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Pre-exogenous treatment of the JA-ABA duo enhances synergistic actions to combat Fusarium wilt-mediated biotic stress in *Piper betel* L.

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Abstract
Piper betel. L is a traditional, medicinal, and economical asset to mankind. Due to its enormous health benefits and delectable taste, it has been a part of heritage cultivation for thousands of years. Unfortunately, the cultivation frequency of this cash crop has declined in recent years due to the challenging setups and abiotic and biotic stresses. *Fusarium* wilt in betel vine caused by *Fusarium* sp. is one of the major causes of huge economic losses every year. This study was designed to observe the effects of pretreatment of the JA-ABA (T) pair on growth and defense in betel vine under pathogenic stress. The treatments, namely N, P, and T, were under observation for 21 days, followed by physiological, biochemical, and statistical analysis. It was observed that T showed a significant increase in shoot length, root length, biomass, and leaf count as compared to P. Biochemical analyses such as RWC, MDA, pigment content, H_2O_2 content, total ascorbate, and total glutathione content revealed the positive impact of the JA-ABA combination on plants even after infection. DPPH radical scavenging activity was found to be highest in leaf extracts of T after standard ascorbic acid. P showed significantly lower antioxidant activity than T and standard N. PCA, correlation matrix, AHC were performed to study the variability between treatment sets using XLSTAT-Student 2025.1.3.1431 software. It was observed that majority of the variance was due to PC1 (79.63%) which included most of the correlated physiological parameters. MDA, H_2O_2 and EL were found to be negatively correlated with others. The above experimental and statistical observations suggest that this combination can be an effective strategy to boost the immune tolerance in betel vine against fusarium wilt disease.

Keywords: Abscisic Acid, Betel vine, *Fusarium Sp.*, Jasmonic acid, Pathogenic stress.

Introduction
Betel vine (*Piper betel* L.) belongs to the Piperaceae family and is a dioecious evergreen perennial creeper. Millions of people in India and many other Asian countries rely on this natural crop as a direct or indirect source of income, and it has become a cash crop [1]. It has anti-inflammatory, anti-cancer, anti-apoptotic, antibacterial, and antioxidant properties [2]. High levels of eugenol-rich essential oil (EO) (1-3%) are found in leaves and are the primary component of medicines, stimulants, antiseptics, tonics, and other ayurvedic compositions [3]. Betel vine fusarium wilt is a damaging, soil-borne fungal disease that is mostly caused by Fusarium sp. It is characterized by the wilting, yellowing, and stunted growth of the affected vines, which frequently begins with the lower leaves [4]. Each year, biotic stress-mediated crop loss is exacerbated by this infamous pathogen [5]. The fungus Fusarium infects the roots of plants and thrives in the soil. After that, it spreads into the vascular system, obstructing the passage of nutrients and water, which causes the plant to wilt and eventually die [6]. Conventional methods for reducing biotic stress in plants rely on integrated pest management (IPM), which combines cultural tactics like crop rotation, intercropping, and proper spacing with physical and mechanical procedures like manual bug removal and traps [7]. Despite their advantages, these methods are expensive, time-consuming, and challenging for farmers to implement in the field [8]. Conventional breeding for resistance is a crucial strategy, in addition to early detection and surveillance [9]. However, using breeding to control disease has several drawbacks, including slow development time, loss of genetic

diversity, the possibility of unintended trait transfer (linkage drag), and restricted ability to breed between unrelated species [10].

The main way that exogenous treatments for biotic stress in plants help is by activating and strengthening the plant's defenses, which makes it possible for it to respond to attacks more quickly and powerfully. Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are examples of these reactions [11]. By increasing the production and activity of antioxidant enzymes such as CAT (catalase), SOD (superoxide dismutase), and APX (ascorbate peroxidase), which detoxify reactive oxygen species (ROS) produced during biotic stress, [12], stimulating the expression of stress-responsive genes (upregulating defense genes and downregulating redundant genes), and influencing hormonal crosstalk (synergistically/antagonistically) [14], exogenous phytohormone treatment improves plant tolerance to biotic stress. Phytohormones have been shown in numerous studies to improve plant growth and reduce stress in plants under biotic stress (Table 1). The application and effects of pre-treating the Jasmonic acid and Abscisic acid (JA-ABA) pair on the growth and defense of betel vines under pathogenic stress (Fusarium wilt disease) are the focus of this study.

Table 1: Previously reported work on the effects of exogenous phytohormones on plants under pathogenic stress.

Plants	Stress	Exogenous hormone	Concentration	Effects	Ref.
Wheat	*Fusarium graminearum*	ABA+JA	ABA at 2.85 and 4.75 mM, JA at 3.57 and 5.95 mM	JA treatment reduced *F. graminearum* growth and fusarium head blight (FHB) symptoms, while an increase in FHB was observed with ABA	[15]
Groundnut	*Helicoverpa armigera*	JA+SA	1 mM each	Higher levels of enzymatic activities and amounts of secondary metabolites were observed in the insect-resistant genotypes pretreated with JA and then infested with H. armigera	[16]
Arabidopsis	*Leptosphaeria maculans*	ABA + GABA	25 µM (ABA) 16 mg/L (GABA)	Treatments enhanced callose depositions and induced resistance to *L. maculans* in oilseed rape, and BABA-induced resistance was found to be independent of salicylic acid.	[17]
Soybean	*Fusarium virguliforme*	Ethephon	0.1, 1, and 4 mM	Activation of genes involved in ethylene biosynthesis, such as ethylene synthase (*ACS*) and ethylene oxidase (*ACO*), and genes involved in soybean defense response, such as pathogenesis-related protein (*PR*), basic peroxidase (*IPER*), chalcone synthase (*CHS*), and defense-associated transcription factors	[18]
Barley	*Fusarium culmorum*	Epibrassinolide	20 mM	Growth in epiBL activates genes related to chromatin remodeling, hormonal signaling, photosynthesis, and pathogenesis.	[19]
Wheat	*Fusarium graminearum*	Ca^{2+} and SA	5-mM $CaCl_2$, or 0.05-mM SA	*F. graminearum* inoculation promoted Carotenoids, stress markers (electrolyte leakage, lipid peroxidation, protein oxidation, hydrogen peroxide, and hydroxyl radical), and antioxidant molecules (APX, CAT, SOD, etc.).	[20]
Betel vine	*Fusarium sp.*	JA+ABA	10 mg/L	Increased RWC, pigment content, and total ascorbate. Total glutathione, MSI. Increased growth parameters (root: shoot length, leaf count, total biomass). Overall better growth as compared to untreated-infected plants	[Current study]

