample's weight after drying was recorded. The moisture percentage was estimated using the following formula: moisture percentage= (wet weight of the samples-dry weight of the samples)/wet weight X 100.

2.2.2 Estimation of chlorophyll

The chlorophyll content of the leaf sample was calculated using Arnon's method (1949). 100 mg of fresh leaf sample was finely ground. The tube was then filled with 10ml of 80% acetone and left at room temperature for 7 days under dark conditions. The green liquid was then collected in a separate test tube, and the colourless leaf particles were discarded. The absorbance was measured at 645 and 663nm wavelengths in a Systronics-105 spectrophotometer against a blank containing only 80% acetone.

2.2.3 Estimation of copper

A 0.1g leaf sample (betel leaf) was obtained. Then, 20 ml of DPTA extract was added and agitated for 2 hours on a mechanical shaker. The leaf sample was filtered via Whatman No.42 filter paper and used to quantify copper using AAS.

2.3 Chemical Analysis

2.3.1 Ascorbic acid content

Ascorbic acid was measured using the 2, 6-dichlorophenol indophenol (DCPIP) titration technique described by Casanas et al. [28]. One gramme of fresh leaf tissue was crushed with 20 millilitres of 4% oxalic acid. The crushed material was centrifuged at 10,000 rpm for 30 minutes. A 10 ml aliquot of the sample was mixed with 10 ml of 4% oxalic acid and titrated against 2,6-dichlorophenol indophenol (DCPIP) dye (V2) until a light pink hue appeared and remained for a few minutes. Another 5 ml of 100 ppm ascorbic acid solution and 10 ml of 4% oxalic acid were prepared and titrated against DCPIP dye (V1). The ascorbic acid concentration was calculated as mg per 100g.

2.3.2 Phenol content (Total Phenol and Free Phenol)

The phenol content of Betel leaf was measured using the Folin-Ciocalteau Reagent (FCR) (Vinson et al., 1998). To evaluate the unconjugated ('free') phenol in Betel leaf, a dry sample (0.1g) was extracted with 15 ml of 50% aqueous methanol and heated at 90ºC with occasional shaking for 2 hours. A 0.1g weighed sample was extracted with 15 ml of 1.2 N HCl in 50% aqueous methanol and heated at 90º C for 2 hours to determine the conjugated and unconjugated ('total') phenol. The extracted material was centrifuged at 10,000 rpm for 30 minutes. The supernatant was decanted into a beaker and evaporated until dry. The crude extract was diluted to 25 ml using 1.2 N HCl in 50% aqueous methanol. To estimate phenol, a sufficient portion was diluted to 3 ml with distilled water and 0.5 ml FCR was added. After three minutes, two millilitres of 10% sodium carbonate were added. The samples were then warmed for 5 minutes in a water bath. The absorbance was measured at 650 nm, and the phenol concentration was calculated using a reference curve generated from gallic acid. The phenol content is reported in mg gallic acid equivalent per gramme dry weight (GAE/g DW).

2.3.3 1-diphenyl-2-pycrylhydrazyl (DPPH) assay

The DPPH radical scavenging activity of the extract was measured by measuring the reduction in absorbance of a methanolic DPPH solution at 517 nm [29]. A 3 ml reaction mixture with 150 µl of sample aliquot and 2850 µl of 0.004% DPPH was stored in tubes at room temperature for 30 minutes in the dark. The absorbance of the reaction mixture was measured at 517 nm against a blank. To create a blank sample, 150 µl of water was combined with 2850 µl of DPPH solution. A standard curve was created by graphing the change in absorbance against different amounts of Trolox. The antioxidant activity of DPPH radical is measured in mg trolox equivalent per gramme dry weight (mg TE/g DW).

