



RESEARCH ARTICLE

Copper-chitosan nanocomposites enhance biochemical defence responses and suppress anthracnose in chilli (*Capsicum annuum* L.)

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Abstract

Anthrachnose caused by *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore is a common fungal disease in chilli (*Capsicum annuum* L.) causing severe reduction in fruit quality and yield. There is a dire need for sustainable solutions to not only suppress the disease-causing agent but also strengthen the host defence responses. In this study, a nano-elicitor in the form of copper-chitosan nanocomposite (Cu-ChNC) was tested as a method to control anthracnose and activate the biochemical resistance in chilli plants grown under greenhouse conditions. Copper-chitosan nanocomposite significantly suppressed anthracnose with lesion formation and disease index to 11.88 % in the prophylactic treatment against copper (22.38 %), chitosan (17.93 %) and without any treatment (47 %). Reduction in anthracnose was observed to be associated with an increase in the activity of defence enzymes. Peroxidase exhibited maximum activity of 14.55 $\Delta OD \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$, whereas polyphenol oxidase displayed its maximum activity of 54.22 $\Delta OD \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ on the 15th day after inoculation. Maximum level of phenylalanine ammonia-lyase (105.84 $\mu\text{g trans-cinnamic acid min}^{-1} \text{ g}^{-1} \text{ FW}$), β -1,3-glucanase (7.67 $\mu\text{g glucose g}^{-1} \text{ FW}$) and chitinase (40.91 $\mu\text{g N-acetyl glucosamine g}^{-1} \text{ FW}$) was observed to be above pathogen controls. Moreover, Cu-ChNC treatment increased the accumulation of phenols (15.09 $\text{mg g}^{-1} \text{ FW}$), tannins (7.52 $\text{mg g}^{-1} \text{ DW}$), proteins (23.56 $\text{mg g}^{-1} \text{ FW}$) and sugars (91.83 $\text{mg g}^{-1} \text{ FW}$) suggesting an induction in phenylpropanoids and other pathways. Correlation analyses revealed highly significant positive correlation among the defence characteristics ($r = 0.85-0.99$) and a high negative correlation between defence enzymes, biochemicals and disease index ($r = -0.88$ to -0.99). Principal component analysis further supported the association between enzyme activity and phenolic compounds with anthracnose control. Overall, Cu-ChNCs demonstrate strong potential as nano-elicitor by enhancing defence enzymes, boosting phenolic accumulation and activating coordinated plant immune responses, offering an eco-friendly strategy for sustainable anthracnose management in chilli.

Keywords: antifungal activity; defence enzymes; induced resistance; nano-elicitor; nano fungicide; particle size

Introduction

Chilli (*Capsicum annuum* L.) is an economically important spice and vegetable crop cultivated worldwide, but it is vulnerable to several fungal pathogens that significantly reduce yield and fruit quality. Among fungal diseases, anthracnose caused by *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore is particularly destructive, affecting fruits at both pre- and post-harvest stages and causing yield losses of up to 50 % under favourable conditions (1, 2). Conventional management of anthracnose relies largely on repeated applications of synthetic fungicides like triazoles and copper oxychloride. Although these chemicals provide short-term disease control, their extensive use has raised serious concerns related to environmental contamination, pathogen resistance and

food safety concern (3). Consequently, there is increasing interest in developing eco-friendly, biodegradable and sustainable alternatives that enhance plant innate immunity rather than relying solely on chemical control (4, 5).

Chitosan, a naturally occurring biodegradable biopolymer derived from chitin, has been widely reported as a plant defence elicitor, capable of activating antioxidant enzymes and pathogenesis-related (PR) proteins. Several studies have shown that chitosan treatment induces defence enzymes such as peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) leading to reduced anthracnose severity in pepper fruits (6). It has been hypothesised that the interaction of chitosan with negatively charged molecules on the fungal cell surface causes leakage of

proteinaceous compounds (7). It is also believed that the interaction between chitosan and fungi leads to the inhibition of mRNA and protein synthesis (8).

Copper-based fungicides have long been used for disease control due to their broad-spectrum antimicrobial activity, yet continuous application of bulk copper formulations often results in environmental accumulation and phytotoxicity (9). Nanotechnology offers an innovative solution to these limitations by improving the efficacy of copper at lower concentrations. Copper nanoparticles exhibit enhanced antimicrobial activity, improved bioavailability and controlled release properties compared with conventional copper formulations (10). Recent advances in nanotechnology enable the integration of copper and chitosan into nanocomposites, improving bioavailability, controlled release and biological efficiency. The integration of copper with chitosan into copper-chitosan nanocomposites (Cu-ChNCs) combines the antimicrobial action of copper with the elicitor and carrier properties of chitosan. Recent studies on chitosan-metal nanocomposites have demonstrated superior disease control and enhanced plant defence responses (11). Although individual effects of copper and chitosan have been reported, comprehensive studies linking disease suppression with coordinated biochemical defence responses and multivariate statistical validation in chilli is limited. However, so far, no reports are available on the induction of resistance by Cu-ChNCs in chilli against anthracnose disease.

Therefore, the present investigation was undertaken to elucidate the biochemical basis of Cu-ChNC-induced resistance in chilli against anthracnose by evaluating defence enzyme activities, biochemical metabolites and disease severity using multivariate statistical approaches including correlation and principal component analyses.

Materials and Methods

Plant material

Seeds of chilli (*C. annuum*) hybrid 'Arka Tejasvi' were procured from a certified seed source and used as the experimental material. Seeds were raised in sterilised potting mixture (soil: sand: farmyard manure (FYM), 2:1:1). Seedlings were raised under nursery conditions and transplanted at the four-leaf stage into plastic bags (30 cm diameter) containing approximately 10 kg of sterilised potting mixture and maintained under greenhouse conditions in the department of Plant Pathology during rabi season. Plants were watered regularly and fertilised following recommended agronomic practices.

Pathogen isolation and inoculum preparation

The anthracnose pathogen *C. truncatum* was isolated from infected chilli fruits collected from the Department's experimental fields. Small sections of infected tissue (approximately 5 mm²) were excised from the advancing margins of lesions and surface sterilised with 1 % sodium hypochlorite solution for 1–2 min, followed by rinsing three times with sterile distilled water. The sterilised tissues were blotted dry using sterile filter paper and transferred onto potato dextrose agar (PDA) medium plates under aseptic conditions. Pure cultures were obtained by hyphal tip method (12) and maintained at 28 ± 1 °C. Conidial suspension was prepared from 10-day-old cultures by flooding plates with sterile distilled water and gently scraping the colony surface with a sterile glass rod. The suspension was filtered through double-layered sterile muslin cloth

to remove mycelial fragments. Spore concentration was adjusted to 1 × 10⁶ conidia mL⁻¹ using a haemocytometer.

Synthesis of copper-chitosan nanocomposites

Preparation of chitosan nanoparticles

Chitosan nanoparticles (CNPs) were synthesised by ionic gelation as given by previous researchers (13) with some changes. Chitosan was dissolved in acetic acid and then sodium tripolyphosphate (TPP) was added (1:1 ratio) dropwise under constant stirring until complete dissolution, forming nanoparticles via electrostatic interaction between positively charged amino groups of chitosan and negatively charged phosphate groups of TPP. The pH was maintained in between acidic range.

Preparation of copper-chitosan nanocomposites

Copper-chitosan nanocomposites were synthesised by incorporating Cu²⁺ ions into the chitosan nanoparticle matrix. Copper sulphate solution was added dropwise to chitosan nanoparticle suspension under continuous stirring. Nanocomposites were stored in airtight containers until use.