2. Materials and methods

2.1 Plant material and experimental design

Betel vine plantlets were grown from the Desawari cultivar found in the Bundelkhand region of Madhya Pradesh state of India, also known as the 'meethi patti' variety native to the Mahoba district of Uttar Pradesh state, and were used as the test plant. The plantlets were transferred to 3-inch pots having an equal mixture of sterile soil, sterile sand, and vermicompost to ensure adequate nutrition for plants. Experiment was conducted during the autumn season (Sep-Oct 2024) growth period. When the plantlets reached the three-four-leaf stage, pest- and disease-free plantlets were selected for the experiment. Previously reported pathogens of betel vine were selected and purchased from MTCC, IMTECH, Chandigarh, India, for imposing biotic stress on plants. *Fusarium* sp. can remain as a fungal endophyte in betel vine and produce mycotoxins that cause health risks in the plant, resulting in wilting afterwards.

2.2 Fungal inoculums preparation

Water suspensions of *Fusarium* fungal culture were sub-cultured on Sabouraud agar medium and incubated at 30°C. Inoculum suspensions were prepared from fresh, mature (8 to 10 days old) cultures. The colonies were covered with 5 mL of sterile distilled water. The inoculation was achieved by carefully rubbing the colonies with a sterile loop and then shaking vigorously for 15s with a vortex mixer and then transferring to a sterile tube. Then, the inoculum was diluted with sterilized distilled water.

2.3 Hormone dose preparation

When the plantlets reached the three-four-leaf stage, pest- and disease-free plantlets from approximately the same growth conditions were selected and exogenously sprayed with 1 µmol/L concentrations of Methyl Jasmonates (85% purity; CAS no. 39924-52-2). JA was purchased from Thylakoid Biotech Pvt Ltd, Gandhinagar, India. A total of 0.1 g of JA was dissolved using 7 mL of anhydrous ethanol, and then the dissolved JA was prepared into a 1 µmol/L solution using distilled water. ABA (85% purity; CAS no. 21293-29-8) was purchased from Thylakoid Biotech Pvt Ltd, Gandhinagar, India. A total of 0.1 g of ABA was dissolved in distilled water to prepare into a 1 µmol/L solution using distilled water. Both hormonal solutions were sprayed individually at the same time.

The plants were set up with no pests, disease, and JA-ABA treatments were taken as negative and will be named as 'N.'. The plants setup infected with the pathogen were taken as positive and will be named as 'P.'. Finally, the plants set up with infection and JA-ABA treatments were taken as a test and will be named as 'T'. The entire experiment was performed in triplicate. The plants were sprayed evenly with the appropriate treatment solution (approximately 20 mL per plant) until the solution dripped off the leaves; then, the

whole plant was covered with transparent plastic bags for 8 h to prevent evaporation of the treatment solution.

2.4 Morphological observations

2.4.1 Anatomical visualization of leaf tissue

Microscopic examination of stomata under a light microscope of 21-day-old leaves from N, P, and T was performed. Using forceps, the cuticle of fresh leaves was delicately peeled off after they had been gently cleaned twice with distilled water. For anatomical studies, the leaf's abaxial/dorsal side was examined using a light microscope with a 400x magnification.

2.4.2 Morphological observations

Morphological parameters such as root length, shoot length, leaf count, and total biomass were recorded from 21-day-old plantlets of N, P, and T, and the difference was represented by the percentage increase in these constraints. The IBM Statistical Package for the Social Sciences (SPSS) software version 30.0.0 Pro (SPSS Incorporation, Chicago, IL, United States) was used to statistically evaluate the collected data using Fisher's ANOVA and compare differences among treatment means using the honestly significant difference (HSD)/Tukey's test at a 5% probability level.

2.4.3 Determination of physiological parameters

A. Relative water content (RWC)

RWC was calculated using each plant's fully expanded leaves across all replicates. Using cork bores, three leaf discs were punched from each plant's interveinal region. To prevent respiratory loss, the fresh weight (FW) of pooled discs per replicate was ascertained right away. After that, leaf discs were left in d/w for four hours at 20°C with low light levels. After blotting the leaf discs to exclude surface water, the water intake was calculated using the turgid weight (TW). The leaf discs were dried for two days at 70°C to calculate their dry weight (DW) [21].

B. Photosynthetic pigments (Chla, Chlb and Carotenoids)

Photosynthetic pigments content was estimated in 1 gram of fresh leaves from both treated and untreated seedlings using 80% (v/v) acetone. After centrifuging the extracts, the pellets were reconstituted in 80% acetone until they lost their color. The absorbance of the resultant solutions was measured using a "Systronics" double beam UV-visible spectrophotometer-108 at 663.2, 646.5, and 470 nm. Using Lichtenthaler's (1983) formulae, the quantity of carotenoids and chlorophylls (chl a and chl b) was determined [22].

$$\text{Chl a } (\mu g/mL) = 12.25\ (A_{663.2}) - 2.79\ (A_{646.5})$$

$$\text{Chl b } ((\mu g/mL) = 21.50\ (A_{646.5}) - 5.10\ (A_{663.2})$$

$$\text{Car } (\mu g/mL) = [(1000\ A_{470} - 1.82\ (\text{Chl a}) - 85.02\ (\text{Chl b})] / 198$$

where, Chl a = Chlorophyll a, Chl b = Chlorophyll b and Car = Carotenoids

C. H_2O_2 content

40 mg of fresh frozen leaves were mixed with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA), and the resulting mixture was centrifuged for 15 minutes at 5,000 rpm. The resulting supernatant was utilized to estimate H_2O_2. 0.5 mL of 10 mM potassium phosphate buffer, 1 mL of 1 M potassium iodide, and 0.5 mL of crude extract were all included in the reaction mixture. The "Systronics" double beam UV-visible spectrophotometer-108 was used to measure the solution's absorbance at 390 nm in comparison to blanks. Each sample's $H2O_2$ content was represented in terms of nmol/gm FW, and the level of $H2O_2$ was determined using a standard curve [23].

