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Botrytis cinerea infection modulates trichome development and secondary metabolite biosynthesis in *Cannabis sativa*

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Abstract

Botrytis cinerea, the causal agent of gray mold or bud rot, is one of the most devastating necrotrophic fungal pathogens of *Cannabis sativa* L.. Its impact on trichome morphology and secondary metabolism is not well studied. The research aimed to characterize the *B. cinerea* infecting *C. sativa* in India and to determine the optimal temperature and humidity for sporulation, as well as its effect on plant trichome morphology and cannabinoid and terpene metabolic profiles. The pathogen was identified using both morphological and molecular methods using ITS and GAPDH. There was the most conidial production at 20°C and 94% relative humidity with a strong correlation of $r = 0.87$, $P < 0.001$. Scanning electron microscopy depicted the pathogen effect on the internal tissue parts, distinguishing prolific colonization and deformation of glandular capitate trichomes. GC-FID/MS and HPLC indicated important biochemical alterations in infected plants. Notably, infected plants exhibited a significant decrease in cannabidiol (CBD) levels (from 1.870% to 0.163%) and myrcene (from 5.788% to 1.206%), and, conversely, an increase in tetrahydrocannabinol (THC) (from 1.037% to 2.253%). Principal component analysis demonstrated the distant differences between infected and non-infected samples, indicating that the pathogen induces metabolic reprogramming. This study, the first report of *B. cinerea* on *C. sativa* in India, reported the pathogen adverse impact on trichome development and secondary metabolite profiling, which ultimately compromises the pharmaceutical quality of cannabis. The findings emphasize the need for integrated disease management and the development of resistant cultivars for sustainable medicinal cannabis production.

KEYWORDS Trichomes, *Botrytis cinerea*, *Cannabis sativa*, Cannabinoids

Introduction

Cannabis (*Cannabis sativa* L.) is a widely cultivated plant valued for its medicinal, industrial, and recreational uses. Cultivars are often classified based on their tetrahydrocannabinol (THC) content, with plants containing less than 0.3% THC in dry weight legally defined as hemp, and those exceeding this threshold referred to as marijuana[1]. The female inflorescence is covered with glandular trichomes and produces a range of secondary metabolites, including cannabinoids, terpenes, and tetrahydrocannabinol (THC), the main psychoactive compounds. To differentiate between cannabis types that have less than 0.3% THC and those that contain more than this threshold, an arbitrary criterion of 0.3% THC in the plants dry weight[2]. The legalized growth and distribution of cannabis for medicinal and potentially recreational

purposes is increasing in India, and this increased cultivation is likely to cause an increase in previously undescribed pathogens that can negatively affect the production and quality of the crop. Cannabis is affected by a range of pre and postharvest diseases, which can lead to yield losses of up to 30%[3]. These losses cause enormous losses in the pharmaceutical industry in the production of these crops for non-psycho- and medicinal purposes. Among the pathogens affecting cannabis, *Botrytis cinerea*, the causal agent of gray mold, is one of the most destructive necrotrophic fungi, infecting a broad range of crops cultivated under both field and protected conditions. This pathogen employs a multifaceted infection strategy, including the secretion of cell wall degrading enzymes to penetrate host tissues, production of phytotoxic metabolites to trigger oxidative stress, and induction of programmed cell death, ultimately leading to tissue maceration and nutrient acquisition[4]. With global control measures costs exceeding one billion euros per year in 2012, *B. cinerea* has become the second most economically important pathogenic fungus worldwide behind *Magnaporthe oryzae*[5]. This is an important gap in our knowledge because environmental factors influence the availability and dispersal of *B. cinerea* inoculum during critical growth stages of cannabis. Conidia are abundant in these tissues and can be spread by seeds, wind, water, rain, and infected pollen[6,7]. Consistent with these epidemiological patterns, *B. cinerea* infections of the top of inflorescences and stems of cannabis has been reported under field conditions previously in the United States and Canada[8–10], where it significantly reduces crop economic value. Recently, bud or gay mold pathogen (BCs) has also been shown to affect in cannabis field in India, indicating it is emerging relevance in new production regions.

Trichomes of the cannabis plant are essential structures that produce a variety of bioactive compounds, such as cannabinoids, terpenes, and flavonoids. However, previous studies reported that different types of glandular trichomes can be found on cannabis flowers, including capitate-sessile, capitate-stalked, and bulbous trichomes, which had been identified through structural assessments by scanning electron microscopy. Understanding the role of trichomes in cannabis physiology is essential for maximizing the potential benefits and economic value of plants[11,12]. Cannabis produces a diverse array of secondary metabolites, including phytocannabinoids and terpenes, which are responsible for its distinctive aroma, flavor, and therapeutic properties[13]. It had been demonstrated that terpenes and phytocannabinoids are synthesized and stored primarily in glandular trichomes located on the abaxial surface of inflorescence bracts[14–16]. Recent studies suggest that cannabinoids and terpenes have synergistic effects, referred to as entourage effects, from a medical perspective[17]. Accurate profiling of volatile terpenes and cannabinoids is typically achieved using gas chromatography-

mass spectrometry (GC-MS), while gas chromatography coupled with flame ionization detection (GC-FID) remains a widely used, cost-effective method for quantification due to its robustness and sensitivity[18]. Studies in other plant pathogen systems have shown that pathogen infections can alter the synthesis and accumulation of secondary metabolites, including terpenes and cannabinoids, underscoring the need for further investigation of how fungal pathogens, such as *Botrytis cinerea*, may influence cannabis phytochemistry. Understanding the effects of *B. cinerea* on the development of trichomes and the quantity of oil, particularly from a medical perspective, is important. For the analysis of volatile terpenes, GC-MS is the most commonly used method[19]. A GC-FID and MS analysis cannot detect these phytocannabinoids due to the high temperature reached, but THC can be easily detected due to its thermolabile nature. Consequently, high-performance liquid chromatography (HPLC) has been employed as a more suitable analytical method for detecting and quantifying cannabinoid compounds[20]. As per this study, *B. cinerea* plays a significant role in the secondary metabolism of *C. sativa*, particularly in the glandular trichomes, where cannabinoids and terpenes are synthesized. Once the pathogen colonized, the capitate trichome's structure and density were compromised, resulting in a significant loss of oil production and changes in phytochemical compounds above all decreased cannabidiol (CBD) and myrcene levels, and increased tetrahydrocannabinol (THC) levels. Therefore, this data suggests that *B. cinerea* affects the overall health of the plant and modifies its metabolic pathways likely through changes in stress signaling, which in turn, can alter the expression of biosynthetic-related genes. Such changes are not only expected based on what is observed with hemp; they fit within the broader context of fungal pathogenesis, where necrotrophic fungi like *B. cinerea* utilize phytotoxic secondary metabolites, such as the botrydial and botcinic acid, not only to also to rearrange host metabolism in favor of the fungal colonization process[21].

Currently, there is no known research, both nationally and internationally, that has investigated the impact of *B. cinerea* on trichome development, terpenes and cannabinoid production in *C. sativa*. Understanding the factors that affect the growth and maturation of trichomes in cannabis can be used to monitor and improve the quality of this important medicinal plant, both before and after harvesting. To address this gap, we initiated a comprehensive study to document the pathogen, its effects, and the resulting biochemical changes. This study is the first report of *B. cinerea* causing graymold (bud rot) on cannabis in India during 2021-2022. Considering the significance of *C. sativa* in India, this study intends to isolate, characterize, and assess the impact of *B. cinerea* on the loss of secondary metabolites. Additionally, we assessed the disease severity, measured the impact of the graymold pathogen on trichome development and bract

trichome density, and analyzed the correlation between environmental factors and the conidial production of *B. cinerea*. To our knowledge, this is the first study to apply both GC/MS and HPLC analysis to infected and non-infected cannabis tissues to observe changes in terpene and cannabinoid production. The effect of *B. cinerea* infection on trichome structures that are responsible for producing terpenes and cannabinoids has not been studied before. While cannabis produces both types of phytochemicals, the interaction between terpenes and phytocannabinoids in response to pathogen attacks is not well understood. This lack of understanding is particularly concerning given the plants use for both medicinal and recreational purposes.

Thus, studies on the potential effects of *B. cinerea* on cannabis plants are needed, as understanding the pathogen development and infection dynamics is crucial for managing this emerging disease and improving mold control strategies.

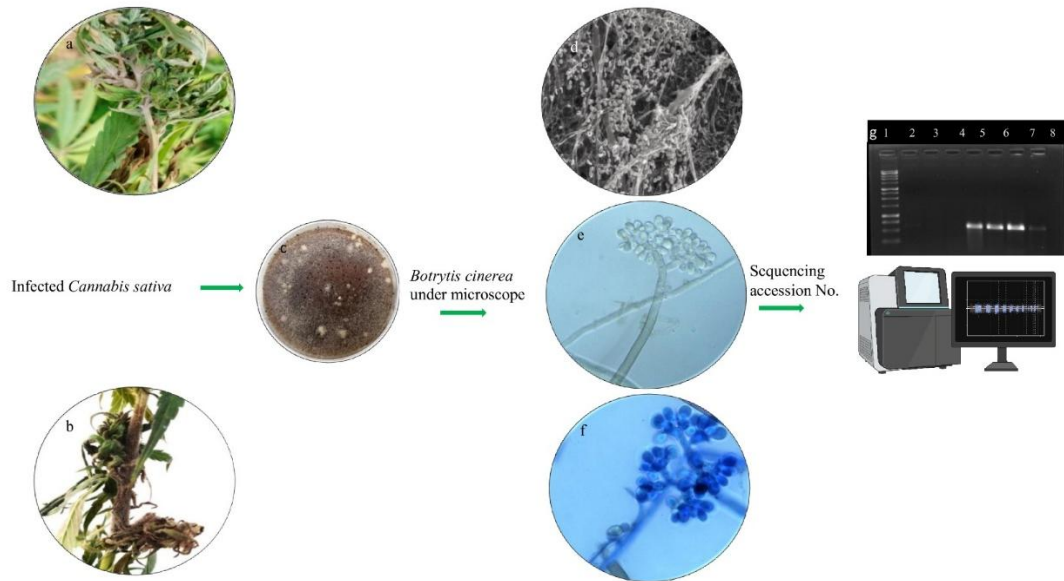
Results

Field observation and isolation of Botrytis bud rot

During a field trial at CSIR-CIMAP, Lucknow, India, from February-March 2022. The first appearance of infection by *Botrytis* on cannabis inflorescence during the five-week flowering period. Inflorescence tissues were observed with fuzzy-like symptoms with gray to brown spore masses. As the disease progressed, symptoms resulted in die-back with necrosis of the tissue and decay of the entire inflorescence (Fig. 1).