Characterisation of nanocomposites

The physicochemical analysis of the prepared Cu-ChNCs was performed to investigate their particle size distribution, morphology and surface chemistry. The particle size and polydispersity index were estimated by dynamic light scattering (DLS). This technique allows for the determination of the hydrodynamic diameter and stability of the nanocomposite dispersion. The morphology and surface architecture of the nanocomposites were characterised by scanning electron microscopy (SEM). It helped in determining the shape of the nanoparticles, aggregation behaviour and surface morphology. Furthermore, Fourier transform infrared spectroscopy (FTIR) was used for confirmation of functional groups and identification of the interaction between copper and chitosan.

Experimental design and treatments

The experiment was conducted using a completely randomised design (CRD) with 5 replications. A total of 12 treatments were evaluated, including preventive treatments, curative treatments and control treatments. The preventive treatments consisted of T₁ - Chitosan (200 ppm), T₂ - CNPs (200 ppm), T₃ - copper sulphate (200 ppm), T₄ - Cu-Ch NCs (200 ppm) and T₅ - Copper oxychloride (2.5 g L⁻¹). The curative treatments included T₆ - Chitosan (200 ppm), T₇ - CNPs (200 ppm), T₈ - Copper sulphate (200 ppm), T₉ - Copper-chitosan nanocomposites (200 ppm) and T₁₀ - Copper oxychloride (2.5 g L⁻¹). In addition, two control treatments were maintained: T₁₁ - pathogen-inoculated untreated control and T₁₂ - healthy control sprayed with sterile distilled water. In the preventive set (T₁–T₅), foliar sprays were applied two days before pathogen inoculation, whereas in the curative set (T₆–T₁₀), sprays were applied two days after pathogen inoculation. The plants were inoculated with the pathogen at 45 days after transplanting, corresponding to the near-mature fruit stage.

Pathogen inoculation

Artificial inoculation of chilli fruits was performed using the pinprick method. Fruits were slightly wounded with a sterile needle to facilitate infection. Approximately 20 µL of conidial suspension (1 × 10⁶ conidia mL⁻¹) of *C. truncatum* was introduced into the wounded site using a sterile syringe. Following inoculation, plants were maintained under greenhouse conditions conducive to disease development.

Disease assessment

Anthrachnose severity was assessed at 40, 50, 60 and 70 days after transplanting using a 0–9 disease rating scale as described previously (14). According to this scale, a score of 0 indicates no visible symptoms on the leaf, branch, or fruit. A score of 1 represents 1–10 % or less infection, while 3 corresponds to 11–25 % infection. A rating of 5 indicates 26–50 % infection, 7 represents 51–75 % infection and a score of 9 denotes 75 % and above infection of the leaf, branch, or fruit area.

Further, the scale was converted to per cent disease index (PDI) using the formula described earlier (15). (Eqn. 1)

$$\text{Percent disease index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of fruits/ Leaves observed}} \times \frac{100}{\text{Maximum disease grade}}$$

Area under disease progress curve (AUDPC) calculation:

n-1

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left\{ \left[\frac{(X_i + X_{i+1})}{2} \right] \times (t_{i+1} - t_i) \right\} \quad (\text{Eqn. 2})$$

i=1

X_i = disease index expressed as a proportion at the i^{th} observation.

t_i = time (days after inoculation) at the i^{th} observation.

Sampling for biochemical analysis

Fruit samples were collected at 1 day before inoculation and 1, 5, 10, 15, 20, 25 and 30 days after inoculation. Fresh samples were used for enzyme assays, while oven-dried samples (35 °C, 24–48 hr) were powdered for biochemical estimations.

Estimation of defence-related enzymes

Enzyme extraction

One gram of fresh fruit tissue was homogenised in 0.1 M extraction buffer and centrifuged (12000 rpm, 20 min, 4 °C). The supernatant served as crude enzyme extract. The biochemical components were assessed by using the following methods:

The assessment of enzymes and their methods

The assessment of enzymes and their activities was carried out using standard methods. Peroxidase activity was determined by the pyrogallol oxidation method (16), while PPO activity was estimated through catechol oxidation (17). The PAL activity was measured based on the formation of trans-cinnamic acid (18). β -1,3-glucanase activity was assayed using laminarin hydrolysis (19) and chitinase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method (20). Enzyme activities were expressed as the change in absorbance per minute per gram of fresh weight or as micrograms of product formed per minute per gram of fresh weight, as given in Equation (3):

$$\text{Enzyme activity} = \Delta A \text{ min}^{-1} \text{ g}^{-1} \text{ FW or } \mu\text{g product min}^{-1} \text{ g}^{-1} \text{ FW}$$

Estimation of biochemical constituents

It was performed using established procedures. Total phenols were quantified using the Folin-Ciocalteu method (21), total sugars by the phenol-sulphuric acid method (22) and reducing sugars by the DNS method (22). Crude protein content was determined using the Micro-Kjeldahl method (23), while tannins were estimated using the Folin-Denis method (24). The results were expressed on either a fresh

weight or dry weight basis, as indicated in Equation (4):

$$\text{Content} = (\text{Amount of constituent} / \text{Sample weight}) \times 100$$

Statistical analysis

All experimental data were analysed using analysis of variance (ANOVA) (25) appropriate for a CRD using SPSS statistical software 16.0. Treatment means were compared using Tukey's Honestly Significant Difference (HSD) test at $p \leq 0.05$. Additionally, correlation analysis was performed to evaluate relationships between biochemical parameters and disease severity (PDI).

Results

Effect of copper-chitosan nanocomposites on defence related enzyme activities in chilli fruits

The results indicated that application of different treatments significantly influenced the activity of major defence-related enzymes such as POD, PPO, PAL, β -1,3-glucanase and chitinase in chilli fruits under greenhouse conditions. Among the treatments, Cu-ChNC exhibited the highest induction of antioxidant and pathogenesis-related enzymes compared with bulk copper, chitosan and untreated controls in both preventive and curative applications, indicating their strong potential in activating plant defence responses against chilli anthracnose.

Peroxidase

Peroxidase activity increased progressively after pathogen inoculation in all treatments as mentioned in Table 1, reaching a peak at 15 days after inoculation (DAI). Preventive application of Cu-ChNC (T_4) recorded the highest POD activity ($14.547 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$ at 15 DAI), which represented approximately 1.9-fold increase in activity (90.5 % increase) compared with the pathogen control ($7.638 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$), followed by copper oxychloride (COC) (13.889) and CNP (13.677). A similar trend was observed in curative treatments where Cu-ChNC (T_9) showed elevated POD activity ($14.147 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$ at 15 DAI) representing about 1.85-fold increase (85.2 %) over the pathogen control.

Polyphenol oxidase

Polyphenol oxidase activity also showed a significant increase in treated plants (Table 2). Preventive application of Cu-ChNC (T_4) recorded the highest PPO activity ($54.224 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$ at 15 DAI), which was approximately 5.77-fold higher (477.4 % increase) than the pathogen control ($9.396 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$), followed by COC (47.58) and CNP (46.245). In curative treatments, Cu-ChNC (T_9) recorded $51.625 \mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FW}$, corresponding to 5.49-fold increase (449.4 %) which was considerably higher than pathogen control (9.396). Enhanced PPO activity promotes oxidation of phenolics into quinones, which are toxic to invading pathogens and contribute to disease resistance.