D. Electrolyte leakage (EL) and Membrane stability index (MSI)

200 mg of fresh leaves from each pair of betel plants were chopped into 5-mm-long pieces and put in test tubes with 20 mL of deionized water at 30ºC for two hours. After centrifuging the samples, a Digital Conductivity Meter (Century CC-607, India) was used to assess the supernatant's initial electrical conductivity (EC1). Following a 15-minute boil at 100ºC to extract all electrolytes, the samples were chilled, centrifuged, and the final electrical conductivity (EC2) of the supernatant was determined [24]. This is how the membrane stability index (MSI) and electrolyte leakage (EL) were computed:

$$EL = EC1/EC2$$

$$MSI = (1\text{-}EL) \times 100$$

E. Malonaldehyde content (MDA)

Using a mortar and pestle, 1 gram of recently collected leaves was ground. 5 milliliters of 2% trichloroacetic acid (TCA) were mixed with 0.6% 2-thiobarbuteric acid (TBA). After adding the powdered paste to this solution, it was heated for 15 minutes at 100°C and then chilled in ice. For ten minutes, the finished mixture is centrifuged at 5000 rpm/min. obtained supernatant's absorbance was measured with a Systronics double beam UV-visible spectrophotometer-108 at 450, 532, and 600 nm [25]. The MDA levels in µmol/gm fresh leaf weight were computed by following formula. MDA content was calculated as follows:

$$\text{MDA } (\mu mol/gm \text{ fresh weight of leaf}) = 6.45(A_{532}\text{-}A_{600})\text{-}0.56\ X\ A_{450}$$

F. Ascorbate content

One gram of frozen leaf tissue was pulverized using a mortar and pestle along with 10 milliliters of 5% (w/v) m-phosphoric acid and inert sand. The homogenate underwent a 15-minute, 12,000-g centrifugation. 200 µL of supernatant, 500 µL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 100 µL of 10 mM dithiothreitol (DTT) were used to convert dehydroascorbate to reduced ascorbate in order to measure the total amount of ascorbate. 100 µL of 0.5% (w/v) N-ethylmaleimide was added to eliminate excess DTT after 10 minutes at room temperature. By adding 400 µL of 10% (w/v) TCA, 400 µL of 44% (v/v) o-phosphoric acid, 400 µL of 2,2'-bipyridyl in 70% (v/v) ethanol, and 200 µL of 30 g L^{-1} $FeCl_3$ to reaction mixtures, color was produced. The absorbance of the reaction mixtures was measured using spectrophotometry at 525 nm after they were incubated for one hour at 40ºC. In the range of 0-100 µg ASA/mL, a standard curve was created [26].

G. Glutathione content

Reduced glutathione (GSH) was measured by mixing 600 µL of 0.5M phosphate buffer, 40 µL of 0.4% (w/v) DTNB (5,5-Dithiobis 2-nitrobenzoic acid)/Ellman's reagent, and 100 µL of supernatant. After five minutes of incubation at room temperature, measure the absorbance at 412 nm. The

0-500 µM standard solution is used to create the standard curve [27].

H. Proline content

About 0.5 grams of plant material were mixed with 10 milliliters of 3% aqueous sulfosalicylic acid, and the resulting mixture was then filtered using Whatman filter paper. The reaction was stopped in an ice bath after 2 mm of filtrate and 2 mL of reagent (30 mL glacial acetic acid, 20 mL phosphoric acid, and 1.25g ninhydrin) were combined in a test tube and heated to 100°C for one hour. Toluene (4 mL) was added to the reaction mixture and thoroughly stirred. Toluene was used as a blank when the chromophore containing toluene was aspirated from the aqueous phase after it had been warmed to room temperature and measured at 520 nm [28].

2.5 Antioxidant assay

Following the guidelines of Gulcin & Alwasel, 2023 [29], the 1,1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging assay was used to assess the antioxidant activity of leaf extracts of N, P, T, and commercial ascorbic acid. We measured the absorbance of the reaction mixtures at 517 nm using a UV-Vis spectrophotometer. The DPPH assay measures the absorbance at 517 nm because it coincides with the maximal absorption wavelength of the DPPH radical in its unreacted state. DPPH is a stable free radical that absorbs significantly at 517 nm and has a characteristic deep violet color. An antioxidant such as ascorbate or glutathione scavenges the DPPH radicals, reducing them and changing their color from violet to yellow. Because it falls in direct proportion to the antioxidant's ability to scavenge free radicals, the absorbance at 517 nm is an ideal testing point for evaluating antioxidant activity. Using the formula, the proportion of DPPH radical scavenging activity was determined:

$$\text{Radical scavenging activity (\%)} = \left(\frac{Ao - As}{Ao}\right) X\ 100$$

Where *Ao* is the absorbance of the DPPH solution without NPs, and *A*s is the absorbance in the presence of antioxidants. Inhibitory concentration 50 (IC_{50}) is an effective concentration of the compound for removing 50% of radicals. The IC50 value was calculated using a straight-line equation. The IC50 value of antioxidants was calculated using the log dose inhibition curve and compared with that of ascorbic acid.

2.6 Statistical correlation analysis (PCA, Correlation matrix, AHC)

PCA, Correlation matrix, AHC were applied to 13 variables (MDA, RWC, H_2O_2, MSI, EL, Chl a Chl b, CRT, ASC, Proline, GSH, Shoot length and Biomass to understand the relationship between different variables. PCA, correlation matrix, AHC computation were done using XLSTAT-Student 2025.1.3.1431 software without variable scaling. PCA type was set to Pearson correlation and maximum filter factors were 5. Five to ten uncorrelated main components, each of which accounts for a part of the overall dataset variance, make up the PCA output. Usually, the majority of the variance is explained by the first principal component. Only principal components with an Eigenvalue above 1.0 were considered significant [30].

3. Results and Discussion

3.1 Plant infection, symptom and hormonal treatment

Plants were grown in triplicates (Fig 1.a), and 100 mL of 10mg/mL (JA-ABA) were sprayed. Afterwards, plants were infected with inoculum of live *Fusarium* culture (Fig. 1b). After 21 days symptoms of Fusarium wilt were observed in 'P' clearly (Fig. 1c). A significant difference was also observed in the leaf quality (Fig. 1d).