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Fig. 1



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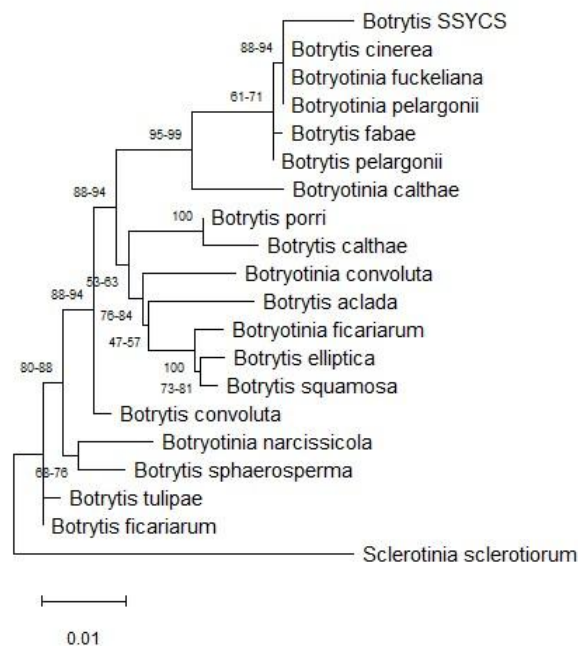
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176 (a-b) Erumpent fuzzy-like mycelium growth with sclerotia formation of *B. cinerea* (c) Colony morphology (d)
 177 The microscopic view of the single and ovoid mature conidia on the bract surface (e-f) Sporulation tip of a
 178 conidiophore bearing grape like cluster of conidia and septate, hyaline to brown mycelia (g) PCR assay
 179 amplification of the internal transcribed spacer (ITS) region of ribosomal DNA of *B. cinerea* from *C. sativa* in
 180 India showing ~700 bp. Lane 1-Marker (1- kb), Lane 2,3 & 4-asymptomatic samples, Lane 5,6, 7 & 8-
 181 symptomatic samples

Mycological characteristics

A causal fungal pathogen was isolated from freshly infected tissues. The mycelium growth and sclerotia formation on PDA was observed at 20 °C after 2 weeks. The conidia were single-celled, ovoid in shape, and colorless and measured 7.0 to 7.5-6.5 to 9.5 µm. The conidiophores were brown, slender, and branched with enlarged apical cells bearing clusters of conidia and produced tree-like conidiophores (Fig. 1). After three weeks, gray fungal colonies developed black, round, or irregular shaped sclerotia that measured 1.5 × 0.6 to 1.5 mm in size. Based on molecular identification, the isolate was identified as *Botrytis cinerea* and obtained the accession no. ITS (OP622939) and GAPDH (PP209529) from NCBI. Neighbor-joining (NJ) and maximum parsimony analyses of the combined DNA sequences of ITS and GAPDH generated a phylogenetic analysis tree (Fig. 2). Sequences included in the phylogenetic analysis were retrieved from the NCBI public database based. A distinct clade with 100% bootstrap support was formed among all *Botrytis* spp., separated from the closely related outgroup *S. sclerotium*. Isolates used

208 Fig. 2



209
210 Phylogenetic position of *Botrytis cinerea* (*Botrytis* SSYCS) within the *Botrytis* genus. Trees were constructed with maximum likelihood
211 methods for the GAPDH, and ITS loci and for the concatenated dataset (the best-fitting substitution model is indicated in parentheses). Bootstrap
212 values >50% are indicated above branches. The sequences of the isolate *Botrytis* SSYCS has been deposited in GenBank under accession
213 numbers PP209529 (GAPDH) and OP622939 (ITS), respectively.

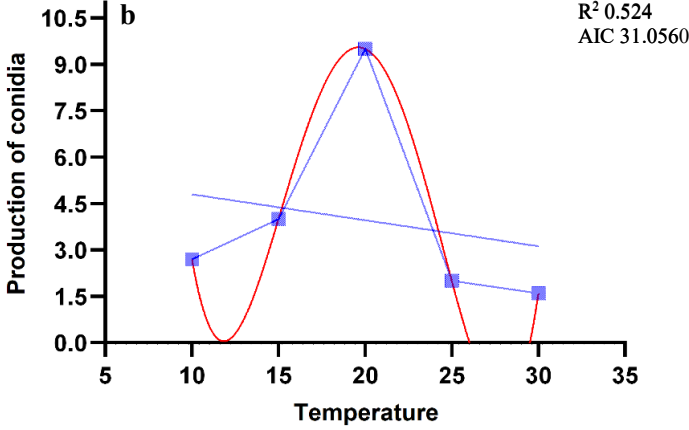
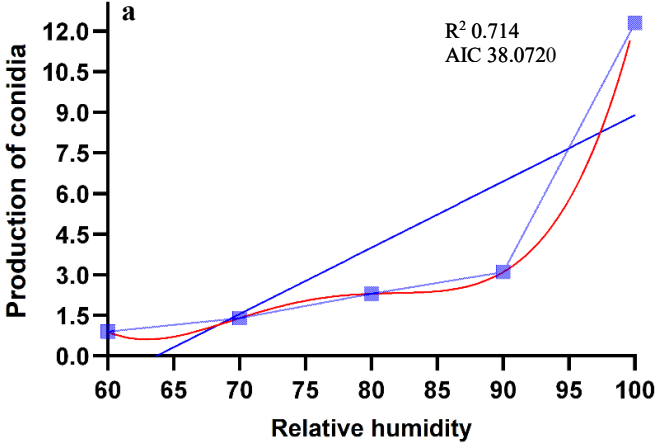
Influence of temperature and relative humidity on conidial productions

Conidial production was estimated at various ranges of temperature (T) and relative humidity (RH). Higher conidia production was observed at 94% RH at 20 °C. The optimal temperature ranged between 15 and 20 °C. The relationships between the temperature and conidial production were fitted with the above equation. The Pearson correlation coefficients between the in-transformed numbers of conidia produced on PDA and inflorescence were $r=0.65$ ($n=30, P<0.001$) and $r=0.87$ ($n=40, P<0.001$) for the temperature and RH experiments, respectively.

The polynomial equation provided a good fit for the data, with a low standard error of the estimated parameters, $R^2 \geq 0.524$ and $AIC = 31.056$ for T, and $R^2 \geq 0.714$ and $AIC = 38.072$ for RH, respectively (Fig. 3).

Conidia were produced on bunch trash at all values of temperature and RH tested: there were $2.7 \pm 0.80 \times 10^6$, $4.0 \pm 0.49 \times 10^6$, $9.5 \pm 0.97 \times 10^6$, $2.0 \pm 0.50 \times 10^6$, and $1.6 \pm 0.46 \times 10^7$ conidia/g developed at the temperature of 10, 15, 20, 25 and 30 °C, respectively. The maximum conidia production was $0.88 \pm 0.32 \times 10^6$, $1.39 \pm 0.40 \times 10^6$, $2.30 \pm 0.39 \times 10^6$, $3.07 \pm 0.50 \times 10^6$, and $12.30 \pm 0.77 \times 10^6$, at 60-100% relative humidity respectively (Tables 1 and 2).

Fig. 3



Equation	Production of conidia= -1015 + 37.05 RH -0.4357 RH ² – 0.001667 RH ³					
R ²	0.714					
R ² adjusted	-0.143					
AIC	38.720					
AICc	53.548					
BIC	28.376					
Parameter	Estimate	95% CI	SE	t	p-value	
Constant	-1015	-16670 to 14639	1232.1	-0.82	0.0825 ¹	
RH	37.05	-569.0 to 643.1	47.699	0.78	0.0886 ¹	
RH ²	-0.4357	-8.127 to 7.255	0.60528	0.72	0.6028 ¹	
RH ³	0.001667	-0.03035 to 0.03368	2.5198E-03	0.66	0.6280 ¹	

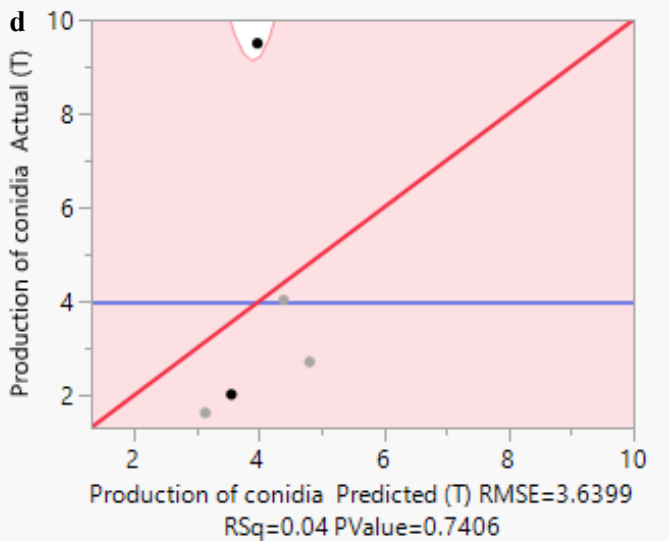
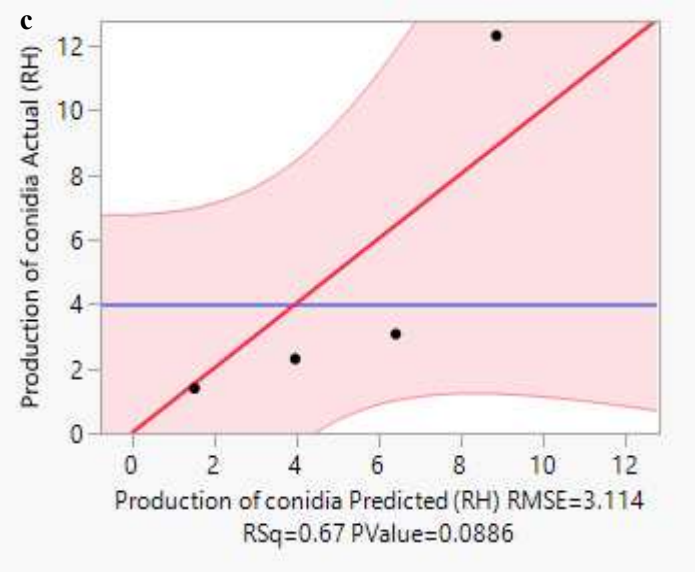
H0: β =0
The parameters is equal to 0.
H1: β ≠ 0
The parameters is not equal to 0.

¹Do not reject the null hypothesis at the 0.5% significance level.

Equation	Production of conidia= -23.02 + 3.96 Temperature – 0.1638 Temperature ² +0.001951 Temperature ³					
R ²	0.524					
R ² adjusted	-0.903					
AIC	31.056					
AICc	55.108					
BIC	29.937					
Parameter	Estimate	95% CI	SE	t	p-value	
Constant	-23.02	-16670 to 14639	1232.1	-0.82	0.5612 ¹	
Temperature	3.963	-569.0 to 643.1	47.699	0.78	0.5796 ¹	
Temperature ²	-0.1638	-8.127 to 7.255	0.60528	-0.72	0.6028 ¹	
Temperature ³	0.001951	-0.03035 to 0.03368	2.5198E-03	0.66	0.6280 ¹	

H0: β =0
The parameter is equal to 0.
H1: β ≠ 0
The parameter is not equal to 0.

