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Arbuscular mycorrhizal colonization alters biochemical, molecular defense responses and root exudate composition against *Phytophthora capsici* infection in black pepper

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ABSTRACT

Mycorrhizal symbiosis benefits the host plants in several aspects. Among the soil microbiota, AM fungi have established a substantial potential to decrease crop damage from plant pathogens in several crops, yet the mechanism of its action in black pepper has not yet been studied. In this study, we investigated the effect of mycorrhizal colonization on biochemical, molecular defense responses, and root exudates composition in the *Phytophthora capsici* -black pepper host-pathosystem. Four biochemical parameters such as total phenols, orthodihydroxy (OD) phenols, peroxidase activity, and lignin were studied to delineate the effect of mycorrhizal colonization upon pathogen inoculation. Total phenol, OD phenol, and lignin content increased consistently upon challenge inoculation with *P. capsici* as well as in AM alone inoculated plants in both leaves as well as roots. Similar peroxidase activity was noticed in AM alone and challenged inoculated plants. The quantitative polymerase chain reaction analysis showed that AM pre-inoculation led to up regulation of pathogenesis related genes *viz*., cAPX, Osmotin and β-1,3-glucanase, phenylalanine ammonia-lyase and NPR 1 in black pepper leaves and roots upon of *P. capsici* inoculation. Sole inoculation of *P. capsici* also positively influenced the copies of most genes studied. Root exudates of AM colonized plants showed higher fatty acid/fatty acyl compounds *viz*., tetradecanoic acid, n-hexadecanoic acid, octadecanoic acid, and nonacos-1-ene compared to *P. capsici* inoculated plants. On the other hand, most of the alkane hydrocarbons such as (z)-3-tetradecene, octylcyclohexane, 4-cyclohexylundecane, and decylcyclohexane were observed majorly in the root exudates of *P. capsici* inoculated plants. Multivariate analysis of the root exudate data revealed that AM colonization in black pepper roots normalized the content of root exudates during *Phytophthora* infection comparable to control plants. Our findings indicates that the AM colonization enhances the resistance of *P. capsici* in black pepper plants by alterations in defense enzymes and gene expression and root exudates composition.

1. Introduction

Black pepper (*Piper nigrum* L.) commonly recognized as the 'King of Spices' belongs to the Piperaceae family and is cultivated for its pungent and fragrant berries which are the main economic produce. Due to higher demand in global markets, black pepper cultivation is expanding both in traditional and non-traditional areas in the past decade. However, soil-borne pathogens are one of the major limitations in the cultivation of black pepper. Among them, *Phytophthora* foot rot is a vastly detrimental disease and brings down productivity in black pepper plantations. This pathogen affects the all parts of plant including the roots, leaves, stems and fruits (Anandaraj, 2000). Foot rot is regularly controlled by extensive usage of fungicides on most farms (Kollakkodan et al., 2020). Another eco-friendly strategy is ability of plant root systems to form mycorrhizal associations, and which are ubiquitous and form mutual relations with most all terrestrial plant root systems, including many horticultural crops (Smith and Read, 2008). AM fungi association improves the nutrients uptake and water in the host plant in exchange for photosynthetic carbon (Bonfante and Genre, 2010). Added advantage deliberated by the AM colonization is an enhanced level of

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resistance to root-borne pathogens (Pozo et al., 2015; Whipps, 2004). The better resistance to root borne pathogens in AM colonized plants is mainly due to the competition for root colonization sites, photosynthates, improved nutrition of the plant, rhizosphere microbial population changes, and plant defense activation mechanisms. It has been observed that, with mycorrhizal colonization, elicitation of specific plant defense compounds occurs at the biochemical and molecular levels which makes the plant more competent against invading deleterious microbes (Gianinazzi-Pearson et al., 1994). Numerous studies have proven the effect of AM on most of the common fungal pathogens belonging to genera *Rhizoctonia, Pythium, Macrophomina, Alternaria, Aphanomyces, Botrytis, Colletotrichum, Cylindrocladium, Fusarium, Erysiphe, Oidium, Phytophthora, Gaeumannomyces, Verticillium* and *Sclerotium* (Ronsheim, 2016). The improved resistance to root borne pathogens in AM colonized plants is mainly due to the competition for root colonization sites, photosynthates, improved nutrition of the plant, rhizosphere microbial population changes, and plant defense activation mechanisms.