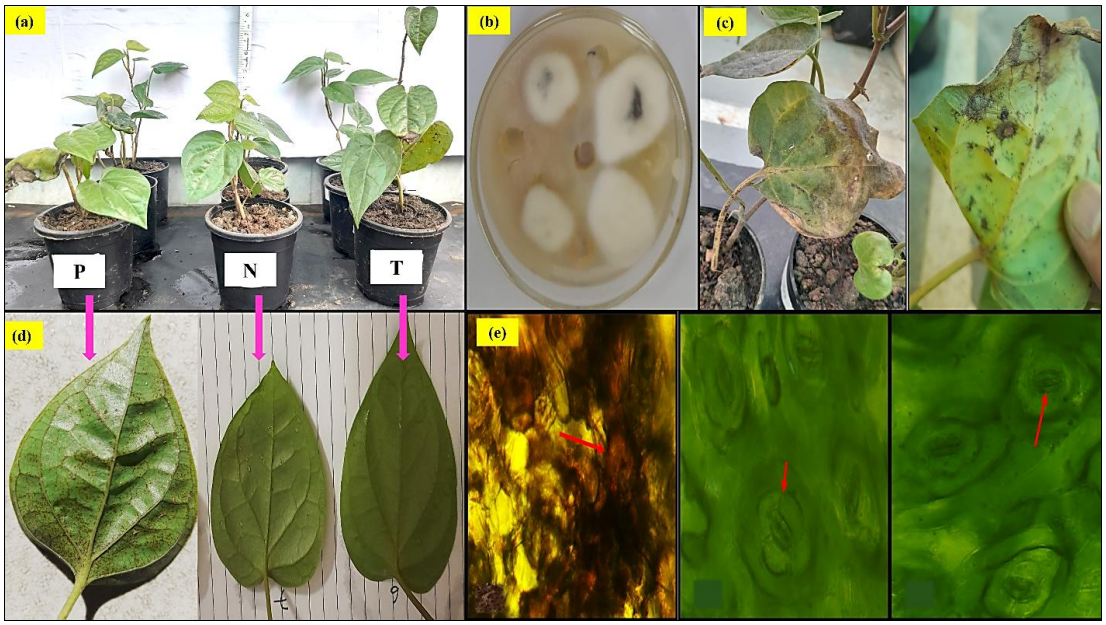

Fig 1: Representation of effect of exogenous pretreatment of JA-ABA pair on betel vine.

3.2 Microscopic analysis of leaf tissue of Betel vine

Microscopic analysis of the transverse sections of the N, P, T leaves reveals that the tissues of T and N leaves showed a healthy, continuous layer of cells with a protective cuticle, frequently with stomata for gas exchange. In contrast to T, infected leaves of P frequently showed disruptions in the morphology and organization of cells such as cell enlargement, cell breakdown, and decreased differentiation leading to necrosis (Fig.1e).

3.3 Physiological and biochemical parameters

3.3.1 Effects of JA-ABA pre-treatment on Plant biomass, height, leaf count, Chlorophyll and Carotenoids content

The effect of T (pre-treatment + infection) on BV Plants resulted in an increase of 19% in Carotenoids content (Fig. 2b), 78% in Chlorophyll a content, 332% in Chlorophyll b content (Fig. 3b), 98 % in leaf count (Fig. 2a), 69.36% in plant height and 164.11% in plant biomass (Fig. 2c) as compared to P (untreated + Infected). The increment in the above parameters was also greater than N (untreated + uninfected). Above parameters were increased as compared to controls due to the synergistic signaling between JA and ABA. Pre-treatment enhanced the immunity of betel vine plants which did not allow the fungus to enter and cause physiological alterations in cells [30].

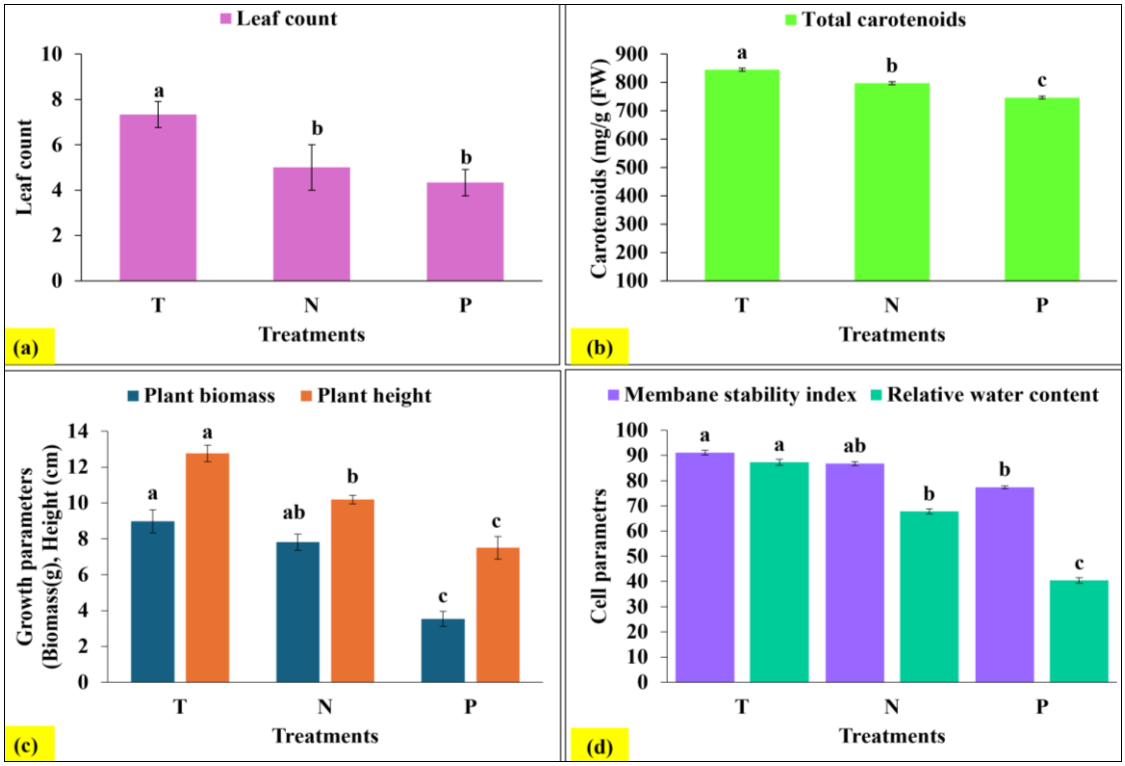

Fig 2: Effects of JA-ABA pre-treatment on Plant biomass, height, leaf count, Chlorophyll and Carotenoids content. The collected data were statistically evaluated by using Fisher's ANOVA and differences among treatment means were compared using the honestly significant difference (HSD)/ Tukey's test at a 5% probability level by using IBM Statistical Package for the Social Sciences (SPSS) software version 30.0.0 Pro (SPSS Incorporation, Chicago, IL, United States).