¹Do not reject the null hypothesis at the 0.5% significance level.



Source	SS	DF	MS	F	p-value	H0: $E(Y X=x) = \mu$ The model is no better than a null model $Y = \mu$
Difference	228.6	3	76.2	0.83	0.6466	H1: $E(Y X=x) = \beta_0 + \beta_1 \times + \beta_2 \times + \beta_3 \times$ The model is better than the null model.
Error	91.4	1	91.4			
Null model	320.0	4	80.0			

Parameters and statistics of the equations describing the relationship of conidial production between different relative humidity (RH = 60 to 100%), incubation temperature (10 to 30 °C), and their interaction of *B. cinerea* on inflorescence bud in vitro. The lines represent the fitted curves. The straight blue line indicates the model trend, while the blue dotted line with squares represents the actual experimental data points of conidial

241 production. The red line depicts the predicted polynomial curve showing the relationship between T and relative RH. The black dots represent the
242 observed experimental data. The line represents the linear fit of observed and predicted values, intercept. Significant differences among temperature
243 were determined values with different letters indicate statistically significant differences ($p < 0.05$)

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245 Parameters and statistics of the equations describing the relationship of conidial production between different relative humidity (RH = 60 to 100%),
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249 observed experimental data. The line represents the linear fit of observed and predicted values, intercept. Significant differences among temperature
250 were determined values with different letters indicate statistically significant differences ($p < 0.05$)

Table 1 Effect of temperature on the relative humidity values obtained using distilled water or salts to prepare saturated saline solutions

*Salt or distilled water	Relative humidity (%) at the indicated temperature					
	10°C	15°C	20°C	25°C	30°C	35°C
NH ₄ NO ₃	60	62	63	63	62	63
NaNO ₂	70	-	72.5	71	73	-
(NH ₄) ₂ SO ₄	80	-	81	82	81.5	80
Na ₂ C ₄ H ₄ O ₆	94	93	94	93.5	92	92
Distilled water	100	100	100	100	100	100

Winston and Bates (1960); Dhingra and Sinclair (1985).

Table 2 *Botrytis cinerea*, inoculum sources and combination of environment conditions

Pathogen	Inoculum source	Environment Conditions	
<i>Botrytis cinerea</i>	Conidia	T: 10, 15, 20, 25, 30	RH: 60, 70, 80, 90, 100

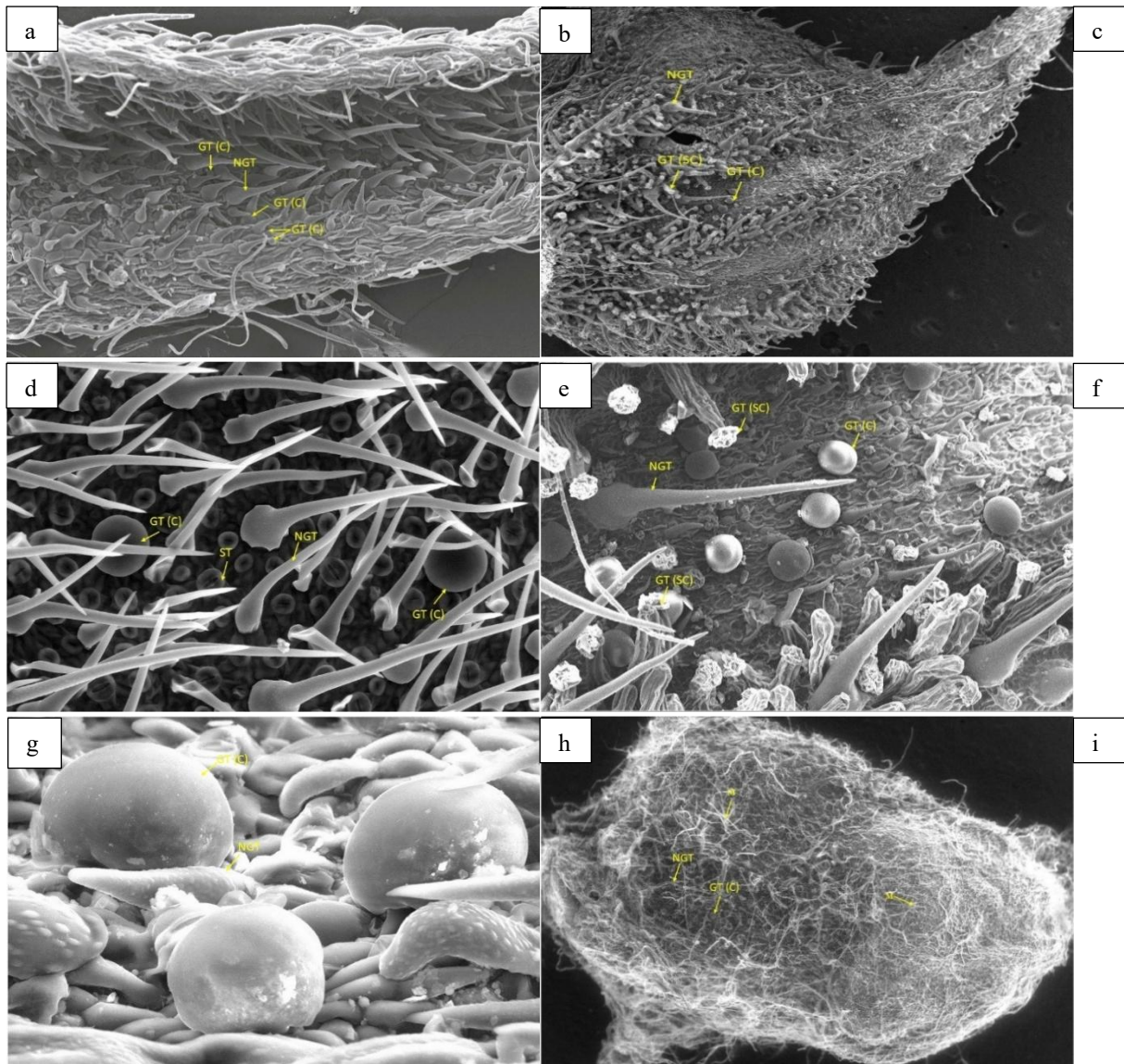
Experiment was performed thrice. T= Temperature, RH= relative humidity. All combinations of T and RH were tested systematically.

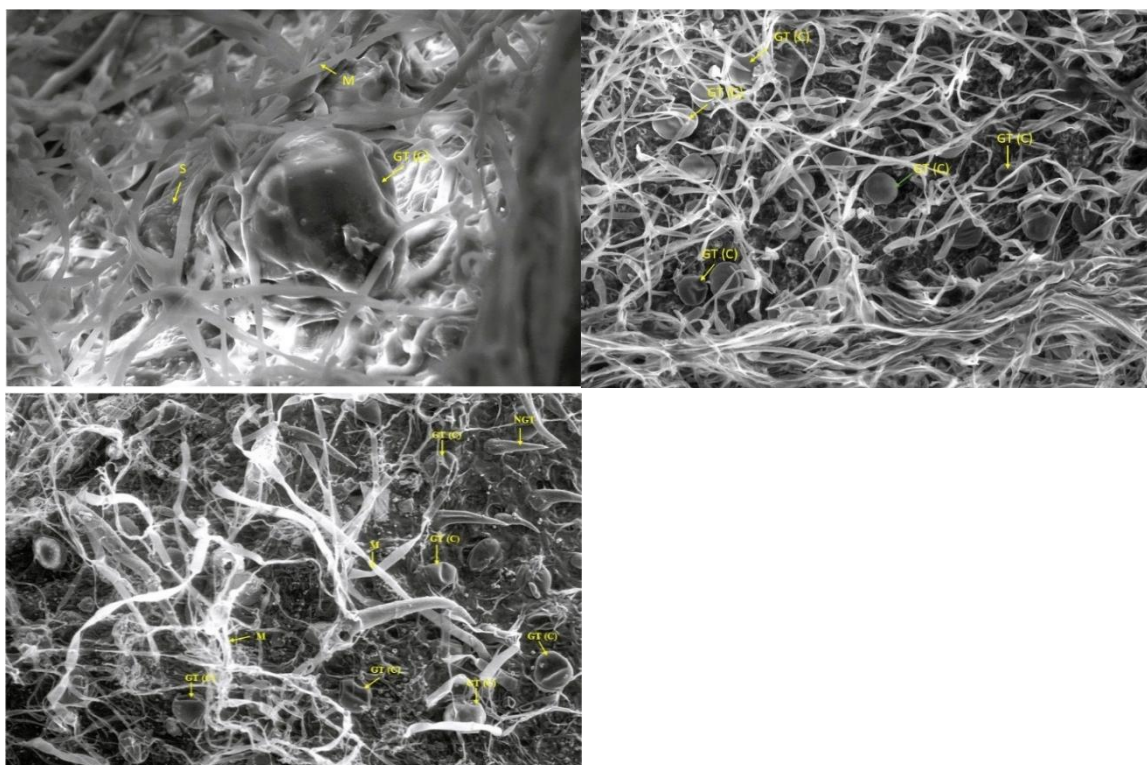
Determination of fungal propagules with the trichome glands

In this study, we observed three types of trichomes on the bracts of both healthy and infected plants. These included nonglandular (NGT) and glandular (GT) (stalk-capitate) trichomes, but we focused on capitate trichomes due to their ability to accumulate cannabinoid compounds and terpenes. Our observations showed that the trichomes were generally damaged, disrupted, and trapped with the mycelium and spores of the bud rot pathogen. These observations showed the invasion of the pathogen in different tissue layers (epidermis, collenchyma, cortical and medullary parenchyma, xylem, and phloem). We also noticed NGT hairs interspersed with sessile-capitate trichomes on seven-week-old bract tissues. The observations revealed remarkable colonization of the fundamental tissues, as well as the stele, with abundant mycelial filaments and microsclerotia of the pathogen. This study depicted the invasion of the pathogen in different tissue layers (epidermis, collenchyma, cortical and medullary parenchyma, xylem, and phloem). Under the scanning electron microscopy of *B. cinerea* infected buds showed extensive mycelial growth over the bud surface. Conidiophores and conidia were also observed by a SEM (FEI Quanta 250 FEG-SEM, Thermo Fisher Scientific). Observations of

275 conidiophore size and branching patterns; spore masses; and conidial size, shape and surface
276 topography were made at various magnifications (Fig. 4).