Phenylalanine ammonia-lyase

The preventive treatment (Table 3) T_4 recorded the highest PAL activity ($105.84 \mu\text{mol trans-cinnamic acid min}^{-1} \text{ g}^{-1} \text{ FW}$ at 15 DAI), followed by COC (101.16) and CNP (83.70). In curative treatments, Cu-ChNC (T_9) also showed elevated PAL activity (104.04). The PAL catalyses the conversion of phenylalanine to trans-cinnamic acid, initiating the synthesis of phenolics, lignin and phytoalexins, which play a critical role in plant defence.

Table 1. Temporal dynamics of peroxidase (POD) activity ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$) in chilli fruits under different treatments

Treatments	1 DBI	1 DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI	30 DAI
T ₁ Chitosan (P)	4.322 ^a	6.392 ^{ef}	7.567 ^{cd}	8.507 ^{abcd}	12.949 ^{bcde}	12.878 ^{ab}	12.573 ^{bcd}	12.197 ^{bcd}
T ₂ CNP (P)	4.073 ^{ab}	6.533 ^{ef}	7.802 ^{bcd}	8.954 ^{abc}	13.677 ^{abc}	13.513 ^{ab}	13.113 ^{abc}	12.737 ^{abc}
T ₃ CuSO ₄ (P)	3.603 ^{bc}	6.698 ^{def}	6.993 ^{de}	8.178 ^{cde}	12.22 ^{de}	11.599 ^c	11.656 ^d	11.092 ^{de}
T ₄ Cu-ChNC (P)	4.23 ^{ab}	6.862 ^{cdef}	8.883 ^a	9.447 ^a	14.547 ^a	13.936 ^a	13.936 ^a	13.583 ^a
T ₅ COC (P)	4.152 ^{ab}	7.003 ^{bcd}	8.108 ^{abc}	9.259 ^{ab}	13.889 ^{abc}	13.748 ^a	13.395 ^{abc}	13.043 ^{abc}
T ₆ Chitosan (C)	3.682 ^{abc}	7.168 ^{abc}	7.403 ^{cde}	8.249 ^{bcde}	12.737 ^{cde}	12.432 ^{bc}	12.291 ^{cd}	11.891 ^{cde}
T ₇ CNP (C)	4.073 ^{ab}	7.332 ^{abc}	7.661 ^{bcd}	8.695 ^{abcd}	13.372 ^{abcd}	13.231 ^{ab}	12.949 ^{abc}	12.479 ^{abc}
T ₈ CuSO ₄ (C)	3.525 ^{bc}	6.345 ^f	6.473 ^e	7.779 ^{de}	11.703 ^e	11.246 ^c	11.327 ^d	10.74 ^e
T ₉ Cu-ChNC (C)	4.23 ^{ab}	7.638 ^{abcd}	8.601 ^{ab}	9.306 ^{ab}	14.147 ^{ab}	13.818 ^a	13.654 ^{ab}	13.184 ^{ab}
T ₁₀ COC (C)	3.995 ^{ab}	7.802 ^{abc}	7.849 ^{bcd}	9.048 ^{abc}	13.513 ^{abc}	13.513 ^{ab}	13.325 ^{abc}	12.667 ^{abc}
T ₁₁ Pathogen	3.055 ^{cd}	7.943 ^{ab}	7.074 ^{de}	7.274 ^e	7.638 ^f	8.186 ^d	7.332 ^e	7.144 ^f
T ₁₂ Water control	2.742 ^d	8.108 ^a	6.98 ^{de}	7.638 ^{de}	8.131 ^f	8.045 ^d	7.074 ^e	6.839 ^f
S Em $\pm$	0.059	0.110	0.117	0.131	0.192	0.188	0.185	0.178
C. D. at 1 %	0.172	0.323	0.342	0.384	0.564	0.553	0.543	0.523

SE m $\pm$: Standard error of the mean CD: Critical difference; DBI: Day before pathogen inoculation; DAI: Days after pathogen inoculation.**Table 2.** Temporal dynamics of polyphenol oxidase (PPO) enzyme activity ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$) in chilli fruits under different treatments

Treatments	1 DBI	1 DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI	30 DAI
T ₁ Chitosan (P)	6.672 ^{bc}	9.355 ^d	11.356 ^c	16.096 ^b	37.808 ^d	33.721 ^d	28.717 ^e	24.45 ^d
T ₂ CNP (P)	7.27 ^b	10.925 ^{bc}	14.234 ^b	17.945 ^a	46.245 ^c	40.741 ^c	38.656 ^{bc}	32.206 ^b
T ₃ CuSO ₄ (P)	6.505 ^{bc}	8.479 ^d	10.786 ^c	11.023 ^c	26.327 ^e	22.462 ^f	20.169 ^g	16.68 ^f
T ₄ Cu-ChNC (P)	8.493 ^a	12.524 ^a	18.376 ^a	18.445 ^a	54.224 ^a	48.233 ^a	41.756 ^a	35.945 ^a
T ₅ COC (P)	8.66 ^a	11.356 ^{bc}	17.139 ^a	18.07 ^a	47.58 ^c	43.813 ^b	40.185 ^{ab}	34.375 ^a
T ₆ Chitosan (C)	6.311 ^{bc}	9.313 ^d	11.106 ^c	15.763 ^b	37.322 ^d	31.317 ^e	26.605 ^f	21.017 ^e
T ₇ CNP (C)	7.172 ^b	10.773 ^c	13.997 ^b	17.723 ^a	45.564 ^c	38.614 ^c	33.624 ^d	26.702 ^c
T ₈ CuSO ₄ (C)	5.949 ^c	8.243 ^d	10.625 ^c	10.786 ^c	28.3 ^e	19.655 ^g	17.111 ^h	13.052 ^g
T ₉ Cu-ChNC (C)	8.437 ^a	12.024 ^{ab}	17.458 ^a	18.251 ^a	51.625 ^b	42.909 ^b	37.85 ^c	30.775 ^b
T ₁₀ COC (C)	8.604 ^a	11.162 ^{bc}	17.055 ^a	17.848 ^a	47.107 ^c	40.074 ^c	36.807 ^c	27.077 ^c
T ₁₁ Pathogen	5.741 ^c	6.477 ^e	6.922 ^d	5.949 ^d	9.396 ^f	9.952 ^h	10.05 ⁱ	10.536 ^h
T ₁₂ Water control	5.908 ^c	6.172 ^e	6.533 ^d	5.866 ^d	8.576 ^f	9.188 ^h	9.535 ⁱ	9.744 ^h
S Em $\pm$	0.111	0.152	0.207	0.232	0.604	0.522	0.467	0.384
C. D. at 1 %	0.325	0.446	0.608	0.680	1.774	1.534	1.371	1.128

SE m $\pm$: Standard error of the mean; CD: Critical difference; DBI: Day before pathogen inoculation; DAI: Days after pathogen inoculation.**Table 3.** Temporal dynamics of phenylalanine ammonia-lyase (PAL) enzyme activity in chilli fruits under different treatments ($\mu\text{mol trans-cinnamic acid min}^{-1} \text{g}^{-1} \text{FW}$)