3.3.2 Effects of JA-ABA pre-treatment on MSI, MDA, H_2O_2 and RWC content

The effect of T (pre-treatment + infection) on BV plants resulted in an increase of 19.74% in MSI, 136% in RWC (Fig. 2d) and 22% in total protein content (Fig. 2b). RWC was increased as compared to P and N suggesting that JA-ABA together played a significant role in water status balance in BV leaves [30]. Although, a decrease of 54% in EL, 76% in MDA content, and 71% in H_2O_2 content (Fig.3d) as compared to P (untreated + Infected) was observed. MSI and EL are reciprocal indicators of stress (when membrane is stable there will be lesser electrolyte leakage) and were observed to be increased and decreased respectively as compared to P. Enzymes such ascorbate peroxidase, glutathione reductase and peroxidase neutralize ROS to protect the cell organelles and their membranes from oxidative damage, consequently, these enzymes raise the %RSA, MSI, and lower the EL [31].

3.3.3 Effects of JA-ABA pre-treatment on Glutathione, Ascorbate, Protein, Proline, Phenolic, alkaloid and flavonoid content

The effect of T (pre-treatment + infection) on BV leaves resulted in an increase of 35% in Ascorbic acid content, 161% in Glutathione content, and 144 % in Proline content (Fig. 3c). A decrease of 18% in phenolic content, 54% in alkaloid content and 35% in flavonoid content was observed (Fig. 3a). Glutathione serves as an antioxidant, scavenging dangerous ROS, and proline acts as an osmolyte, assisting in maintaining cell turgor and preventing water loss [32][33]. MDA and H_2O_2 content were found to be significantly low in T as compared to P indicating the stress-alleviating effect of JA-ABA application. Total phenolic, alkaloid and flavonoid contents were observed to be high as compared to P and N and This suggests that the production of secondary metabolites as a defense response was high to prevent the pathogenic infection. Secondary metabolites serve as the plant's primary chemical defense against herbivores, pathogens, and environmental stresses by acting as toxins, deterrents, or by signaling defense responses. These compounds, such as alkaloids, terpenoids, and phenolics, can directly harm attackers, repel them, attract beneficial organisms that prey on pests, or act as precursors for physical defenses [34].

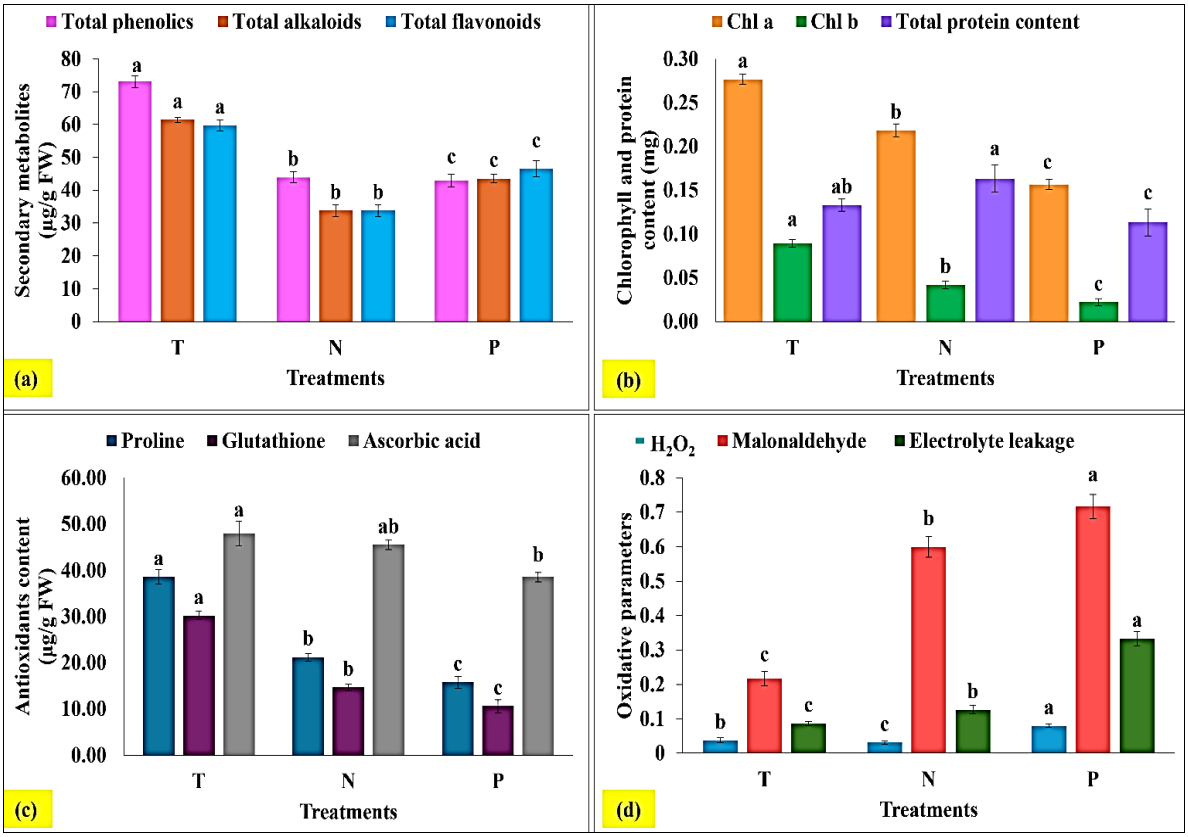

Fig 3: Effects of JA-ABA pre-treatment on MSI, MDA, H_2O_2, RWC, Glutathione, Ascorbate, Protein, Proline, Phenolic, alkaloid and flavonoid content. The collected data were statistically evaluated by using Fisher's ANOVA and differences among treatment means were compared using the honestly significant difference (HSD)/ Tukey's test at a 5% probability level by using IBM Statistical Package for the Social Sciences (SPSS) software version 30.0.0 Pro (SPSS Incorporation, Chicago, IL, United States).