Fig. 4





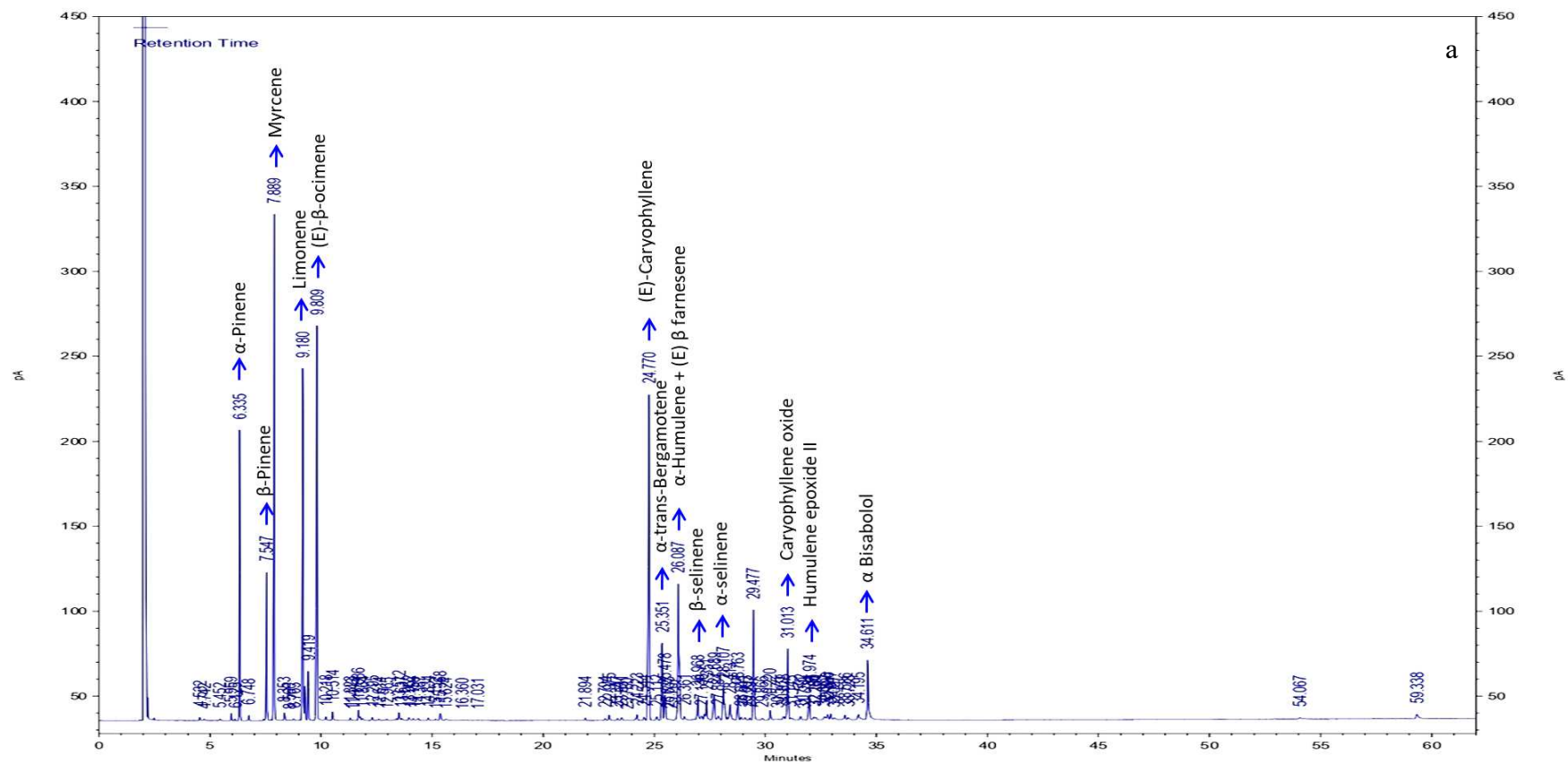
Microscopic images of bract and trichomes types (a- b) Healthy seven-week mature bract and seed (c) Healthy abundant Sessile- capitate and non- glandular trichomes with stomata (d) Stalked-capitate formed along with sessile-capitate and non- glandular trichomes (e) Clear view of sessile-capitate in healthy bract (f) Seven-week mature infected seed covered from *B. cinerea*. g-i) Mass of mycelium of *B. cinerea* that cover the surface of cannabis tissue. GT (StC)-Glandular trichomes (Stalked-capitate), NGT-Non- glandular trichomes, St- Stomata, M- Mycelium, GT (SC)-Glandular trichomes (capitate), S- Spores

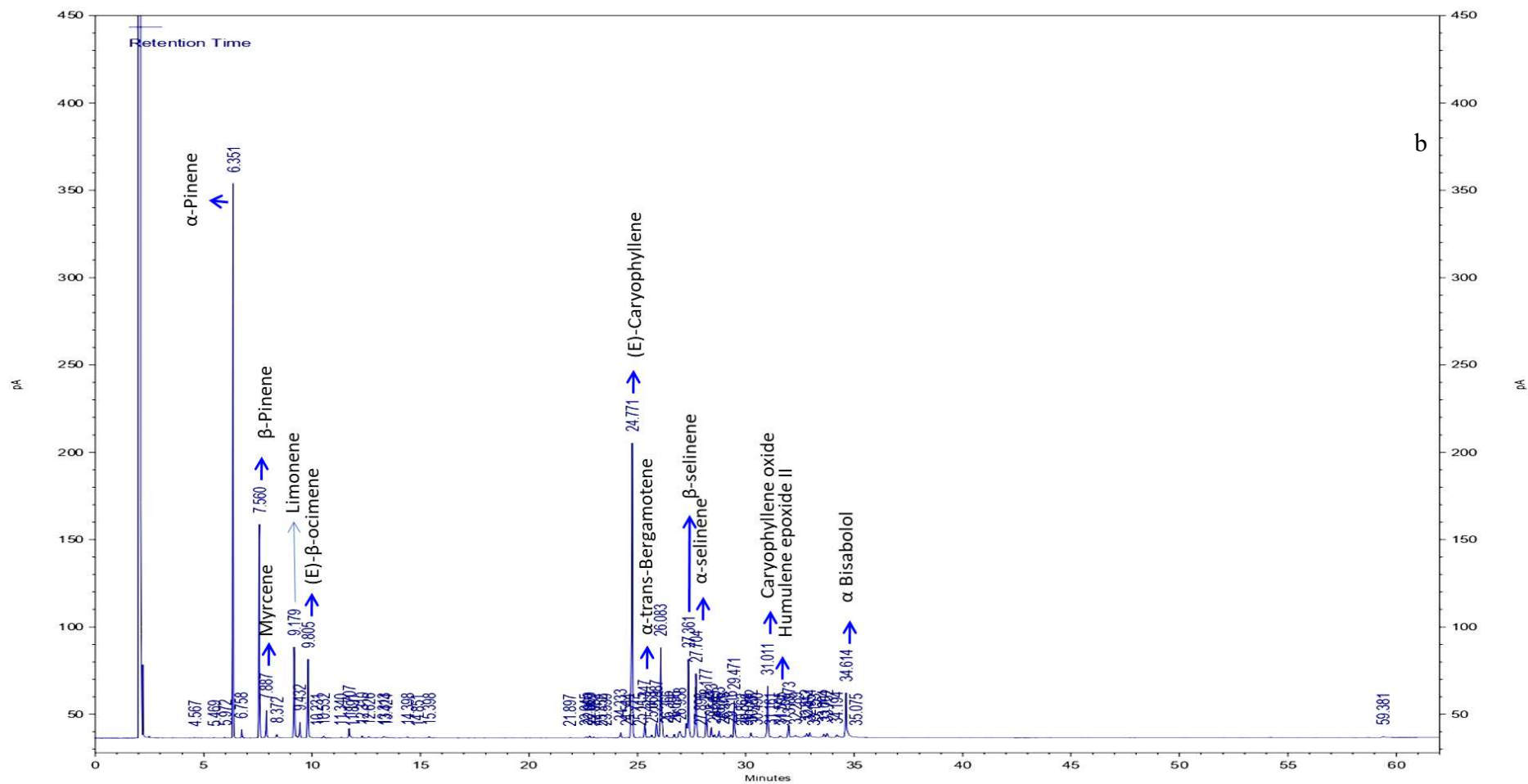
GC/GCMS

In this study, we analyzed the volatile organic compounds (VOCs) of the *C. sativa* Indian accession in a greenhouse and field level. The chromatogram of the extracted oil, depicted in Fig.5, displays the maximum proportion and concentration of thirteen significant phytochemical compounds. These phytochemicals were identified in both non infected and infected plants of greenhouse, comprising 77.517% to 92.385% of the total essential oil composition (Table 3). The non-infected plant has high levels of β -caryophyllene (18.541%) and myrcene (5.788%), both of which synergize with CBD content to enhance therapeutic properties. The limonene level was 37.073%, while the infected plants presented lower VOC emissions, including myrcene (1.206%) and reduced limonene levels. In the field, non-infected plants exhibited strong VOCs, particularly high concentrations of limonene (13.214%) and β -caryophyllene (11.384%), indicating robust health benefits and potential synergy with CBD. Conversely, the infected plant displayed diminished VOCs diversity and lower limonene (1.325%), reflecting a reduced defense capacity.

Overall, non-infected plants presented relatively high levels of beneficial VOCs, including limonene and myrcene, which may enhance CBD efficacy, whereas infected plants exhibited compromised profiles, highlighting the need for monitoring VOC emissions in disease management strategies. Additionally, high levels of limonene and myrcene are known to have synergistic effects with high CBD contents and low THC contents, further enhancing the therapeutic potential of these plants.