Treatments	1 DBI	1 DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI	30 DAI
T ₁ Chitosan (P)	36.36 ^{cd}	54.36 ^{de}	63.36 ^d	71.82 ^{de}	79.56 ^{de}	75.24 ^d	69.48 ^e	61.2 ^e
T ₂ CNP (P)	39.96 ^a	61.2 ^c	71.64 ^c	77.04 ^c	83.7 ^c	81.9 ^c	77.76 ^c	74.34 ^d
T ₃ CuSO ₄ (P)	36.18 ^{cd}	56.52 ^d	59.04 ^{ef}	61.2 ^f	69.12 ^f	63.18 ^f	60.48 ^g	43.2 ^g
T ₄ Cu-ChNC (P)	45.82 ^{cd}	76.68 ^a	86.76 ^a	93.42 ^a	105.84 ^a	101.16 ^a	96.48 ^a	93.06 ^a
T ₅ COC (P)	46.36 ^{cd}	74.52 ^a	84.78 ^{ab}	90.18 ^b	101.16 ^b	98.1 ^{ab}	95.04 ^a	87.84 ^{bc}
T ₆ Chitosan (C)	35.28 ^{cd}	52.38 ^e	61.02 ^{de}	69.84 ^e	77.4 ^e	71.46 ^e	64.08 ^f	57.96 ^f
T ₇ CNP (C)	39.96 ^a	59.76 ^c	69.12 ^c	73.44 ^d	81.18 ^{cd}	79.56 ^c	74.52 ^d	71.64 ^d
T ₈ CuSO ₄ (C)	36.9 ^{bc}	54.36 ^{de}	57.06 ^f	59.58 ^f	67.5 ^f	61.38 ^f	58.86 ^{gh}	40.14 ^h
T ₉ Cu-ChNC (C)	38.7 ^{ab}	74.7 ^a	86.04 ^{ab}	90.72 ^{ab}	104.04 ^{ab}	98.28 ^{ab}	93.42 ^{ab}	89.82 ^b
T ₁₀ COC (C)	40.5 ^a	71.28 ^b	83.34 ^b	88.38 ^b	100.98 ^b	95.94 ^b	90.36 ^b	86.58 ^c
T ₁₁ Pathogen	36.36 ^{cd}	41.4 ^f	45.18 ^g	49.5 ^g	53.82 ^g	55.44 ^g	56.7 ^{hi}	58.5 ^{ef}
T ₁₂ Water control	34.56 ^d	37.98 ^g	40.5 ^h	43.56 ^h	50.76 ^g	52.56 ^g	53.64 ⁱ	56.88 ^f
S Em $\pm$	0.570	0.930	1.054	1.132	1.272	1.216	1.158	1.079
C. D. at 1 %	1.673	2.729	3.095	3.323	3.734	3.571	3.399	3.168

SE m $\pm$: Standard error of the mean; CD: Critical difference; DBI: Day before pathogen inoculation; DAI: Days after pathogen inoculation.

Table 4. Temporal dynamics of β -1,3-glucanase activity in chilli fruits under different treatments (μg glucose released g^{-1} FW)

Treatments	1 DBI	1 DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI	30 DAI
T ₁ Chitosan (P)	1.092 ^{ab}	1.224 ^a	2.016 ^{bc}	2.844 ^{bc}	3.624 ^{bc}	3.54 ^{bc}	3.288 ^{bc}	3.06 ^{bc}
T ₂ CNP (P)	1.128 ^{ab}	1.38 ^a	2.424 ^b	3.288 ^b	4.068 ^b	3.936 ^b	3.78 ^b	3.624 ^b
T ₃ CuSO ₄ (P)	0.84 ^b	1.188 ^a	1.536 ^{cd}	2.004 ^{de}	3.024 ^c	2.94 ^{cd}	2.784 ^c	2.676 ^{cd}
T ₄ Cu-ChNC (P)	1.116 ^{ab}	1.536 ^a	4.068 ^a	5.268 ^a	7.668 ^a	7.464 ^a	7.344 ^a	7.284 ^a
T ₅ COC (P)	1.176 ^{ab}	1.452 ^a	3.624 ^a	5.016 ^a	7.452 ^a	7.212 ^a	7.212 ^a	7.14 ^a
T ₆ Chitosan (C)	1.068 ^{ab}	1.164 ^a	1.86 ^{bcd}	2.532 ^{cd}	3.3 ^{bc}	3.384 ^{bcd}	3.18 ^{bc}	2.94 ^{bc}
T ₇ CNP (C)	1.128 ^{ab}	1.332 ^a	2.376 ^b	3.132 ^{bc}	3.888 ^b	3.78 ^b	3.696 ^b	3.492 ^b
T ₈ CuSO ₄ (C)	1.08 ^{ab}	1.14 ^a	1.452 ^{cd}	1.896 ^{de}	2.976 ^c	2.664 ^d	2.676 ^c	2.568 ^{cd}
T ₉ Cu-ChNC (C)	1.092 ^{ab}	1.464 ^a	3.912 ^a	5.1 ^a	7.584 ^a	7.296 ^a	7.2 ^a	6.936 ^a
T ₁₀ COC (C)	1.164 ^{ab}	1.416 ^a	3.576 ^a	4.932 ^a	7.38 ^a	7.104 ^a	7.008 ^a	6.744 ^a
T ₁₁ Pathogen	1.188 ^{ab}	1.224 ^a	1.332 ^d	1.536 ^e	1.608 ^d	1.692 ^e	1.776 ^d	1.92 ^d
T ₁₂ Water control	1.248 ^a	1.308 ^a	1.404 ^d	1.62 ^e	1.716 ^d	1.788 ^e	1.836 ^d	2.028 ^d
S Em $\pm$	0.017	0.020	0.040	0.054	0.077	0.075	0.073	0.071
C. D. at 1 %	0.050	0.060	0.119	0.160	0.227	0.220	0.215	0.208

SE m $\pm$: Standard error of the mean; CD: Critical difference; DBI: Day before pathogen inoculation; DAI: Days after pathogen inoculation.

β -1,3-glucanase activity

β -1,3-glucanase activity was significantly enhanced in Cu-ChNC treated fruits (Table 4). Preventive application (T₄) recorded 7.668 μmol glucose g^{-1} FW, representing approximately 4.77-fold increase (376.8 %) over the pathogen control (1.608 μmol glucose g^{-1} FW) at 15 DAI, followed by COC (7.452). Similarly, curative treatment T₉ (Cu-ChNC) recorded 7.584 μmol glucose g^{-1} FW, corresponding to 4.71-fold increase (371.7 %) compared with the pathogen control.

Chitinase activity

Chitinase activity showed the most pronounced response among the defence enzymes (Table 5). Preventive Cu-ChNC treatment (T₄) recorded 40.905 μmol N-acetyl glucosamine g^{-1} FW, which was approximately 16.3-fold higher (1529 % increase) than the pathogen control (2.51 μmol NAG g^{-1} FW). Similarly, curative application T₉ recorded 40.69 μmol NAG g^{-1} FW, representing about 16.2-fold increase (1520 % increase) compared with the pathogen control.

Effect of treatments on biochemical constituents of chilli fruits

Significant variations were observed in biochemical constituents including phenols, sugars, proteins and tannins among treatments.

Total phenols

Total phenol content increased significantly in treated fruits, with Cu-ChNC preventive treatment (T₄) recording the highest value (15.089 mg catechol equivalent g^{-1} FW at 15 DAI) (Fig. 1), which represented a substantial increase compared with the pathogen control, indicating activation of phenylpropanoid metabolism.

Crude protein

Crude protein content was significantly higher in treated plants. The Cu-ChNC treatment (T₄) recorded the highest protein content (23.562 mg g^{-1} FW), suggesting enhanced synthesis of defence-related proteins following nanoparticle treatment (Fig. 1).

Total and reducing sugars

Total soluble sugars and reducing sugars increased significantly in Cu-ChNC treatments (Fig. 1), indicating enhanced metabolic activity during defence response. Preventive Cu-ChNC (T₄) recorded the highest total soluble sugars (91.832 mg g^{-1} FW) followed by CNP (77.948) and reducing sugars (35.204 mg g^{-1} FW) at 15 DAI.