2.3.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP, which is based on the reduction of Fe (III) by the TP extract, was calculated using the technique published by (Benzie and Strain 1996). In the FRAP experiment, the change in absorbance at 593 nm caused by the production of a blue-colored Fe(II)-tripyridyltriazine molecule from a colourless oxidised Fe(III) form in the presence of a certain concentration of total phenol extracts is preserved. The FRAP reagent was prepared by combining 0.1 M acetate buffer (pH 3.6), 10 mM 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ), and 20 mM ferric chloride (10:1:1, v/v/v). The reaction mixture (150µl neutralised sample extract and 2850µl FRAP reagent) was maintained at room temperature for 30 minutes. The absorbance of the solution was measured at 593 nm. The standard curve was created using trolox and results were expressed as mg trolox equivalent per gram dry weight (mg TE/g DW).

2.4 Enzyme Analysis

2.4.1 Polyphenol oxidase (PPO) activity

The activity of polyphenol oxidase was determined using the Mayer et al., 1979 technique. The leaves were extracted by grinding 1gm fresh tissue in 10ml of 0.1 M Sodium Phosphate buffer, pH 7.5, adding 2% PVP (polyvinylpyrrolidone) and 0.25% Triton-X. The extracted material was centrifuged at 10,000 rpm for 30 minutes at 4ºC, and the supernatant served as the enzyme source. PPO activity was determined by combining 0.05 ml of cooled enzyme extract with 2 ml of phosphate buffer (0.1 M), pH 7.5, and 0.5 ml of catechol (0.01 M). The activity was detected at 410 nm. Enzyme activity were given as µmol catechol oxidized/g/min.

2.4.2 Peroxidase (POD) activity

Peroxidase was calculated using Shannon et al.'s 1966 technique. The experiment was performed by grinding 0.1g of fresh leaf tissue with 10ml of 0.1 M Sodium Phosphate buffer, pH 7.5, containing 2% PVP (polyvinyl pyrrolidone) and 0.25% Triton-X. The extracted material was centrifuged at 10,000 rpm for 30 minutes at 4ºC, and the supernatant served as the enzyme source. Peroxidase was measured by combining 0.05 ml cooled enzyme extract with 2.8 ml reaction mixture (4% Guaiacol dissolved in methanol - 0.15 ml and 2.65 ml sodium potassium buffer (0.1M) pH 7.5). The reaction was started by adding 0.15ml of H2O2 (1%). The activity was measured at 470 nm. Enzyme activity was measured as µmol guaiacol oxidized/g/min.

2.4.3 Superoxide dismutase (SOD) activity

The experiment was based on the extract's ability to block photochemical reduction of nitro-blue-tetrazolium (NBT) in the riboflavin-light NBT system (Beauchamp and Fridovich, 1971). The extraction experiment involved grinding 1g of fresh bulb tissue with 10 ml of 0.05 M phosphate buffer (pH 7.8). The extracted material was centrifuged at 10,000 rpm for 30 minutes, and the supernatant was employed as an enzyme source. Each 6 ml reaction mixture included 4.0-4.4 ml of 0.05 M phosphate buffer (at pH 7.8), 0.4 ml of 20 µM methionine, 0.4 ml of 1.12 µM NBT, 0.4 ml of 1.5 µM EDTA, 0.1-0.4 ml of enzyme extract, and 0.4 ml of 75 µM riboflavin. The response was initiated by turning on the light and left to run for ten minutes. After ten minutes, the response was halted by turning off the light. The absorbance of the reaction mixture was measured at 560 nm. The superoxide dismutase (SOD) activity was determined using the following equation: SOD activity (% inhibition) = [(A0 - Ae)/A0] X 100, where Ae is sample absorbance and A0 is control absorbance. The percentage inhibition was plotted versus the concentration of the enzyme extract. The graph was used to calculate the value of the enzyme extract that produced 50% inhibition (IC50).

2.4.4 Lipid peroxidation

Dhindsa et al. (1982) defined lipid peroxidation as the quantity of malondialdehyde (MDA) generated by the thiobarbituric acid (TBA) reaction. 1g of fresh leaf tissue was crushed with 10 mL of 20% trichloroacetic acid (TCA). The triturated material was centrifuged at 10,000 rpm for 15 minutes. Mix 1ml of sample aliquot, 3ml of 20% TCA with 0.5% TBA, and 0.2ml of 4% butylated hydroxytoluene (BHT) well before incubating at 95º C for 30 minutes. The mixture was then cooled to ambient temperature and centrifuged at 10,000 rpm for 15 minutes. The supernatant's absorbance at 532 nm was measured.