Fig. 5

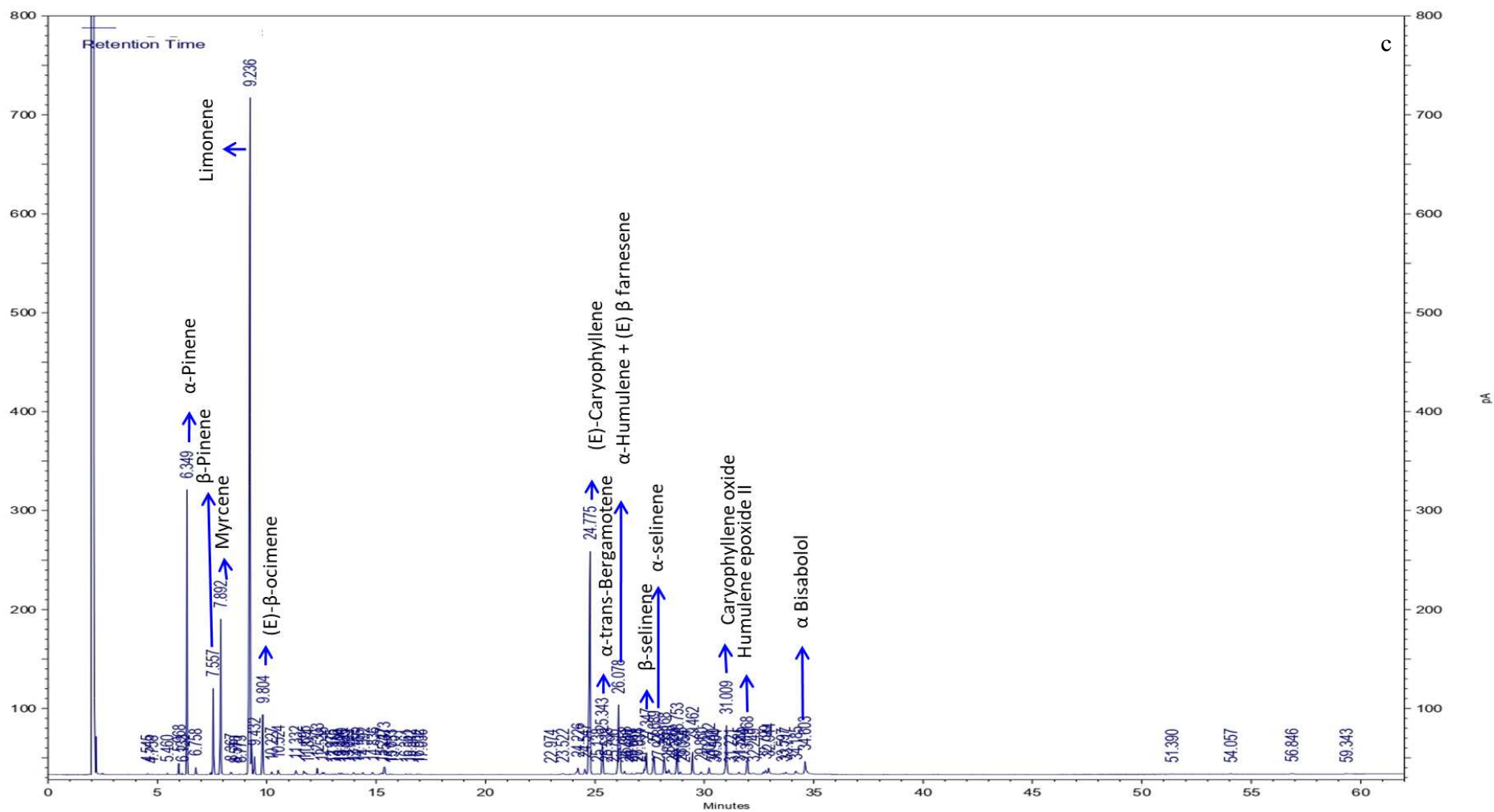


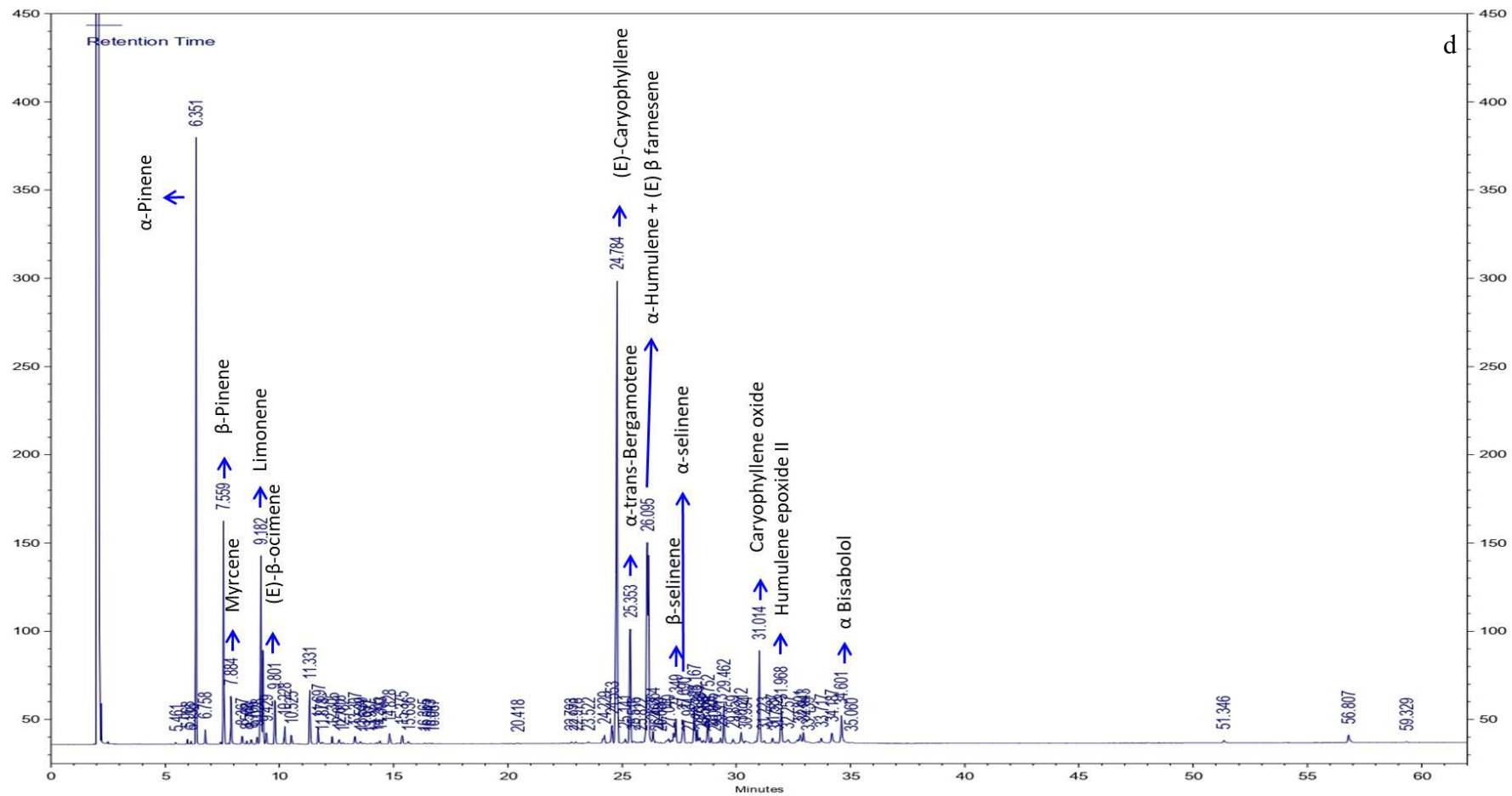


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337 Gas chromatogram (GC-FID) of essential oil isolated from *Cannabis sativa* (a) Non-infected (GH) (b) Infected (GH) (c) Non-infected (Field) (d)
 338 Infected (Field)

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340 Table 3 Total mean of essential oil composition and their concentrations in *Cannabis sativa*
341 in greenhouse and field

Compounds (%)	Non-infected (F)	Infected (F)	Non-infected (GH)	Infected (GH)
α -Pinene	6.864 $\pm$ 1.485	14.47 $\pm$ 0.021	9.738 $\pm$ 0.049	21.311 $\pm$ 0.001
β -Pinene	3.934 $\pm$ 0.118	5.825 $\pm$ 0.035	3.327 $\pm$ 0.002	8.945 $\pm$ 0.001
Myrcene	3.934 $\pm$ 0.221	5.825 $\pm$ 0.004	5.788 $\pm$ 0.002	1.206 $\pm$ 0.002
Limonene	13.214 $\pm$ 0.201	1.325 $\pm$ 0.004	37.073 $\pm$ 0.001	4.465 $\pm$ 0.003
(E)- β -ocimene	11.195 $\pm$ 0.003	8.055 $\pm$ 0.002	2.381 $\pm$ 0.002	3.767 $\pm$ 0.001
β -Caryophyllene	11.384 $\pm$ 0.014	1.249 $\pm$ 0.001	18.541 $\pm$ 0.001	13.225 $\pm$ 0.003
α -trans-Bergamotene	20.534 $\pm$ 0.013	13.287 $\pm$ 0.001	1.625 $\pm$ 0.006	0.843 $\pm$ 0.003
α -Humulene + (E) β farnesene	2.841 $\pm$ 0.004	4.153 $\pm$ 0.002	5.427 $\pm$ 0.020	- $\pm$ -
β -selinene	6.701 $\pm$ 0.001	14.113 $\pm$ 0.002	1.59 $\pm$ 0.010	5.827 $\pm$ 0.001
α -selinene	1.068 $\pm$ 0.014	1.495 $\pm$ 0.002	1.99 $\pm$ 0.000	4.382 $\pm$ 0.001
Caryophyllene oxide	1.284 $\pm$ 0.003	1.165 $\pm$ 0.001	2.922 $\pm$ 0.010	3.405 $\pm$ 0.003
Humulene epoxide II	2.806 $\pm$ 0.004	3.912 $\pm$ 0.003	0.879 $\pm$ 0.006	0.853 $\pm$ 0.001
α Bisabolol	0.980 $\pm$ 0.032	1.504 $\pm$ 0.001	1.110 $\pm$ 0.000	3.751 $\pm$ 0.001
Total concentration	86.749	76.381	92.385	77.517
RI ^a Experimental retention index, determined with reference homologous series of n-alkanes (C7-C30); F; Field and GH; Greenhouse. - $\pm$ -; Not detected				

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Cannabinoid's profiling

The HPLC analysis was conducted to examine the variation of cannabinoids in non-infected and infected inflorescences of Indian accessions (Table 4). The results indicated a lower content of total CBD in the infected plants (0.163%) compared to non-infected plants (1.870 %). In contrast, THC levels increased from 1.0437% in non-infected plants to 2.253% in infected plants. In greenhouse conditions, CBD content decreased form 1.417% in non-infected inflorescences to 0.033% in infected plants, whereas, THC levels increased from 0.820% in non-infected plants to 1.290% in infected plants.