Antioxidant potential of T (IC_{50} = 170.50 µg/mL) was found to be significantly higher than N (230.31 µg/mL) and P (289.2 µg/mL) but remained lower than standard ascorbic acid (73.67 µg/mL). The graph revealed that ascorbic acid showed lower IC_{50} values followed by T and N. This might be due to potent antioxidant nature of ascorbic acid and enhanced production of antioxidant compounds in abundance in T due to the treatment of JA-ABA pair. The primary action of the JA-ABA combination here was observed to manage the production and scavenging of reactive oxygen species such as superoxide anions and hydrogen peroxide. High ROS levels are a common result of environmental stress and cause oxidative damage to cellular components like lipids and proteins. By regulating ROS, the JA-ABA interaction protects the plant from damage [35]

3.4 DPPH radical scavenging activity

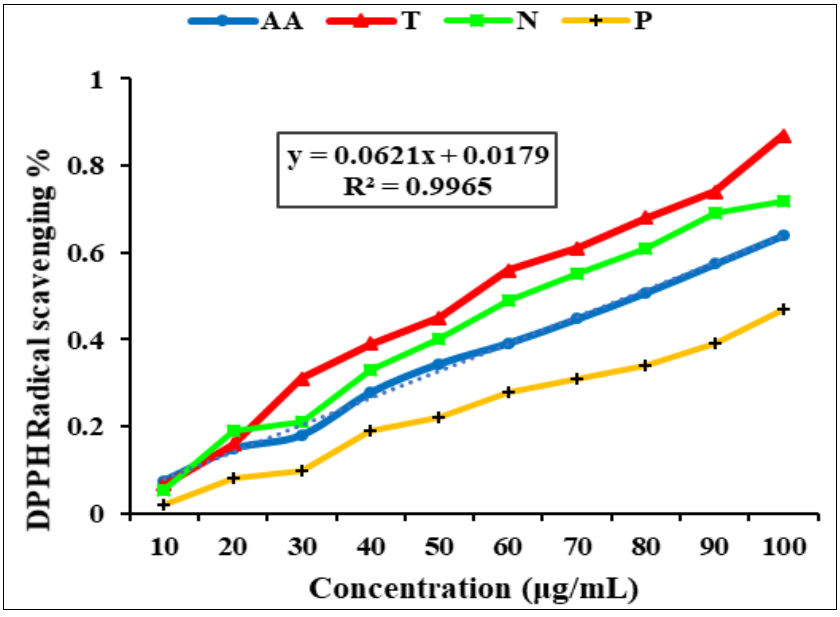

Fig 4: Represents the radical scavenging activity in N, P, and T compared with standard ascorbic acid.

3.5 Correlation matrix, Principal component analysis (PCA), Agglomerative hierarchical clustering (AHL)

When an experiment is set up, the observations of the experiment depend on various factors. To comprehend the complexities of biological phenomena all the factors mut be considered, which is not possible at once. Statistical correlation analysis leads to the systematism in complex data. Pearson correlation matrix was performed to understand relationships between different variables such as MSI, RWC, Chlb, CRT, ASC, Proline, GSH, Phenolics, Flavonoids, Alkaloids, Biomass, Leaf count, Shoot height, EL, H2O2, MDA and represented in the form of Heat map (Fig. 5). The coefficient correlation quantifies the strength and direction of a linear relationship between two variables, typically ranging from -1 (indicating inverse correlation) to +1 (indicating positive correlation). Red color indicates positive correlations and darker red suggests a stronger positive relationship. Blue indicates negative correlations, and darker blue suggests a stronger negative relationship. Yellow color indicates weaker correlations, closer to zero. White cells indicate no or very weak correlation.

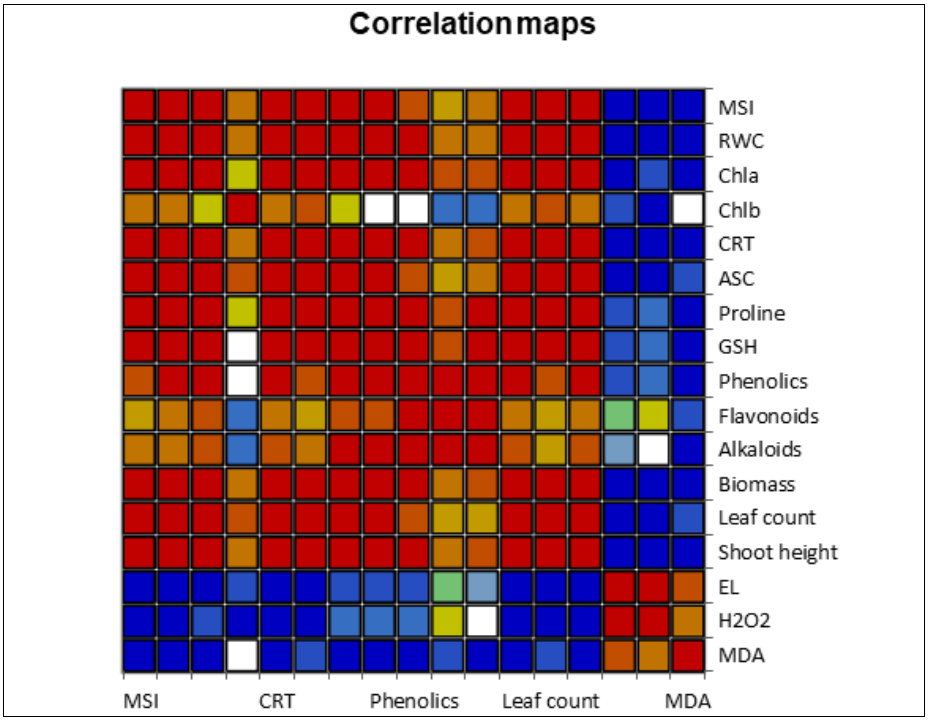

Fig 5: Illustration of correlation matrix displaying the correlation coefficients between physiological parameters (variables) and treatments.