2.5 Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics software, version X.X (IBM Corp., Armonk, NY, USA). Initially, descriptive statistics were used to summarise the research sample's demographic characteristics. Continuous data were reported as means ± SD, while categorical variables were given as frequencies and percentages. Furthermore, the Spearman's rank correlation coefficient was calculated to investigate the relationship between continuous variables. A two-tailed p-value of less than 0.05 was used to determine statistical significance.

3. RESULTS

3.1 Physiological Analysis

3.1.1 Moisture content

Table 1 depicts the moisture content of the Betel vine leaves. The greatest moisture percentage (81.30%) was discovered on dRFD (45th DAFS). On the other side, the lowest moisture percentage (75.57%) was found in dRFD (30th DAFS). In the instance of RFD, the 15th DAFS had the greatest moisture content (81%), while the 30th DAFS had the lowest (76.51%).

3.1.2 Chlorophyll content in betel vine leaf

Table 2 revealed that fungicide spraying had a modest effect on chlorophyll concentration.

3.1.3 Copper (Blitox) content

Copper content increased gradually, although not always significantly. Fungicide (Blitox) caused a gradual rise in the amount of copper in betel leaf, albeit this was not always significant over the growth period from 0 to 75th DAFS (Table 3). The highest copper level (0.010 mg/g) was detected in dRFD at the 75th DAFS. On the other hand, the lowest copper level (0.002 mg/g) was recorded in Control at 0 DAFS. Fungicide (dRFD) significantly increased the level of copper in Betel Vine leaf, followed by RFD and control. The findings revealed that dRFD produced the highest amount of copper content in Betel Vine leaves. This might be because a larger dosage of copper-containing fungicide was sprayed.

3.2 Antioxidant Activity Analysis

3.2.1 Ascorbic acid (Vitamin- C)

The amount of ascorbic acid in the betel vine leaf increased gradually, if not always significantly, from 0 to 30 DAFS after the initial spraying. Then there was a decline at the 45th DAFS. It then

Table 1. Moisture content in Betel leaves

	Moisture Percent (%)		
	T0 (Control)	**T1 (Single Dose)**	**T2 (Double Dose)**
S1(0 DAFS)	81.23	79.40	81.20
S2(15 DAFS)	80.50	81.00	79.90
S3(30 DAFS)	76.07	76.57	75.57
S4(45 DAFS)	80.33	80.87	81.30
S5(60 DAFS)	80.35	80.66	81.23
S6(75 DAFS)	79.12	80.22	80.21

Table 2. Effect of fungicide(Blitox) on the level of Chlorophyll(mg/g) of fresh tissue in the leaf of betel vine

	T0 (Control)	**T1 (Single Dose)**	**T2 (Double Dose)**	**Mean**
S1(0 DAFS)	0.004	0.005	0.005	0.005
S2(15 DAFS)	0.005	0.005	0.005	0.005
S3(30 DAFS)	0.004	0.005	0.004	0.004
S4(45 DAFS)	0.003	0.006	0.005	0.005
S5(60 DAFS)	0.005	0.006	0.004	0.005
S6(75 DAFS)	0.004	0.005	0.005	0.005
Mean	0.004	0.005	0.005	

Factors	**C.D.**
Spray(S)	N/A
Treatment(T)	0.000
Spray X Treatment	0.001

Table 3. Effect of fungicide (Blitox) on the level of COPPER (mg/g)residue in the leaf of betel vine

	T0 (Control)	**T1 (Single Dose)**	**T2 (Double Dose)**	**Mean**
S1(0 DAFS)	0.002	0.004	0.006	0.004
S2(15 DAFS)	0.004	0.005	0.007	0.005
S3(30 DAFS)	0.004	0.008	0.009	0.007
S4(45 DAFS)	0.005	0.007	0.009	0.007
S5(60 DAFS)	0.004	0.008	0.014	0.009
S6(75 DAFS)	0.005	0.009	0.016	0.01
Mean	0.004	0.007	0.01	

Factors	**C.D.**
Spray(S)	0.001
Treatment(T)	0.001
Spray X Treatment	0.002

climbs again to the 75th DAFS, regardless of therapy. The greatest ascorbic acid content (0.042 mg/100g) was discovered in Control at 0 DAFS (Table 4). The lowest ascorbic acid level (0.010 mg/100g) was found in RFD at the 45th DAFS.