Table 4. Total mean of cannabinoids content and terpenes phytoconstituents production in infected and non-infected inflorescences accessions

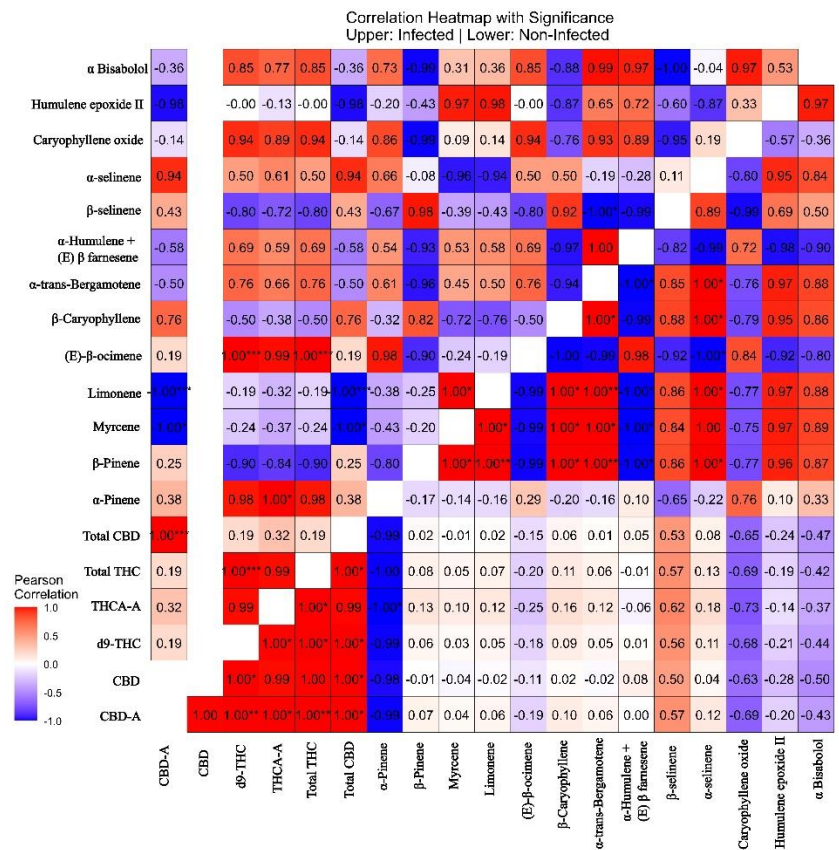
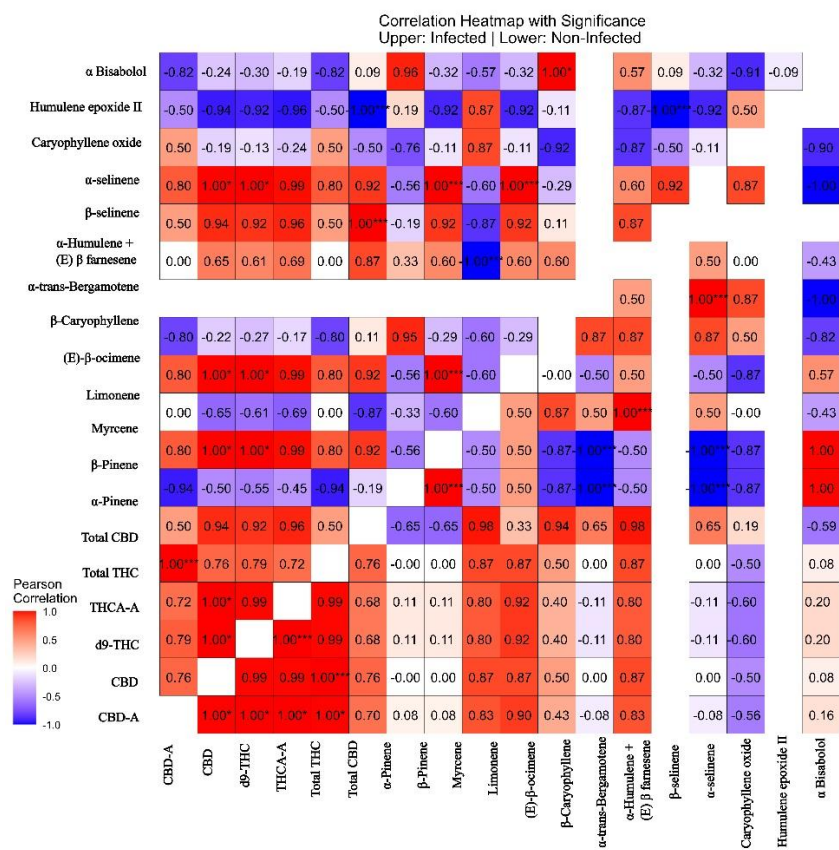
Cannabinoid compounds (%)	Non- infected (F)	Infected (F)	Non- infected (GH)	Infected (GH)
CBD-A	1.867±0.127	0.183±0.015	1.607±0.067	0.033±0.006
CBD	0.237±0.040	0.000±0.000	0.010±0.000	0.000±0.000
THCV	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
CBN	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
d9-THC	0.353±0.072	0.313±0.012	0.077±0.006	0.137±0.015
THCA-A	0.773±0.108	2.203±0.038	0.850±0.044	1.320±0.066
Total THC	1.037±0.171	2.253±0.046	0.820±0.044	1.290±0.072
Total CBD	1.870±0.147	0.163±0.015	1.417±0.058	0.033±0.006

F, Field GH, Greenhouse

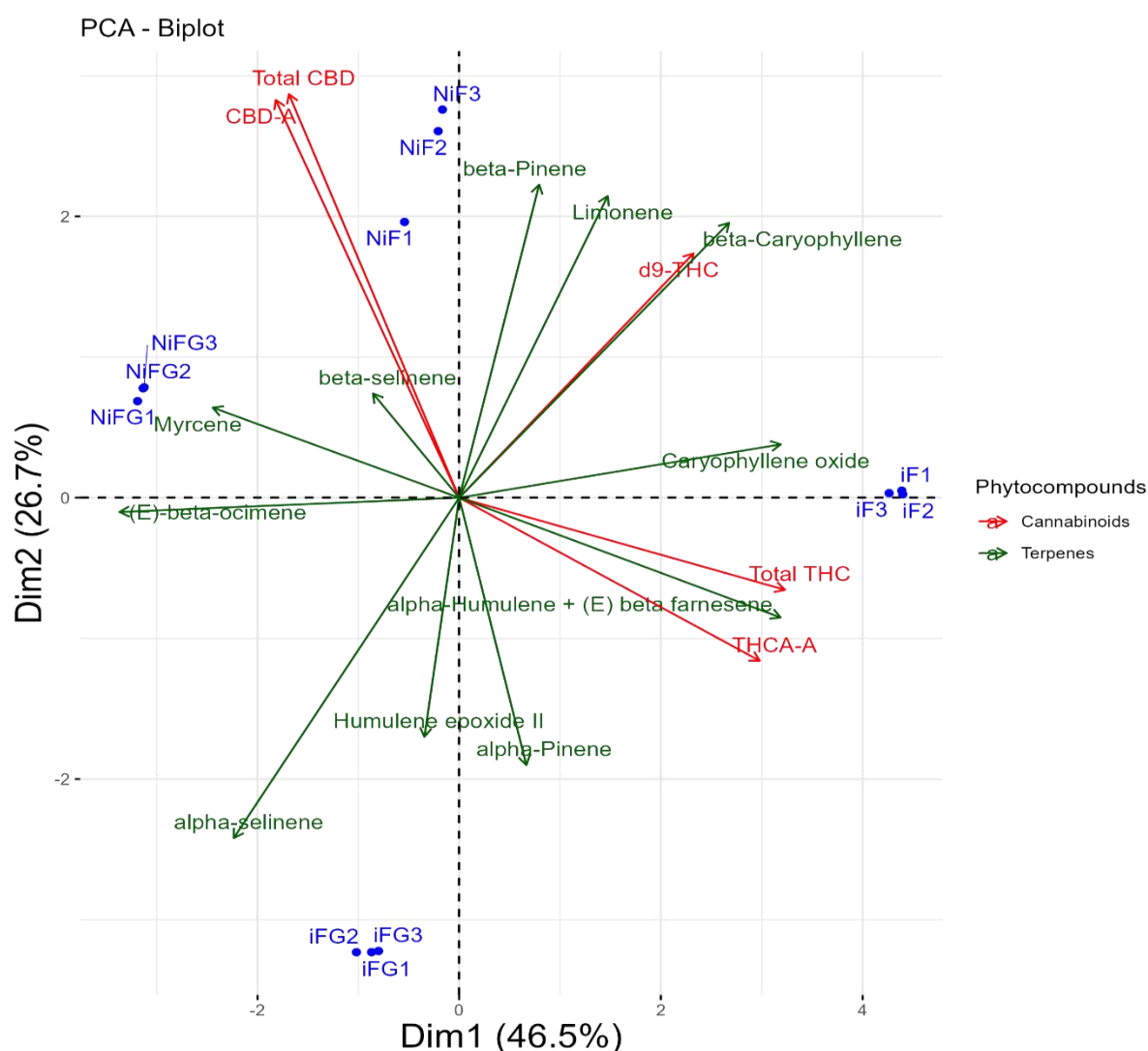
The dynamic between cannabinoids and essential oil elements

In this study, the interplay between cannabinoids and essential oil components in *C. sativa* was examined using HPLC, GC–MS, and GC analyses. These approaches quantified the major phytochemicals presents in the inflorescences. The localization of cannabinoid and terpene biosynthesis within glandular trichomes, particularly the capitate sessile and capitate stalked types is well established in the literature, and our SEM observations qualitatively supported trichome morphological alterations, potentially disrupting their biosynthesis activity following *B. cinerea* infection (Fig. 7). Although our chemical analyses cannot directly determine trichome specific biosynthesis, the observed changes in metabolite profiles correspond with infection induced structural disruptions. Further PCA analysis showed distinct clustering of infected and non-infected samples, with monoterpenes and sesquiterpenes, such as myrcene, β-caryophyllene, and limonene, showing positive correlated with total cannabinoid content, especially CBD (Fig. 8). The first two principal components (PC1 and PC2) explained 73.2 %

378 of the total variance. The biplot revealed distinct grouping of non-infected field (NiF) and
379 greenhouse (NiFG) samples in the second quadrant of the plot. Similarly, infected field (iF)
380 and greenhouse (iFG) samples were grouped differently into first and third quadrants,
381 respectively. The THC and CBD contents are clearly displaying opposite directions aligning
382 with the infected and non-infected samples, respectively.



387 Fig. 8



388

389 PCA biplot illustrating the distinction between infected and non-infected *C. sativa* samples
 390 based on their terpene and cannabinoid profiles. NiFG- non-infected greenhouse, NiF- non-
 391 infected field, iF- infected field and iFG- greenhouse.

392 Pathogenicity and disease assessment

393 The pathogenicity of the recovered representative isolate of *B. cinerea* was tested with three
 394 replicates. After seven days of inoculation, mycelium colonization and rapid necrosis
 395 developed on intact plants, detached inflorescences, and leaves. Gray sporulation of the
 396 pathogen was gray to black at the margins and pale gray at the centers, resembling the original
 397 bud rot symptoms observed in the sampled field. The inoculated fungus was reisolated from
 398 the newly developed plant tissue on PDA, fulfilling Koch's postulates and confirming the
 399 pathogenic behavior of *B. cinerea*. Mock-inoculated intact plants, detached inflorescences, and
 400 leaves in the control plants remained symptomless and disease-free, and no fungus was
 401 recovered (Fig. 6). The disease rating scores confirmed that pathogen was the most virulent

402 and caused the most severe bud rot. Inoculation of flower buds with pathogen revealed that
403 most extensive necrosis and mycelial growth were caused by *B. cinerea* under humid
404 experimental conditions, and the buds were completely colonized after 7 days, with a disease
405 severity rating of 4 (significantly different at $P < 0.01$). The disease incidence was estimated
406 to be approximately 65-70% in severely infected fields on the basis of the plant population.
407 The data from all the experiments were averaged, and the standard deviation was calculated
408 from the replications and repetitions of each experiment.