Tannins

Tannin accumulation was also significantly higher in Cu-ChNC treated fruits (Fig.1), with 7.52 mg tannic acid equivalent g^{-1} DW at

Table 5. Temporal dynamics of chitinase enzyme activity in chilli under different treatments (μg N-acetyl glucosamine released g^{-1} FW)

Treatments	1 DBI	1 DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI	30 DAI
T ₁ Chitosan (P)	1.414 ^a	4.462 ^c	6.981 ^d	9.157 ^e	15.043 ^c	14.471 ^d	12.962 ^d	11.829 ^d
T ₂ CNP (P)	1.443 ^a	6.962 ^b	13.49 ^b	20.11 ^b	29.824 ^b	28.414 ^b	27.533 ^b	26.019 ^b
T ₃ CuSO ₄ (P)	1.305 ^a	2.2 ^d	3.314 ^e	5.581 ^f	6.971 ^d	5.586 ^e	4.586 ^e	4.233 ^e
T ₄ Cu-ChNC (P)	1.538 ^a	8.733 ^a	16.795 ^a	29.29 ^a	40.905 ^a	39.824 ^a	37.919 ^a	35.219 ^a
T ₅ COC (P)	1.511 ^a	6.538 ^b	12.986 ^{bc}	18.6 ^c	28.762 ^b	27.629 ^{bc}	26.471 ^{bc}	24.514 ^{bc}
T ₆ Chitosan (C)	1.324 ^a	3.871 ^c	6.505 ^d	8.533 ^e	14.586 ^c	13.505 ^d	12.143 ^d	11.19 ^d
T ₇ CNP (C)	1.433 ^a	6.667 ^b	12.843 ^{bc}	19.352 ^{bc}	29.376 ^b	27.676 ^{bc}	26.91 ^{bc}	25.062 ^{bc}
T ₈ CuSO ₄ (C)	1.257 ^a	2.086 ^d	3.095 ^{ef}	5.062 ^f	6.876 ^d	4.571 ^e	4.462 ^e	3.771 ^e
T ₉ Cu-ChNC (C)	1.524 ^a	8.205 ^a	16.014 ^a	28.762 ^a	40.69 ^a	38.967 ^a	37.3 ^a	33.633 ^a
T ₁₀ COC (C)	1.491 ^a	6.319 ^b	12.176 ^c	16.967 ^d	28.41 ^b	26.471 ^c	25.519 ^c	23.614 ^c
T ₁₁ Pathogen	1.329 ^a	2.033 ^d	2.076 ^f	2.219 ^g	2.51 ^e	2.571 ^f	2.719 ^f	2.819 ^e
T ₁₂ Water control	1.213 ^a	1.886 ^d	1.986 ^f	2.1 ^g	2.452 ^e	2.552 ^f	2.667 ^f	2.743 ^e
S Em $\pm$	0.021	0.085	0.160	0.254	0.375	0.358	0.343	0.317
C. D. at 1 %	0.063	0.249	0.471	0.746	1.102	1.052	1.007	0.930

SE m $\pm$: Standard error of the mean; CD: Critical difference; DBI: Day before pathogen inoculation; DAI: Days after pathogen inoculation.

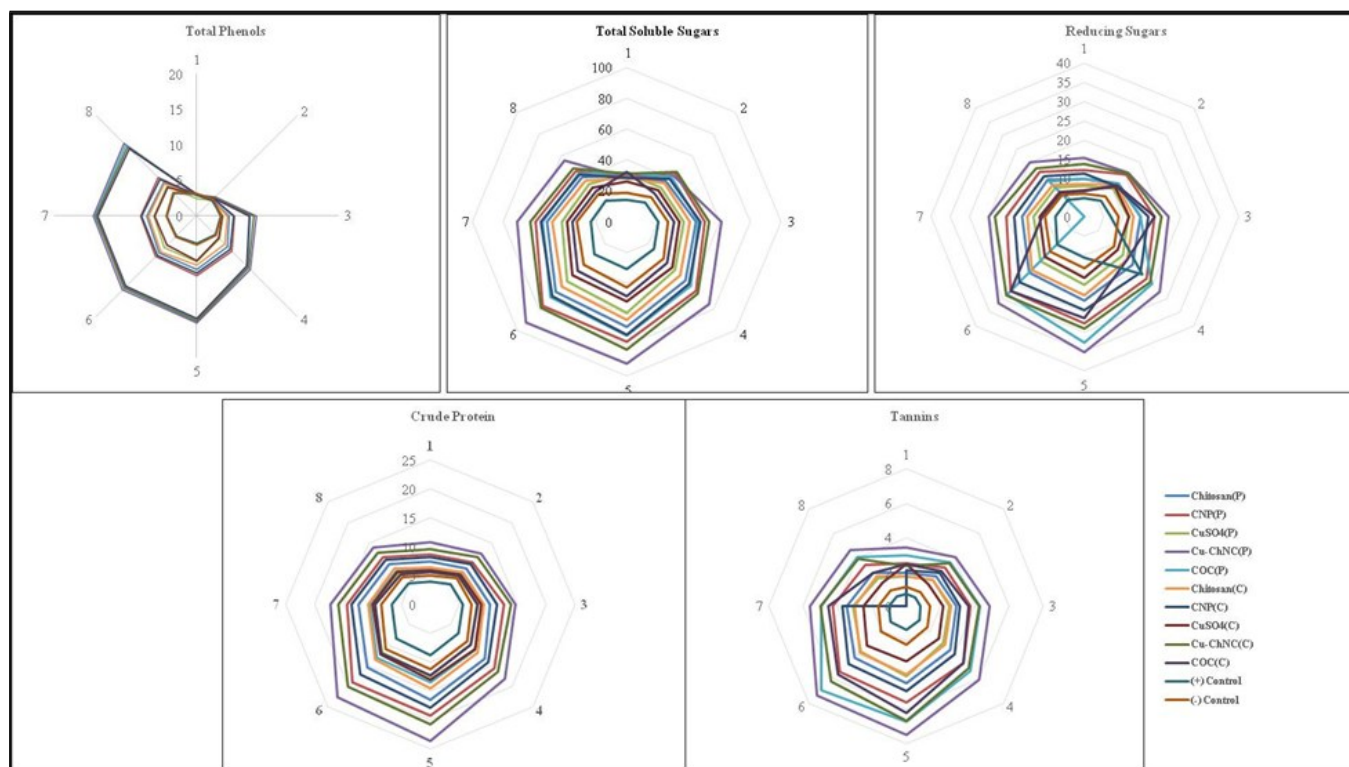


Fig. 1. Effect of different treatments on biochemical constituent's activity in chilli fruits following *Colletotrichum* inoculation.

15 DAI, indicating enhanced production of secondary metabolites involved in plant resistance by inhibiting pathogen enzymes and reducing fungal growth.

Disease severity and lesion development

Copper-chitosan nanocomposite treatments significantly reduced anthracnose severity (Fig. 2). Preventive application T_4 (Cu-ChNC) recorded the lowest mean PDI (11.875) with 74.73 % disease reduction over pathogen control and the lowest lesion length (4.425 cm). Highest AUDPC was observed at T_{11} (Pathogen control)

treatment 1065, while the lowest was recorded in the T_4 Cu-ChNC (P) was 347.5. The reduced disease severity can be attributed to dual action of Cu-ChNC, involving both direct antifungal activity of copper ions and elicitation of host defence mechanisms through chitosan. A radar plot (Fig. 2) integrating normalised mean PDI, lesion length and AUDPC clearly demonstrated that Cu-ChNC (P) (T_4) exhibited the lowest disease intensity across all parameters, followed by COC (P) and CNP (P). In contrast, the pathogen control and water control treatments recorded the largest radar area, indicating maximum disease severity.