3.2.2 Total phenol and free phenol content

Tables 5-6 summarises the effect of fungicide (Blitox) treatment after RFD and dRFD application on the change in free and total phenol during the experiment. Blitox, an inorganic fungicide, when applied at RFD and dRFD dosages, increased the accumulation of free and total phenol in betel vine leaf compared to the control (Tables 5-6). However, the impact of fungicides was stronger with dRFD, and the sixth spray (75 DAFS) was shown to be effective in the buildup of free and total phenols in leaves. When the interaction impact of treatment and spray was evaluated, there was a significant

difference in leaf. Free phenol concentration was raised, as was total phenol content in the leaf, to achieve dRFS >RFD>Control (Tables 5,6). The maximum total phenol concentration (9.938 mg GAE/g D.W.) and free phenol content (8.787 mg GAE/g D.W.) were found in dRFD at the 75th

Table 4. Effect of fungicide (blitox) on the level of ascorbic acid (mg/100gm) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.042	0.03	0.037	0.036
S2(15 DAFS)	0.024	0.025	0.024	0.024
S3(30 DAFS)	0.022	0.012	0.015	0.016
S4(45 DAFS)	0.017	0.01	0.015	0.014
S5(60 DAFS)	0.019	0.011	0.016	0.015
S6(75 DAFS)	0.019	0.018	0.017	0.018
Mean	0.024	0.018	0.021	

Factors	C.D.
Spray(S)	0.004
Treatment(T)	0.003
Spray X Treatment	N/A

Table 5. Effect of fungicide (Blitox)on the level of FREE PHENOL(mg GAE/g D.W) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	6.645	7.235	7.852	7.244
S2(15 DAFS)	6.637	7.557	7.955	7.383
S3(30 DAFS)	6.657	7.848	8.116	7.54
S4(45 DAFS)	7.047	7.976	8.342	7.788
S5(60 DAFS)	7.253	8.047	8.785	8.028
S6(75 DAFS)	7.447	7.947	8.787	8.06
Mean	6.947	7.768	8.306	

Factors	C.D.
Spray(S)	0.004
Treatment(T)	0.003
Spray X Treatment	0.006

Table 6. Effect of fungicide(Blitox)on the level of TOTAL PHENOL(mg GAE/g D.W) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	7.024	7.657	8.996	7.892
S2(15 DAFS)	7.038	7.944	8.921	7.968
S3(30 DAFS)	7.647	8.905	9.144	8.565
S4(45 DAFS)	7.847	8.529	9.938	8.771
S5(60 DAFS)	7.887	8.685	9.821	8.797
S6(75 DAFS)	7.954	8.808	9.745	8.836
Mean	7.566	8.421	9.427	

Factors	C.D.
Spray(S)	0.063
Treatment(T)	0.045
Spray X Treatment	0.110

Table 7. Effect of fungicide(Blitox)on DPPH(mg TE/g) radical scavenging activity in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.084	0.094	0.193	0.124
S2(15 DAFS)	0.101	0.206	0.237	0.182
S3(30 DAFS)	0.111	0.231	0.351	0.231
S4(45 DAFS)	0.112	0.361	0.399	0.291
S5(60 DAFS)	0.11	0.408	0.435	0.318
S6(75 DAFS)	0.121	0.451	0.499	0.357
Mean	0.107	0.292	0.352	

Factors	C.D.
Spray(S)	0.002
Treatment(T)	0.001
Spray X Treatment	0.003

DAFS. Untreated leaves had the lowest total phenol concentration (7.024 mg GAE/g D.W.) and free phenol content (6.637 mg GAE/g D.W.) at 0 DAFS and 15 DAFS percentages, respectively.