Principal Component Analysis (PCA) biplot, which is a powerful statistical visualization technique used to simplify and understand complex datasets with multiple variables. It reduces the dimensionality of the data while retaining as much variance (information) as possible, allowing for visualization of relationships between variables and observations simultaneously. Scree plot shows PC1 (79.63%) and PC2 (20.37%) indicating that majority of the variance in the entire data was due to PC1 and then PC2 (Fig. 6). Eigenvalues above 1 were considered significant. Here, Eigenvalue for PC1 and PC2 was observed to be 13.53 and 3.4 respectively. High eigenvalue means that components capture a large portion of original data's variation. Cumulative variance is the total percentage of the original data's variability explained by a selected number of principal components. Cumulative variance % was observed to be 79.63% and 100% for PC1 and PC2 respectively [30].

Positive correlations between variables are indicated by red vectors pointing in comparable directions such as Chla and Proline are positively associated. A negative correlation is indicated by the vectors pointing in opposing directions (for example, MDA and leaf count are negatively correlated). Those that are around 90 degrees apart are mostly uncorrelated (MDA and Chlb). The longer the vector, the more variation that variable explains and the more strongly it affects the principal components. The comparatively long vectors of variables such as MDA, Chlb, shoot height, and flavonoids show that they play a major role in the observed variation. Blue dots represent individual observation (N, P, and T) in the data. Positioned in the upper center, observation 'N' indicates that it has greater values for variables such as Chlb and Leaf count than either 'P' or 'T'. MDA levels may be higher in observation 'P' on the left, whereas Chla, Proline, and Phenolics may be higher in observation 'T' on the right (Fig.7).

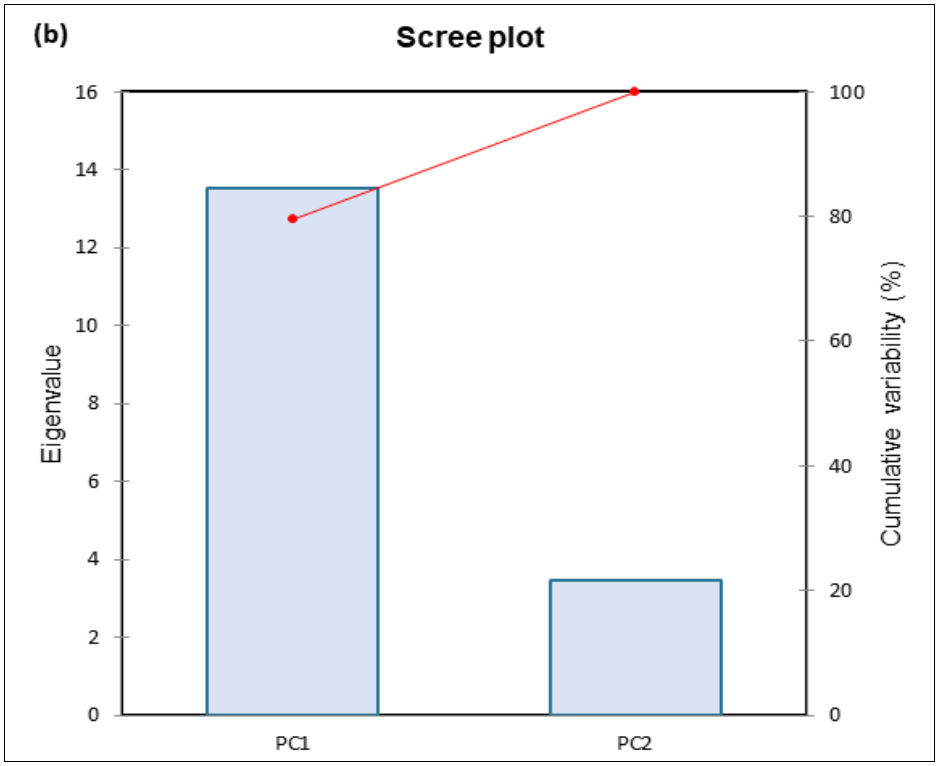

Fig 6: Representation of Scree plot showing Eigenvalue and % Cumulative variability of two principal components (PC1 and PC2).

In Agglomerative Hierarchical Clustering (AHC) dendrogram, initially N, P, and T were considered as separate clusters when data points were clustered based on dissimilarity (Fig. 8). AHC broadly indicated that N and T are clustered closely after merging data points. The effect of JA-ABA pre-treatment allowed the plants to behave normally even under pathogenic stress whereas P showed a separate and correlated to N and T to a lesser extent. It is clear that MDA, H_2O_2 and EL are negatively correlated with all the growth and redox parameters. MDA is a marker of lipid peroxidation and cellular damage which increases in plants under biotic stress [38]. Due to the exogenous pre-treatment of JA-ABA, the impacts of biotic stress caused by *fusarium* infection were significantly reduced as depicted in physiological analysis section. JA-ABA synergistically triggered the downstream signaling of defense related genes which activated the production of antioxidant enzymes to maintain the redox status of the cell and secondary metabolites to prevent the systemic infection [36]. Consequently, levels of H_2O_2 dropped down and the electrolyte leakage was prevented as the membrane integrity was maintained. It is also evident from PCA analysis that majority of the variables fall in the same quadrant (right) and are strongly correlated with each other except H_2O_2, EL and MDA which settled in the left quadrant. On the other hand, Plant height, biomass, leaf count was significantly increased in T as compared to P due to enhanced JA-ABA pre-treatment effects.