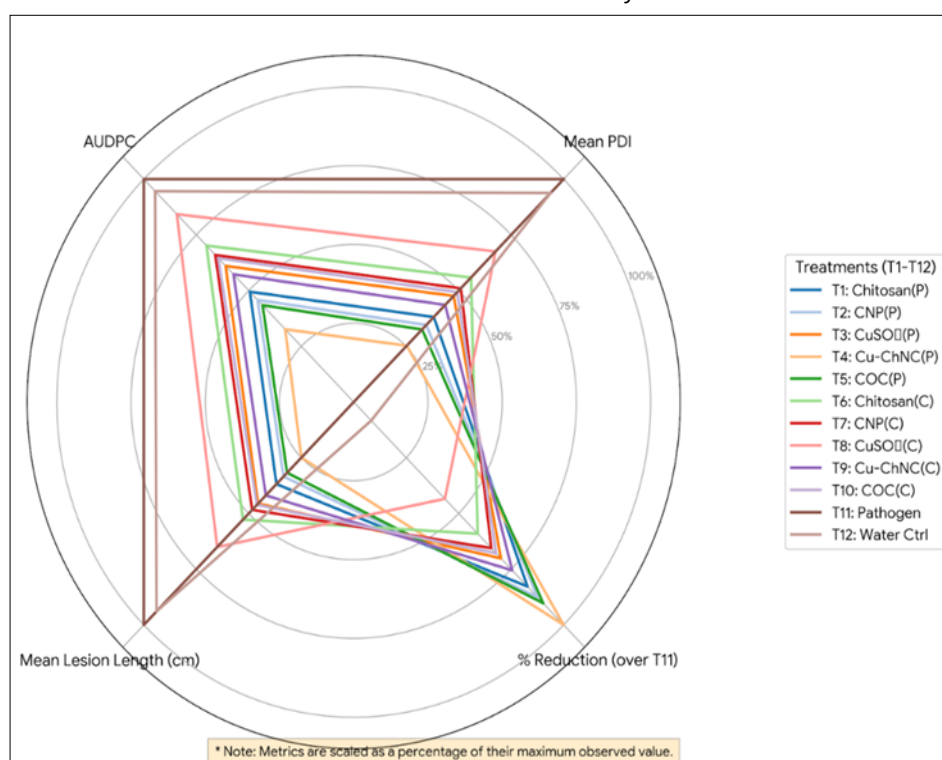


Fig. 2. Radar plot showing normalised values of mean percent disease index (PDI), mean lesion length, area under disease progress curve (AUDPC) and % reduction over control for different treatments against fruit anthracnose.

Correlation analysis between biochemical parameters and disease severity

The Pearson's correlation analysis was used to find out the relationship between defence enzymes and biochemical components as well as between biochemicals and PDI in the infected chillies with anthracnose (Fig. 3). From the analysis, it was noted that strong and significant positive correlations among various biochemicals which suggest a coordinated induction of the defence mechanism. Defence enzymes like POD, PPO, PAL, β -1,3-glucanase and Chitinase had positive correlations with one another ($r = 0.78$ to 0.97 , $p < 0.001$) (Pearson's correlation). The PAL had very strong correlations with β -1,3-glucanase ($r = 0.97$), total phenols ($r = 0.97$) and tannins ($r = 0.97$). It was seen that there was an association between phenylpropanoid pathway and pathogenesis related proteins because of pathogen infection. Likewise, PPO had strong correlations with POD ($r = 0.97$), PAL ($r = 0.95$) and tannins ($r = 0.95$). Metabolites like TSS, TRS, crude protein and tannins also had positive correlations with defence enzymes ($r = 0.68$ - 0.98) because of infection. The most notable correlation was seen between TRS and tannins ($r = 0.98$). On the other hand, PDI had strong negative associations with all biochemicals ($r = -0.72$ to -0.95 , $p < 0.001$). The highest negative correlation was observed for POD ($r = -0.95$), while PPO and tannins showed second strongest negative correlation ($r = -0.90$). These results indicate that increased activities of defence enzymes and accumulation of biochemical metabolites were associated with reduced anthracnose severity in chilli.

Hierarchical cluster analysis

Hierarchical cluster analysis based on correlation coefficients grouped the studied variables into two major clusters as mentioned in Fig. 4. Disease severity (PDI) formed a distinct cluster, clearly separated from biochemical parameters, indicating a strong negative association between disease incidence and defence-related traits. The remaining biochemical variables, including β -1,3-glucanase, phenols, chitinase, PAL, reducing sugars, tannins, proteins, total sugars, POD and PPO, clustered closely together. It is suggesting a strong positive correlation among defence enzymes and biochemical constituents. The clustering pattern indicates that enhanced biochemical defence responses are associated with reduced anthracnose severity in chilli.

The network diagram showed strong interconnections among most biochemical parameters, indicating coordinated activation of multiple defence pathways during pathogen infection (Fig. 5a). Defence enzymes including POD, PPO, PAL, β -1,3-glucanase and chitinase were closely interconnected with phenolic compounds, tannins and sugar metabolites, forming a dense interaction network. This suggests that oxidative enzymes, PR-proteins and phenylpropanoid metabolites function together as part of the induced defence mechanism. Total sugars and reducing sugars also showed strong associations with several defence enzymes, indicating their role in supporting metabolic activities during plant defence responses.