3.2.3 Antioxidant activity of leaves of betel vine measured by DPPH

DPPH activity was highest (0.499 mg TE/g) in dRFD at 75th DAFS and lowest (0.084 mg TE/g) in untreated at 0 DAFS. Regardless of treatment, DPPH activity in betel vine leaves increased gradually over time. In terms of spray, the sixth spray was shown to be the most effective in increasing DPPH activity in the leaf. The treatments differed significantly in terms of DPPH activity. dRFD produced the maximum DPPH activity in betel vine leaves, followed by RFD and Control. There was a direct correlation between phenolic chemicals and antioxidant activity (Kubade et al., 2021). A similar connection was seen in our study (Table 7).

3.2.4 Antioxidant activity of leaves of betel vine measured by FRAP

FRAP activity gradually increased in betel vine leaves from 0 to 75th DAFS, regardless of treatment (Table 8). Treatment dosages showed a substantial impact. The highest activity (1.347 mg TE/g D.W.) and lowest activity (0.706 mg TE/g D.W.) were seen in betel vine leaf in terms of dRFD at 75th DAFS and RFD at 0 DAFS, respectively. The highest FRAP activity was seen in the dRFD therapy and the lowest in the control group. In the current investigation, there was a link between phenolic levels and their contribution to FRAP activity (0.834 significant at 0.01%).

Table 8. Effect of fungicide (Blitox)on FRAP (mgTE/g) radical scavenging activity in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.709	0.826	0.995	0.843
S2(15 DAFS)	0.777	0.977	1.107	0.953
S3(30 DAFS)	0.823	1	1.157	0.993
S4(45 DAFS)	0.822	1.147	1.207	1.058
S5(60 DAFS)	0.832	1.217	1.307	1.118
S6(75 DAFS)	0.836	1.267	1.347	1.15
Mean	0.8	1.072	1.186	

Factors	C.D.
Spray(S)	0.013
Treatment(T)	0.009
Spray X Treatment	0.022

Table 9. Effect of fungicide(Blitox) on POD (mM Guiacol oxidized/g/min) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.039	0.063	0.050	0.051
S2(15 DAFS)	0.042	0.068	0.103	0.071
S3(30 DAFS)	0.052	0.079	0.219	0.116
S4(45 DAFS)	0.052	0.088	0.328	0.156
S5(60 DAFS)	0.057	0.217	0.396	0.223
S6(75 DAFS)	0.042	0.228	0.774	0.348
Mean	0.047	0.124	0.311	

Factors	C.D.
Spray(S)	0.002
Treatment(T)	0.001
Spray X Treatment	0.003

Table 10. Effect of fungicide(Blitox)on PPO (mM catechol oxidized/g/min) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.102	0.179	0.127	0.136
S2(15 DAFS)	0.093	0.157	0.109	0.119
S3(30 DAFS)	0.091	0.115	0.096	0.100
S4(45 DAFS)	0.085	0.082	0.092	0.086
S5(60 DAFS)	0.077	0.074	0.080	0.077
S6(75 DAFS)	0.083	0.041	0.055	0.059
Mean	0.088	0.108	0.093	

Factors	C.D.
Spray(S)	0.002
Treatment(T)	0.001
Spray X Treatment	0.003

Table 11. Effect of fungicide(Blitox) on SOD(protein/ g/min) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	0.005	0.006	0.008	0.006
S2(15 DAFS)	0.006	0.007	0.009	0.007
S3(30 DAFS)	0.007	0.014	0.015	0.012
S4(45 DAFS)	0.009	0.013	0.014	0.012
S5(60 DAFS)	0.009	0.011	0.013	0.011
S6(75 DAFS)	0.011	0.012	0.013	0.012
Mean	0.007	0.011	0.012	

Factors	C.D.
Spray(S)	0.001
Treatment(T)	0.000
Spray X Treatment	0.001

Table 12. Effect of fungicide (Blitox) on LIPID PEROXIDATION (mM/g) in the leaf of betel vine