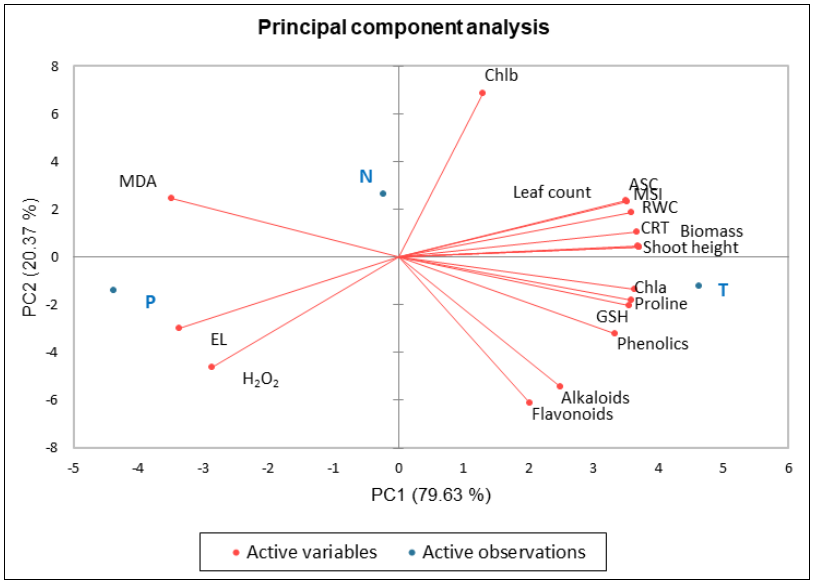

Fig 7: Representation of Principal component analysis (PCA) for dimensionality reduction identifying key underlying factors that explain the most variation in the physiological responses to the treatment.

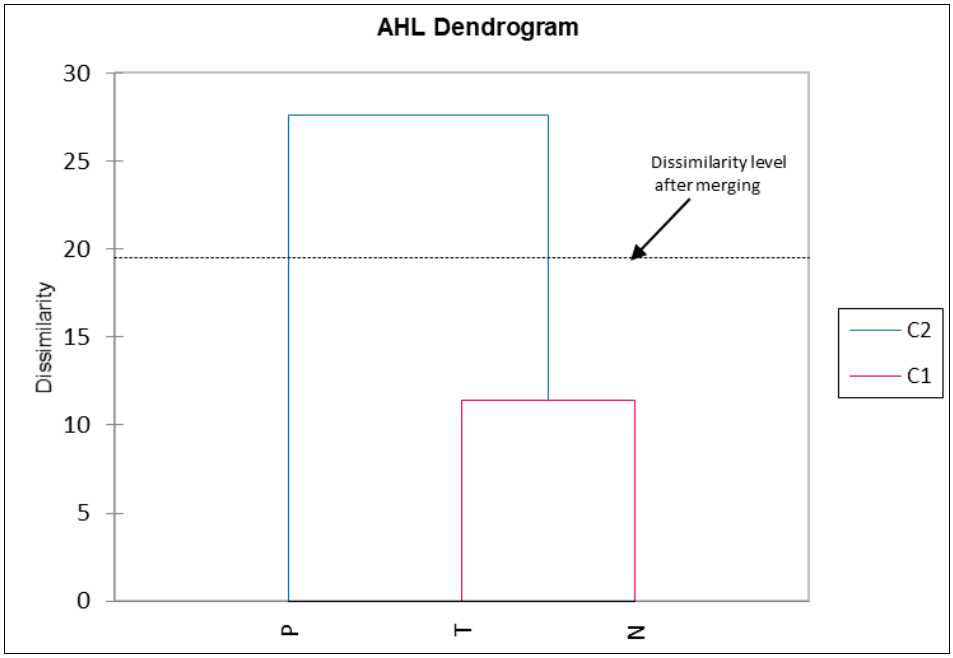

Fig 8: Shows the Agglomerative Hierarchical Clustering (AHL) indicating close relationship between three treatments (P, T, N) in terms of dissimilarities between physiological parameters.

4. Conclusion

Betel vine cultivation has been facing diverse challenges, one of them being biotic stress. Reports suggest that Fusarium wilt cause huge losses in Betel vine. To address this problem limited efforts have been made including use of pesticides, IPM, and breeding, with very low success rate. However, exogenous treatments combating biotic stress in plants offer benefits by triggering and enhancing the plant's natural defense mechanisms, allowing for a stronger and faster response to attacks. The study found that pre-treatment of JA-ABA enhanced plant tolerance to biotic stress by modulating stress-responsive gene expression, boosting the antioxidant system, and influencing hormonal crosstalk. The results showed that pre-treatment enhanced the immunity of betel vine plants, preventing the fungus from entering and causing physiological alterations in cells. JA-ABA also played a significant role in water status balance in BV leaves, increasing MSI, MDA, H2O2, and RWC content. It also increased Glutathione, Ascorbate, Protein, Proline, Phenolic, alkaloid, and flavonoid content, suggesting that secondary metabolites as a defense response were high to prevent the pathogenic infection. In conclusion, pre-exogenous treatment of JA-ABA duo can enhance synergistic actions to combat biotic stress in *Piper betel* L. The antioxidant potential of T was found to be significantly higher than N and P, but lower than standard ascorbic acid. This could be due to the potent antioxidant nature of ascorbic acid and the enhanced production of antioxidant compounds in T due to the treatment of the JA-ABA pair. The primary action of the JA-ABA combination is to manage the production and scavenging of reactive oxygen species, such as superoxide anions and hydrogen peroxide, which are common results of environmental stress and cause oxidative damage to cellular components like lipids and proteins. The Agglomerative Hierarchical Clustering (AHL) analysis indicated close relationships between three treatments (P, T, N) in terms of dissimilarities between physiological parameters. Pearson correlation matrix was performed to understand relationships between different variables, and Principal Component Analysis (PCA) biplot was used to simplify and understand complex datasets with multiple variables. The results showed that MDA, H2O2, and EL are negatively correlated with all growth and redox parameters, and that the exogenous pre-treatment of JA-ABA significantly reduced the impacts of biotic stress caused by fusarium infection. JA-ABA synergistically triggers the downstream signaling of defense-related genes, activating the production of antioxidant enzymes to maintain the redox status of the cell and secondary metabolites to prevent systemic infection.

In conclusion, the JA-ABA combination has shown potential in managing reactive oxygen species and protecting plants from damage.

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Author contribution declaration

SKC and CA were involved in experimentation; SKC and CA analyzed data and wrote the manuscript. JKP critically revised and finalized the manuscript. All authors have read, commented, and approved the final manuscript.

Declarations

1. **Ethics Approval:** All authors declare Ethics approval.
2. **Consent to Participate:** All authors Consent to participate.
3. **Consent for Publication:** All authors Consent for publication.
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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use AI and AI-assisted technologies, the author(s) reviewed and edited the content as needed and took(s) full responsibility for the content of the published article.

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