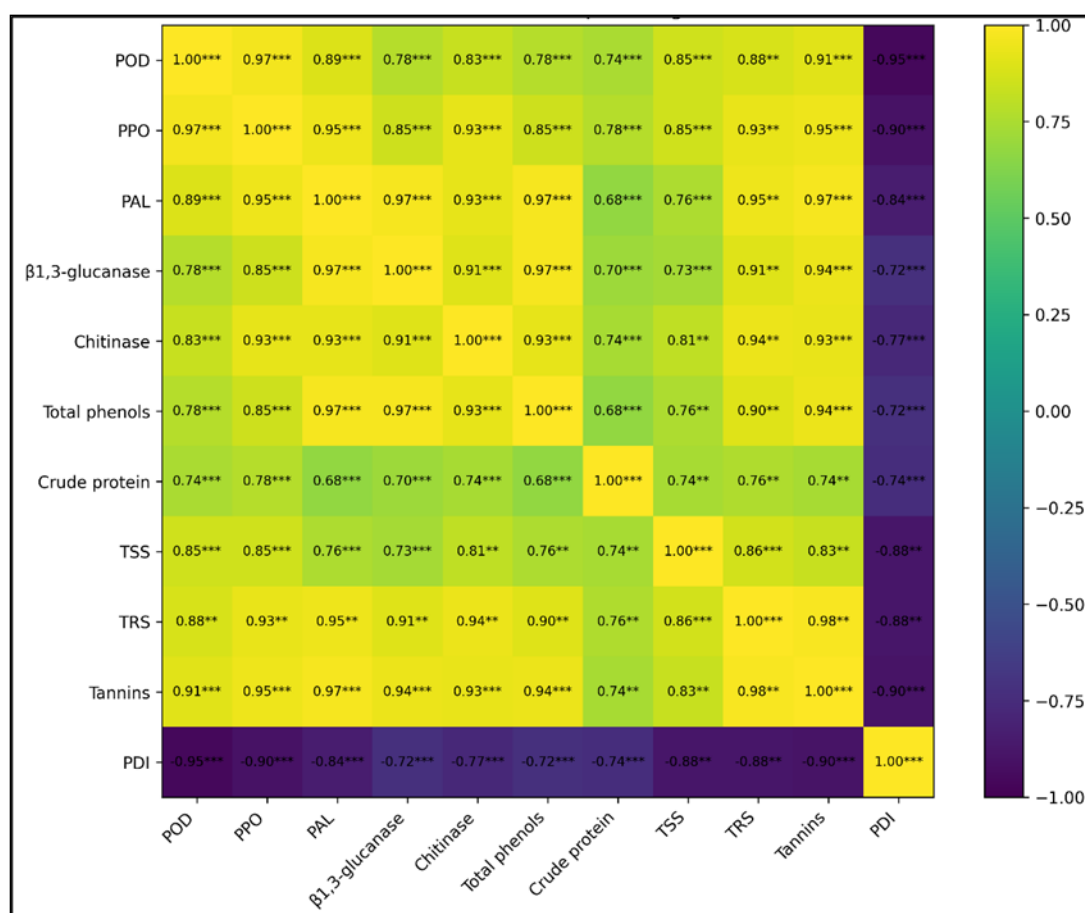


Fig. 3. Pearson correlation matrix heatmap showing the relationship between plant defence enzymes, biochemical constituents and disease severity (PDI) in treated plants.

The heatmap illustrates Pearson correlation coefficients among peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), β -1,3-glucanase, chitinase, total phenols, crude protein, total soluble solids (TSS), total reducing sugars (TRS), tannins and percent disease index (PDI). Positive correlations are indicated by yellow to green colours, whereas purple shades indicate negative correlations. The intensity of colour represents the strength of correlation. Asterisks denote statistical significance levels ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$).

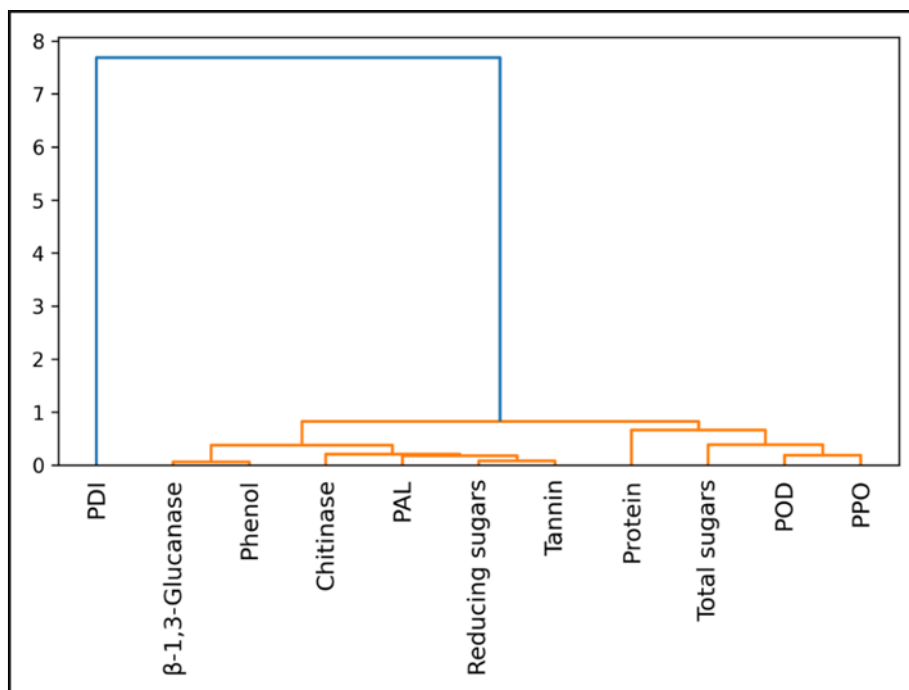


Fig. 4. Hierarchical cluster dendrogram.

Showing the correlation among biochemical parameters and disease severity (PDI) in chilli fruits. Defence-related enzymes and biochemical metabolites formed closely related clusters, whereas disease severity showed a distinct separation, indicating its negative association with biochemical defence responses.

In contrast, crude protein appeared relatively less connected with other variables, indicating a comparatively weaker association with the main defence network. Overall, the network structure highlights the coordinated interaction among enzymatic and biochemical components contributing to enhanced resistance against anthracnose in chilli.

Path coefficient analysis

Path coefficient analysis was performed to evaluate the direct and indirect effects of biochemical parameters on disease severity (PDI) in chilli anthracnose (Fig. 5b). The results indicated that PAL, chitinase and β -1, 3-glucanase exerted strong direct negative effects on disease severity, highlighting their key role in restricting pathogen development through activation of phenylpropanoid metabolism and degradation of fungal cell walls. The POD and PPO contributed indirect negative effects on disease severity through their association with phenolic accumulation and oxidative defence responses. Similarly, biochemical metabolites such as total phenols, tannins and sugars also showed indirect negative effects by supporting enzymatic defence mechanisms and collectively contributing to reduce disease severity.

Principal component analysis

Principal component analysis (PCA) was performed to determine the relationships among biochemical parameters and treatments and to identify the major variables contributing to disease resistance (Fig. 5c). First two principal components (PC1 and PC2) explained a large share of the total variance observed across treatments. The PC1 was mainly linked with defence enzymes such as POD, PPO, PAL, chitinase and β -1,3-glucanase, along with phenolic compounds, indicating their key role in resistance to anthracnose. The PC2 was largely associated with metabolites like sugars, proteins and tannins, reflecting metabolic changes under pathogen stress. Treatments with Cu-ChNCs clustered with defence-related variables, suggesting enhanced biochemical defence, whereas the pathogen-inoculated control was distanced from these, indicating lower activity and higher disease severity. These results suggest that Cu-ChNC treatments play a significant role in activating biochemical defence mechanisms in chilli plants against anthracnose disease.

Discussion

The present study demonstrated that Cu-ChNC significantly

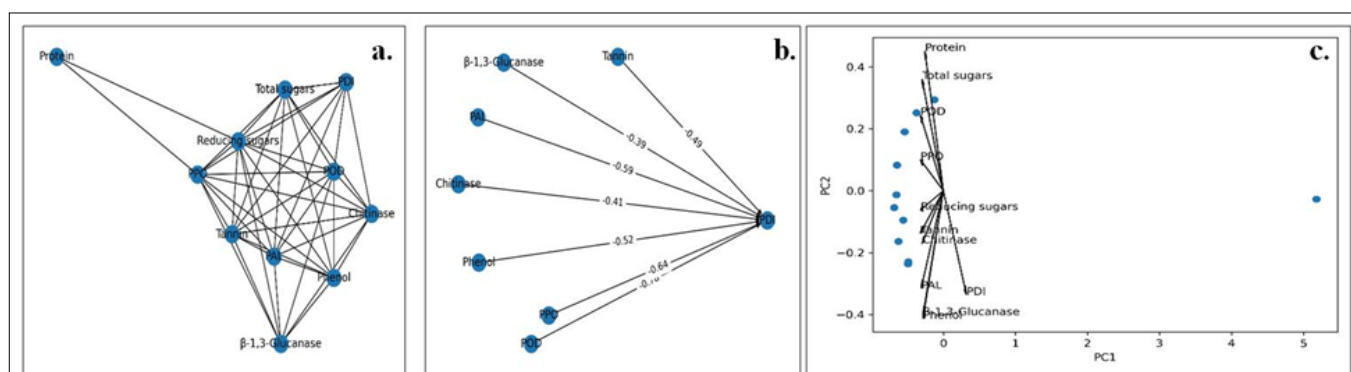


Fig. 5. Network correlation analysis and PCA illustrating relationships among defence enzymes, biochemical constituents and disease severity (PDI). (a) Correlation network among biochemical variables; (b) Correlation network between biochemical parameters and PDI (c) PCA biplot showing variable contribution to principal components.

enhanced biochemical defence responses in chilli plants and effectively reduced anthracnose severity caused by *C. truncatum*. The increased activities of defence-related enzymes, including POD, PPO, PAL, β -1,3-glucanase and chitinase, together with the accumulation of phenolic compounds and other metabolites, indicate that Cu-ChNC treatments activate multiple plant defence pathways.