	T0 (Control)	T1 (Single Dose)	T2 (Double Dose)	Mean
S1(0 DAFS)	16.087	12.957	2.107	10.383
S2(15 DAFS)	13.087	8.867	1.667	7.873
S3(30 DAFS)	9.667	4.827	1.033	5.176
S4(45 DAFS)	1.123	10.107	19.347	10.192
S5(60 DAFS)	1.913	16.767	20.833	13.171
S6(75 DAFS)	2.113	19.287	25.737	15.712
Mean	1.659	12.135	17.459	

Factors	C.D.
Spray(S)	0.178
Treatment(T)	0.126
Spray X Treatment	0.308

Table 13. Correlation matrix due to biochemical characteristics of betel vine leaf

	Chlorophyll	Ascorbic Acid	Copper	Lipid Peroxidation	Total Phenol	Free Phenol	POD	PPO	SOD	DPPH	FRAP
chlorophyll	1	-0.351^{NS}	0.107^{NS}	0.307^{NS}	0.262^{NS}	0.234^{NS}	0.194^{NS}	-0.301^{NS}	0.267^{NS}	0.332^{NS}	0.151^{NS}
Ascorbic Acid		1	-0.753^{NS}	-0.833*	-0.876*	-0.755^{NS}	-0.560^{NS}	0.799^{NS}	-0.909*	-0.818*	-0.082^{NS}
Copper			1	0.966**	0.915*	0.967**	0.948**	-0.980**	0.827*	0.969**	0.625^{NS}
Lipid Peroxidation				1	0.918**	0.979**	0.907*	-0.986**	0.823*	0.992**	0.598^{NS}
Total Phenol					1	0.922**	0.806^{NS}	-0.940**	0.955**	0.946**	*0.834
Free Phenol						1	0.924**	-0.974**	0.787^{NS}	0.984**	0.704^{NS}
POD							1	-0.943**	0.687^{NS}	0.924**	0.791^{NS}
PPO								1	-0.859*	-0.996**	-0.621^{NS}
SOD									1	0.854*	0.198^{NS}
DPPH										1	0.623^{NS}
FRAP											1

** significance at 0.01 %; ** significance at 0.05%*

3.3 Enzyme Activity Analysis

3.3.1 POD activity

Table 9 shows that POD activity rose gradually throughout the days from 0 DAFS to 75 DAFS. The 75th spraying had the highest POD activity (0.774 mM guiacol oxidized/g/min). On the other hand, 0 DAFS resulted in the lowest POD activity (0.059 mM guiacol oxidized/g/min).

3.3.2 PPO activity

Regardless of treatment, polyphenol oxisdase activity decreased progressively in betel vine leaves. The highest PPO activity (0.179 mM catechol oxidized/g/min) was seen in RFD at 0 DAFS. On the other hand, the lowest (0.041 mM catechol/oxidized/g/min) with RFD therapy at 75th DAFS (Table 10). Overall, RFD produced the maximum PPO activity in betel vine leaves, followed by dRFD and control.

3.3.3 SOD activity

The results shown in Table 11 for the change in SOD activity in betel vine leaf demonstrated a gradual rise, but not always significant, independent of dosages from 0-DAFS to 15th DAFS. The maximum remained very constant between the 45th and 75th DAFS. On the 30th DAFS, SOD activity was reported at twice prescribed levels, whereas the control (T0) had the lowest activity on the 0-DAFS.

3.3.4 Lipid peroxidation

Lipid peroxidation gradually reduced from 0 to 30 DAFS, followed by a steady rise in the betel vine leaf regardless of treatment (Table 12). The maximum lipid peroxidation (25.73 mM/g) was reported in dRFD on the 75th DAFS. On the 45th DAFS, the control group showed the lowest lipid peroxidation (1.033 mm/g).

4. DISCUSSION

4.1 Physiological Analysis

Jørgensen and Olesen [30] discovered that varied fungicide treatments had a small impact on wheat moisture content. The same phenomenon was observed in this study, which indicated that moisture content in betel vine leaves increased in both dRFD and RFD. In the case of Chlorophyll, minute alterations occurred. Because Bliotox is a copper-containing fungicide, it has been shown that the concentration of copper is greatest in the case of dRFD when compared to RFD or control. Copper in trace levels is needed for numerous metabolic activities in the plant, but at greater concentrations, it causes physiological stress through the creation of free radicals, including the synthesis of reactive oxygen species (ROS) [31].