Fig. 6



Symptoms development on cannabis leaves, inflorescences, mature plants with *B. cinerea* inoculation. (a-b) detached leaf assays (d-e) intact plants (g-h) detached the inflorescences (c, f, i) control

Discussion

Among the diseases previously reported to affect cannabis inflorescences grown under field or greenhouse conditions, gray molds[8,22-26] as illustrated in figure 1 and 2. In the present study, the isolate was morphologically and molecularly identified as *B. cinerea*, confirming previous reports. Its occurrence on cannabis inflorescences represents the first report of what is likely an opportunistic infection on this host[9,10,27]. Gray mold (bud rot), caused by *B. cinerea*, is a common problem during the autumn season, when cooler nights and humid daytime conditions favor the disease, as previously observed on other crops. Trichomes are produced in large numbers on the bracts of cannabis inflorescences and lead to defense mechanisms in plants[1,28]. In the present study, pathogen infection altered both the production and morphology of trichomes, increasing tissue susceptibility to pathogen attack. Gray mold causes dark brown discoloration and internal rot of buds. Cannabis strains with large inflorescences and tightly clustered bracts that retain moisture are considered more susceptible to infection[23].

The main source of inoculum for colonizing cannabis inflorescences is airborne conidia. Spores of *B. cinerea* have been detected in field, greenhouse and post-harvest areas such as harvesting and drying rooms. These airborne spores can be a common cause of allergic reactions in humans[29,30]. Infection of cannabis inflorescences may take place during periods of high spore release, although no prior studies have been confirmed this, and further research is needed to determine the environmental conditions that favor successful infection and disease development. In tomato, airborne inoculum levels associated with 50% flower infection were reported to be 170, 295 and 178 conidia/m³ at 15 °C, 20 °C and 25 °C, respectively[31]. Information on the relationships of spore and climatic conditions to disease incidence in cannabis inflorescence was currently lacking. In this present study, disease incidence of gray mold was recorded on cannabis inflorescences under controlled conditions at 20 °C. A conidial suspension of 5.0×10^4 conidia mL⁻¹ was highly virulent and produced disease at 20 °C and 94% RH. This study revealed high conidia production occurred at 20 °C, whereas no conidia were produced at 10°C. The impact of high relative humidity at specific temperatures on spore germination may be used to predict disease risk in the field. Our current results on relative humidity and conidial germination *in vitro* align with previous findings regarding *B. cinerea* infection in chickpeas and other crops [32].

Figures 4, 5, and 6 illustrate the results related to bud rot symptoms, which typically develop during the final 2-3 weeks of the flowering period in the late summer and autumn seasons. Symptoms are rarely observed in spring or early summer, when greenhouse temperatures exceed 25 °C, a condition unfavorable for pathogen growth. Similarly, in field conditions, bud

rot symptoms are most common in the autumn season. The former species was recently isolated from bud rot-infected hemp flowers in Oregon at a low frequency (two out of 23 isolates), and was shown to be equally virulent to *B. cinerea*[8]. In this study, relative humidity higher than 94% was critical for the conidial germination of *B. cinerea*.

As illustrate in Tables 3 and 4, these pathogens pose serious concerns for cannabis industry and pharmaceutical sector because cannabis inflorescences produce economically important volatile terpenoid compounds. Previous research has shown that cannabis trichomes synthesize monoterpenes and sesquiterpenes, such as β -myrcene, limonene, α -pinene, β -caryophyllene, and α -humulene[1,33]. These terpenes have been reported to exhibit therapeutic properties, including anti-inflammatory, antimicrobial, and analgesic effects. Our findings support previous reports that volatile mono- and sesquiterpene compounds produced in aerial plant tissues possess medicinal properties. In this study, we conducted a comprehensive analysis of bioactive compounds in healthy and infected cannabis inflorescences. These terpenoids are produced in stalked glandular trichomes produced on the bracts [2]. Our research focused on terpenoid and cannabinoids production, and alterations in stalked glandular trichomes. Terpenes are industrially important components of essential oils used in nutritional, cosmetic, and medicinal products. Few studies have examined the production of terpenoids in response to pathogen invasion. A deeper understanding of their biological activity remains needed. Only a few studies have examined terpenoid production in response to pathogen invasion, and our study contributes new insights into these interactions. Results shown in Figure 7 indicate that terpenoid and cannabinoids synthesis differed between healthy and infected samples. Infection suppressed several characteristic cannabinoids while altering terpene profiles, perhaps because the pathogen interferes with the legitimate secondary metabolic pathways of the stressed plant[24,34]. Also, the injury inflicted on glandular trichomes may obstruct cannabinoid and terpene biosynthesis, thus restricting compound accumulation[35]. Our findings suggest that *B. cinerea* infection can alter terpene production and trichome structure, potentially reducing the market value of cannabis flowers. This pathogen can completely cover the inflorescence of plants, rendering them unsuitable for pharmaceutical and industrial use. Understanding the impact of the bud rot pathogen on inflorescence development is crucial, as bud rot can destroy flowers within a week. In the present study, SEM images illustrated, for the first time, the morphological changes that take place during the infection of plants.

A novel aspect of this study is the observation that *B. cinerea* infection significantly impacts trichome integrity. *B. cinerea*, a necrotrophic fungal pathogen known to infect a wide range of plant species, has been previously reported to compromise glandular structures and impair

secondary metabolism[36]. According to our findings, this pathogen affects both the morphology of capitate trichomes and their biosynthesis capacity, potentially altering the composition of terpenoids and cannabinoids. These structural changes may impair the plants chemical defense mechanisms, which are largely mediated by trichome-derived compounds. Furthermore, our data revealed significant positive correlations between cannabidiol (CBD) levels and specific terpenes, particularly myrcene, β -caryophyllene, and limonene. Previous research suggests that the pathways for cannabinoid and terpene biosynthesis are coordinated. Terpenes such as β -caryophyllene have also been shown notable pharmacological effects, including anti-inflammatory and analgesic properties, that may synergize with cannabinoids via the "entourage effect"[33]. As a consequence, the observed correlations may have both physiological and therapeutic significance. The interplay between pathogen infection, trichome function, and secondary metabolism in *C. sativa* presents an important area for further research. Further transcriptomic and metabolomic studies are needed to clarify the molecular mechanisms involved and to support breeding efforts aimed at improving resistance and maintaining phytochemical quality.

This occurrence on cannabis inflorescences represents the first report of *Botrytis cinerea* in India, which is likely an opportunistic infection of this host at the maturity stage and postharvest. Cannabis flowers become vulnerable to *B. cinerea* when the plant's maturity coincides with specific environmental conditions and the presence of the pathogen. Graymold affects bract leaves, leading to a decrease in terpenoid production and impacting the development of trichomes. In this pioneering research, we conducted a comprehensive analysis of the bioactive compounds present in cannabis-infected and healthy inflorescences. Our findings provide valuable insights into the disparities between the two and reveal the detrimental impact on terpene production. The results of this study lay the foundation for future research into the metabolic regulation of medicinally important secondary metabolites in *C. sativa* glandular trichomes. Future research should focus on controlling and managing bud rot pathogens at specific times during inflorescence development. *Botrytis* disrupted the cuticle of the glandular and caused a reduction in essential oil content compared with that in uninfected cannabis plants. These significant findings hold substantial relevance within a rapidly expanding industry, wherein the quality of cannabis flowers is increasingly imperative for both medicinal and recreational applications.

In conclusion, this study is of utmost importance since it represents the first report of *B. cinerea* on *C. sativa* in India and its repercussions upon trichomes and secondary metabolite profiles. Infection altered cannabinoid (CBD↓, THC↑) and terpene profiles, that ultimately affect the oil

quality. The production of conidia was apparently highest at 20°C and 94% RH, on *C. sativa*. Further, transcriptomic studies must be done in order to dissect this interaction, followed by breeding programs and biocontrol application. These findings provide crucial information for developing disease management strategies and for ensuring quality control in the expanding cannabis pharmaceutical sector.

Materials and Methods

Isolation of pathogen from cannabis tissues

During the plant disease survey from February-March 2022, severe symptoms of graymold and bud rot were observed on the inflorescence of *C. sativa* Indian accession in a field trial at CSIR-Central Institute of Medicinal & Aromatic Plants, Lucknow (N 26° 53' 41.1396", E 80°13 58' 56.2512"), India. The disease symptoms were mostly observed on inflorescences and stems, with gray or brownish masses of spores produced in the infected tissue. The flowers were necrotic and covered by graymold. The disease progressed and led to complete necrosis of the inflorescence. Additionally, infected flower parts fell and became attached to adjacent leaves, and new fungal colonies developed from these fallen residues, leading to the formation of necrotic spots on leaves. Plants with visible symptoms of the disease were photographed. A small portion of the diseased inflorescence tissue segment (~1 mm²) was excised and surface sterilized in 5% (v/v) NaOCl solution for 30 seconds, rinsed three times in sterile distilled water, placed onto potato dextrose agar (PDA) supplemented with 100 mg/L streptomycin sulfate, and incubated at 19-22 °C for ten days[9]. Emerging colonies were transferred to fresh PDA for subsequent identification at the genus level via general morphological criteria, including colony color and size and microscopic examination of spores. Hyphal tip subcultures were transferred and stored on PDA slants at 4 °C prior to identification and used for molecular identification.

Cultural and morphological characterization and histopathological observation

A pathogenic strain of graymold was isolated and characterized on the basis of the cultural, morphological and microscopic features of the isolates reported in available references, such as the descriptions provided in previously reported literature[10,37]. Under *in vitro* conditions, fungal colony features such as color (surface) and growth were evaluated on PDA and OMA culture media after 7 days of incubation at 22 °C with alternating UV-A light (315–400 nm; Philips TL-D 18W BLB) (12-h light/12-h dark) with the wavelength range of approximately

320-400 nm to induce sporulation, after which the fungal structure was scraped with water to harvest conidia as previously described[38]. Morphological identification was based on the morphological features of the produced conidia and sclerotia (shape and size) in the culture media. Microscopic slides of the fungal isolates were prepared via lactophenol and lactophenol cotton blue, and the slides were subsequently examined under an optical microscope (Euromex, magnification $\times 40$) with an Olympus BH2 light microscope[24].