Peroxidase and polyphenol oxidase are key oxidative enzymes involved in plant resistance to pathogen invasion. In the present investigation, Cu-ChNC treatment markedly enhanced POD and PPO activities, suggesting activation of oxidative defence mechanisms. These enzymes contribute to the formation of structural barriers through lignification and oxidative polymerisation of phenolic compounds, thereby strengthening plant cell walls and restricting pathogen penetration. Similar increases in oxidative enzyme activity have been widely reported as indicators of induced resistance in plants exposed to biotic stress (26, 27). Previous studies have also shown that chitosan-based nanoparticles stimulate oxidative defence enzymes through reactive oxygen species (ROS) signalling and activation of mitogen-activated protein kinase (MAPK) pathways, resulting in enhanced resistance against fungal pathogens (28, 29). It was also reported that 1.5–2-fold increase in POD activity in maize leaves treated with Cu-ChNPs (30), while a three-fold enhancement of POD in date palm seedlings treated with copper-chitosan nanocomposites (31).

The increased PAL activity observed in the present study further suggests activation of the phenylpropanoid pathway, which plays a central role in plant defense. The PAL catalyses the first step in the biosynthesis of phenolics, lignin, flavonoids and phytoalexins that contribute to antimicrobial defence and structural reinforcement of plant tissues (32). Enhanced PAL activity following nanoparticle treatment has been reported in several crop-pathogen systems. For instance, demonstrated that green-synthesised silver nanoparticles significantly increased PAL activity in rice plants infected with *Magnaporthe oryzae* (33). Similarly, two- to three-fold increase in PAL activity was reported in maize leaves treated with Cu-ChNPs compared with bulk chitosan treatments (30). Increased PAL activity in the present study likely contributed to the higher accumulation of phenolic compounds and tannins, which are known to inhibit pathogen growth and enhance plant resistance.

In addition to oxidative enzymes and phenolic metabolism, the activities of PR proteins such as β -1, 3-glucanase and chitinase were also significantly enhanced in Cu-ChNCs treated plants. These hydrolytic enzymes directly degrade structural components of fungal cell walls, thereby inhibiting pathogen growth and spread. The increased activities of these enzymes observed in the present study confirm the activation of PR-protein mediated defence responses. Earlier studies have also reported that CNPs act as effective elicitors that stimulate the production of PR proteins and enhance plant immunity (34, 35). Significant increases in chitinase and β -1, 3-glucanase activities were observed in finger millet plants treated with CNPs, highlighting the role of these enzymes in enhancing pathogen resistance through degradation of fungal cell walls and activation of plant defence mechanisms (36). Previous studies generally reported 2–5-fold increases in chitinase enzymes following nanoparticle treatments. The 16.3-fold increase observed in the present study is substantially higher, indicating a strong elicitation effect of Cu-ChNCs. Nanoparticle-based systems can

enhance the functional efficiency and stability of chitinase enzymes, as demonstrated in engineered nanomaterials such as UiO-66 frameworks (37).

The enhanced accumulation of phenolic compounds observed in Cu-ChNC treated plants further supports the activation of phenylpropanoid metabolism. Phenolic compounds contribute to plant defence through antimicrobial activity and by strengthening plant cell walls through lignin deposition. Similar increases in phenolic content following nanoparticle treatment have been reported in several studies. A two-fold increase in the total phenolic content was observed in date palm seedlings treated with copper-chitosan nanocomposites, indicating stimulation of plant immune responses (31).

Furthermore, increased crude protein content in treated plants suggests enhanced synthesis of defence-related proteins involved in stress responses. Similar observations were reported by previous researchers, who found that silver nanoparticle treatment increased total protein levels and other stress-related metabolites in chilli plants infected with *Colletotrichum capsica* (38). In their study, nanoparticle treatment mitigated pathogen-induced damage and restored protein synthesis, thereby improving plant defence responses. Sugars act as energy sources and signalling molecules during plant defence responses (39).

Correlation analysis in the present study further confirmed the relationship between biochemical parameters and disease resistance. Strong positive correlations among defence enzymes and phenolic compounds indicated coordinated activation of plant defence pathways, whereas negative correlations between biochemical parameters and disease severity suggested that enhanced biochemical responses contributed to suppression of anthracnose infection. Similar relationships between increased defence enzyme activity and reduced pathogen infection have been reported in several crops. For instance, enhanced activities of POD, PPO and PAL were correlated with increased resistance to *Colletotrichum* infection in chilli, tomato (40, 41). Likewise, elevated chitinase and β -1, 3-glucanase activities associated with enhanced immunity have been reported in finger millet following chitosan nanoparticle treatment (36).

Principal component analysis supported these findings by identifying defence enzymes and phenolic metabolites as the major variables associated with disease resistance. The Cu-ChNC treatments clustered closely with these biochemical variables, indicating their strong influence on plant defence responses. Similar multivariate patterns have been reported in studies on nanoparticle-mediated disease resistance where defence enzymes and phenolic metabolites were identified as major contributors to pathogen suppression (30, 36).

Path coefficient analysis indicated that defence enzymes such as PAL, chitinase and β -1,3-glucanase play a major role in reducing anthracnose severity in chilli by enhancing phenylpropanoid metabolism and degrading fungal cell walls (42). While biochemical metabolites provide supportive roles in strengthening plant defence responses (34).

Conclusion

Copper-chitosan nanocomposites (Cu-ChNCs) enhanced biochemical defence responses in chilli plants and reduced

anthracnose severity. The treatment increased activities of key defence enzymes and promoted the accumulation of phenolics and related metabolites, indicating activation of multiple defence pathways. Correlation and multivariate statistical analyses further revealed strong positive associations among defence enzymes and biochemical metabolites, while disease severity showed strong negative correlations with these parameters. Principal component and clustering analyses consistently identified defence enzymes and phenolic metabolites as the principal determinants of anthracnose resistance, highlighting the coordinated activation of biochemical defence networks in Cu-ChNC-treated plants. Overall, Cu-ChNC treatments effectively induced resistance against *Colletotrichum*-caused anthracnose, likely acting both as an antimicrobial agent and a defence elicitor that triggers ROS-mediated and systemic resistance in plants. Future research should focus on detailed molecular studies to unravel the underlying defence mechanisms, particularly gene expression, signalling pathways and ROS-mediated systemic resistance.

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Authors' contributions

MS carried out the study, participated in drafting the original manuscript, data analysis and statistical analysis. NSP contributed to the conceptualisation of the study, provided supervision, assisted in data interpretation and carried out the final validation of the manuscript. NKK, NUK, JM, BD, RS and KHP were involved in supervision, reviewing the manuscript and providing critical corrections and improvements to enhance its quality. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interests to declare.

Ethical issues: None

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