4.2 Antioxidant Analysis

Ascorbic acid is a key component of the plant's antioxidant system. Untreated fungicide (Control) produced the greatest amount of ascorbic acid, followed by RFD and dRFD. There was no significant difference between RFD and dFRD in terms of ascorbic acid concentration in betel vine leaves. Ascorbic acid content increased minimally among treatments. According to Broadley et al. [32] there is a strong positive association between copper level in leaf tissue and ascorbate oxidase activity. The administration of dRFD increased the amount of copper, which increased the activity of ascorbate oxidase. As a result, the Control group had the lowest concentration of ascorbic acid in betel vine leaves.

The activity of different enzymes regulates phenol buildup. Because dRFD had the highest phenol content, it was suggested that the PAL enzyme was activated, as it is a key enzyme for the biosynthesis of phenols [33]. and there was a significant difference between treatment RFD and dRFD in terms of the influence of spray. According to Soobrattee et al. [34] phenolic compounds are significant plant elements with redox characteristics that contribute to antioxidant action. DPPH, a stable neutral process involving the removal of hydrogen atoms from phenolic compounds where oxygen is radical, acts as an antioxidant [34]. Thus, the capacity of phenolic extracts to neutralise the DPPH radical serves as an indicator of antioxidant activity. Thus, this mechanism should be powerful enough to initiate defence free radicals. There has always been a high association between DPPH and FRAP assays for possible reducing properties [35]. The current inquiry data showed that FRAP free radical scavenging capabilities were greater than those of DPPH, which did not support the findings of Rathee et al., 2006. The absolute value of antioxidant activity obtained from the FRAP experiment is larger than that of DPPH, indicating that the phenolic chemicals found in

betel vine leaves react primarily via single electron transfer (SET) rather than hydrogen atom transfer.

4.3 Enzyme Activity Analysis

Overall, dRFD produced the highest POD, PPO, and SOD activity in betel vine leaves, followed by the prescribed dosage and control. The activation of peroxidase activity shows fungicide tolerance (oxidative stress) and the level of stress to which the treated plant is subjected [36]. Table 13 shows a strong association between increased peroxidase activity and phenolic compound content. Under stress circumstances, phenolic compounds build in crop plants, and these harmful molecules are oxidised and degraded by the activity of polyphenol oxidases. These increases in per capita PPO activity may have resulted in phenolic oxidation and hence a loss in their regulation [37]. The current results differ from previous findings. The activity of antioxidant enzymes are typically enhanced when exposed to oxidative stress. When free radicals, such as superoxide anions, are formed in treated plants, the plant cells are harmed owing to the greater concentration, thus the plant stimulates the activities of free radical scavenging enzymes like SOD for self-defense [37]. Lipid peroxidation is connected to antioxidant enzyme activity, and when SOD levels increase, oxidative stress tolerance improves [38]. Furthermore, lipid peroxidation has been employed to assess oxidative stress in betel vines [39]. At 30 DAFS, the lowest lipid peroxidation and greatest SOD activity (Table 11) were seen at the 15th DAFS, suggesting that SOD protected the betel plant from oxidative stress at the start. After increased lipid peroxidation levels were reported without interfering with plant growth, additional antioxidants may have functioned [40].

5. CONCLUSION

Except for chlorophyll, the biochemical investigation found significant variance in all parameters studied in betel vine leaves. The highest concentration of antioxidant component, free and total phenol, was found in the dRFD therapy. In addition, the dRFD had the greatest activity of antioxidant enzymes such as POD, PPO, and SOD. Furthermore, dRFD had the highest free radical scavenging activity, as determined by the DPPH and FRAP assays. In contrast, the control had the highest levels of ascorbic acid. Based on the comparison of the aforementioned parameters, dRFD appears to be the most effective of the three treatments given to betel vine leaves in West Bengal's gangetic alluvial zone. It stimulates the plant's defence mechanisms.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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