Molecular identification

Total DNA was extracted from the isolate via the method of CTAB and DNA used as a template in different PCR assays [39,40]. Genus-level identification was initiated using the internal transcribed spacer (ITS) region, which serves as the universal DNA barcode for fungi but lacks species-level resolution within the *Botrytis* genus[40,41]. Because of recent work showing the high extent of diversity within the *Botrytis* genus, the analysis was extended using sequences for nuclear protein-coding genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) due to its higher phylogenetic resolution at the species level and provide a distinguishable closely related *Botrytis* species[42]. The resulting sequences of the ITS and GAPDH genes were deposited in GenBank to obtain accession numbers. A phylogenetic tree was built with the concatenated sequences of ITS and GAPDH using ClusterX and MegaX[40,43]. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) were shown next to the branches, as described by branch support[44]. *Sclerotinia sclerotiorum* was used as the outgroup.

Effects of temperature and relative humidity on conidial production

A bud was inoculated with a conidial suspension (10^4 conidia mL^{-1}) prepared from five day old colonies of *B. cinerea*. The concentration of conidia was measured using a hemocytometer under a light microscope. The suspension was placed on the bud equator, and inoculated buds were incubated inside humidity chambers (plastic boxes) under dark conditions for one week at different combinations of temperatures. To create various relative humidity values, 100 ml of different saline solutions or distilled water (Table 1) was placed in each plastic box. To evaluate the effects of temperature, relative humidity, and their interactions on conidial production, were evaluated using linear or nonlinear regression models. Further analysis was conducted to study the interaction between temperature, relative humidity, and conidial production via the following equation[45]:

$y = [a \times \text{Teq}^b \times (1 - \text{Teq})]^c / [1 + \exp(d - e \times \text{RH}/100)]$ (equation 1) and $y = [a \times \text{Teq}^b \times (1 - \text{Teq})]^c \times \exp[-d \times \exp(-e \times \text{WD})]$ (equation 2), where y is the rescaled infection incidence; Teq is the equivalent of T calculated as $(T - T_{\min}) / (T_{\max} - T_{\min})$, and T is the temperature regime (in °C), and $T_{\min}$ and $T_{\max}$ are the minimal and maximal T for infection, respectively, which are considered equation parameters. A completely randomized experimental design was used for each treatment. Temperature effects were compared using one-way ANOVA followed by Tukey's HSD post hoc test. Each RH level was replicated in three separate humidity chambers.

The production of conidia increased as the relative humidity (RH) increased from 70% to 95%. The following polynomial equation was used to fit the relationship between RH and conidia production:

The following formula was used to determine the relationship between rescaled conidial production (y) and the relative humidity (RH):

$$y = f(\text{RH})$$

Profiling of terpenes using GC-FID and GC–MS analysis

Fresh female inflorescence of *C. sativa* was collected at 4 weeks old from greenhouse (GH) and field (F) plots. The samples were extracted using hydro distillation in a Clevenger apparatus to produce essential oils. The oil yield was estimated as relative percentage units on the basis of fresh weight (200 g), and the oils were transferred into 10 mL headspace vials. After extraction, the oil was dried over anhydrous sodium sulfate (Na_2SO_4) and stored in sealed glass vials in a refrigerator before analysis. Terpene profiling was carried out using gas chromatography with flame ionization detection (GC-FID) and gas chromatography–mass spectrometry (GC-MS). To perform this analysis, an Agilent GC 8890 series system with a flame ionization detector (FID) and an HP-5 fused silica capillary column (5% phenyl-polymethyl siloxane stationary phase; 30 m length $\times$ 0.32 mm internal diameter; 0.25 μm film thickness) were used. The oven temperature was programmed to hold at 60 °C for 3 min, followed by a linear gradient of 20 °C/min to 290 °C. The temperature was then held constant for 8 min[46,47]. Nitrogen was used as the carrier gas at 1.5 mL/min, and the injection volume was 0.2 μL (diluted in acetone: 1:1) with a split ratio of 1:130. Quantification of major terpenes was conducted using external calibration curves generated from certified reference standards

injected in triplicate, and only linear regressions with $R^2 > 0.99$ were accepted. Sample concentrations were calculated by interpolating peak areas into the calibration equations and corrected for dilution. For compound identification, GC-MS analysis of *C. sativa* was carried out on a Clarus 680 GC interfaced with a Clarus SQ 8C Quadrupole mass spectrometer with an electron ionization source at 70 eV in positive mode, as per the reported method[47,48]. The contents of phytochemicals present in the test samples were identified on the basis of comparisons of their retention time (min), peak area, peak height and mass spectral patterns with those in the spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) library[49].

Phytocannabinoids analysis

For HPLC analysis, cannabinoids (CBDA, CBD, THCV, THCA, d9-THC and CBN) concentrated stock standard were used (Cayman Chemical, USA). These stock standards were prepared into working concentration in methanol (HPLC Grade) and stored at -20 °C for further use. Standard stock solutions were serially diluted in methanol to obtain a five-point calibration range (0.5-200 µg/mL). Each concentration was injected in triplicate under identical HPLC conditions. Calibration curves were generated by plotting peak area versus concentration, and linear regression was applied. Only calibration curves with $R^2 \geq 0.999$ were accepted. Female inflorescences from both healthy and infected plants were shade-dried, finely ground, and extracted using a solvent mixture of acetonitrile and ethanol (9:1, v/v). HPLC analysis was conducted using a Prominence-i LC-2030C-3D Plus system (Shimadzu, Japan) equipped with a quaternary pump, autosampler, column oven, and photodiode array (PDA) detector. Cannabinoid compounds were separated on a Phenomenex Luna C18(2) column (150 × 4.6 mm, 5 µm) under isocratic conditions with a mobile phase comprising acidified water (0.25% acetic acid), acetonitrile, and methanol in a 2:7.5:0.5 (v/v/v) ratio. The flow rate was maintained at 1.0 mL/min, with the column temperature set at 35 °C. A 10 µL sample was injected for each run, and chromatographic separation was completed within 30 minutes. The chromatographic data were acquired at 210-400nm for quantitative analysis of the cannabinoids. The total THC and CBD compounds were calculated based on the following formula:

$$\% \text{ Total THC} = \% \text{ THC} + (\% \text{ THCA} \times 0.877)$$

$$\% \text{ Total CBD} = \% \text{ CBD} + (\% \text{ CBDA} \times 0.877)$$

Occurrence of fungal association with inflorescence tissue

To evaluate whether the formation and density of trichomes were affected by pathogen, seven-week-old, infected bracts of inflorescence were harvested for scanning electron microscopic observation at eight weeks. For this purpose, naturally infected adaxial surface of the bract tissue from an inflorescence bearing pistils and trichome was segmented ($0.5 \times 0.5 \text{ mm}^2$) and immersed in 4% glutaraldehyde at 4 °C overnight. After vacuum infiltration for 30 min, the infected tissues were washed five times for 5, 10, 15, 20, and 20 min, respectively, with 0.1 M sodium phosphate buffer (PBS, pH 6.8). The specimens were dehydrated in a graded series of ethanol: 30%, 50%, 70% and 80% for 15 min each; 90% for 20 min each; and 100% alcohol twice for 30 min. Finally, the specimens were incubated in acetone for 30 min and isoamyl acetate twice for 15 and 30 min in three biological replicates. The specimens were desiccated using CO₂ and coated with gold in a sputter coater for 30 s at a current of 10 mA. The object being examined was placed inside the SEM chamber, and then, an electron beam with a current of 50 pA at 3 kV was used. High-resolution images were then captured with a scanning resolution of 3072×2207 at a working distance of 4 millimeters. To ensure accuracy, the images were collected over 128 low-dose scanning passes, and drift correction was applied to the 15 kVBC SEM image[40,50]. The experiment was conducted twice with a replicate batch of samples.

Detached pathogenicity assay and disease assessment trials

Healthy ten-week-old cannabis detached inflorescences (buds), leaves, and intact plants were harvested arbitrarily to conduct pathogenicity tests for a representative *B. cinerea* isolate[25] detached inflorescence and leaves were surface sterilized in 10% bleach solution for 20-50 s and then washed twice in sterile deionized water. The buds were placed inside a sterile plastic container lined with sterile moistened filter papers. The leaves were subsequently placed on PDA plates. Healthy inflorescence was inoculated with mycelial plugs (8 mm in diameter) and mock inoculated with noncolonized PDA plugs. The containers were sealed and wrapped in a plastic bag to maintain high humidity. The containers and petri dishes were incubated under ambient conditions for lesion development. The inoculation of intact plants was conducted by spraying a spore suspension of 10^6 spores/mL with some modifications[25]. Disease symptoms were observed after 7 and 14 days. Typical gray mold symptoms developed on the inoculated plants, whereas the control plants were asymptomatic. Pathogenicity trials revealed no significant differences in symptoms and disease severity from those described in the literature[22]. A rating scale of 0-5 was used, where = healthy bud, no mycelium visible, tissues

green; 1 = <10% mycelial growth on the bud surface and/or browning of bud tissues; 2 = mycelial growth in one or multiple spots, with 10-30% coverage; 3 = >30-50% of the bud surface covered with mycelial growth; 4 = >50-80% of the bud surface covered with mycelial growth; and 5 = the entire bud covered with mycelium. The percent leaflet area of infected leaves was measured via FIJI open-source software[51]. This experiment was repeated five times. *B. cinerea* was reisolated from symptomatic tissues and identified to confirm pathogenicity and fulfill Koch's postulates, following the methods described in the literature[27]. Disease rating data were averaged from all replicates and repetitions of the experiments and subjected to analysis via ANOVA followed by Fisher's LSD test at $P < 0.01$.

Statistical analysis

The data obtained from the experiments on pathogen, temperature (T) and relative humidity (RH) were analyzed using the software GraphPad Prim 10. To make the variance homogenous, the number of conidia was transformed using the function " $\ln(n+1)$ ". The effects of temperature and humidity in the *in vitro* experiments were analyzed using linear and non-linear regression. Different regression models were used and compared based on the Akaike information criterion (AIC). The model with the smallest AIC value was considered the most likely to be correct[52]. Principal component analysis (PCA) and heatmap were performed using factoextra and complexHeatmap packages of R version 4.1.0.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

All research procedures involving species at risk of extinction were conducted in accordance with the guidelines provided by the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Authors' contributions Birendra Kumar supervised the experiment, provided the facility for the experiment; Sujata Singh Yadav designed the experiment, conducted the experiment, prepared the manuscript; Nashra Aftab contributed to perform the experiment. All authors read and approved the manuscript.

Data availability

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the primary accession code OP622939 and PP209529.

<https://www.ncbi.nlm.nih.gov/nuccore/OP622939>

<https://www.ncbi.nlm.nih.gov/nuccore/PP209529.1/>

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