It has been observed that, with mycorrhizal colonization, elicitation of specific plant defense compounds occurs at the biochemical and molecular levels which makes the plant more competent against invading deleterious microbes (Gianinazzi-Pearson et al., 1994). Plant-AM interaction effects on changing host plant nutrition and physiology have largely been the vital point of investigation in the past decade. Plant roots exude a plethora of biochemical compounds, which play an essential role in signaling an array of rhizosphere microorganisms. In AM-colonized plants, established both localized and induced systemic resistance against plant pathogens (Pozo et al., 2015; Sarathambal et al., 2022). Through root exudation around 21% of the photosynthesized carbon passes into the rhizosphere region, forming a zone around the root system varying in biochemical, ecological, and physical properties (Walker et al., 2003; Vivanco et al., 2002). Root exudates consist of low-molecular-weight compounds, including phenolics, organic acids, amino acids, sugars, and numerous secondary metabolites, and are altered by mycorrhizal symbiosis (Vivanco et al., 2002; Jones et al., 2004). The composition of the root exudates is particularly important for improving the plant defense as well as boosting crop performance and yield. The initial signal exchange between arbuscular mycorrhizal fungi and host plants was facilitated through an exchange of diffusible compounds from root exudates. To the best of our knowledge, studies on root exudates profiling of AM colonized black pepper are limited. The roles of mycorrhizae in plant defense are largely ignored compared with their nutrient effects. Mycorrhizal infections induce various morphological, physiological, biochemical, molecular, and signaling changes in the host plants, yet rarely the experiments been done to deduce a correlation between induction and the enhancement of disease resistance to soil-borne pathogens. In the present work, we aimed to evaluate the i) Defense related biochemical parameters to unravel the possible correlation with disease resistance traits of mycorrhizae. ii) Variations in the expression of defense related pathway genes using qPCR analysis and iii) profiling of root exudates to study AM colonization in response to *P. capsici* infection.

2. Materials and methods

2.1. a.m. inoculum preparation

The inoculum of AM fungi, *Rhizophagus irregularis* was prepared with vermiculite as the carrier which contained 100 propagules per gram of the carrier material in the form of spores, hyphae, and mycorrhizal roots. Since, AM need 21–30 days to colonize in black pepper roots, 20 g of inocula was mixed with the potting mixture at the time of planting as per the treatments. The AM spores used for the inoculation are shown in Fig. 1A.

2.1.1. Inoculation of pathogen

Phytophthora capsici was cultured using carrot agar media and incubated at 24 ± 1 °C for 72 h (see Fig. 2). The mycelial discs of 5 mm^2 size from the edge of the actively growing culture were cut and kept for sporulation under incessant light conditions at 24 ± 1 °C for 48 h (Fig. 1B). The 10 mL of spore suspension containing 10^8 spore ml^{-1} was prepared in sterile distilled water and it was inoculated to black pepper cuttings near the root zone. The inoculated cuttings were maintained at a temperature of 25–28 °C with relative humidity 75–90% in a green house and uninoculated plants served as control (Bhai et al., 2007).

2.1.2. Pot experiment

Single node cuttings of black pepper (variety Sreekara) were maintained in the sterile potting mixture (soil: sand: FYM, 2:2:1) in polythene bags (20 × 10 cm) under greenhouse conditions. The plants were multiplied adopting the serpentine propagation method. For conducting the experiment, 4–5 leaf stage plants were used. Pots with a capacity of 10 kg were used for the pot culture experiment. AM inoculum and *P. capsici* were applied alone or in combination as per the treatments. The treatments were as follows: T1-control; T2-AM; T3-AM + *P. capsici*; T4- *P. capsici*. In order to see the changes in biochemical and molecular defense responses at early stages of infection, the leaf and root samples were collected from 0 to 4 days and 0–3 days after inoculation (DAI) of *P. capsici* at 24 h intervals and were subjected to biochemical and quantitative polymerase chain reaction analyses respectively.

2.2. Biochemical analysis

Defense related biochemical parameters *viz*., phenols, lignin, and peroxidase were studied to find out the possible link with disease resistance characteristics of mycorrhizae.

For the estimation of phenols, 200 mg of tissue was two times extracted with 1.5 mL of 80% methanol at room temperature for 1 h

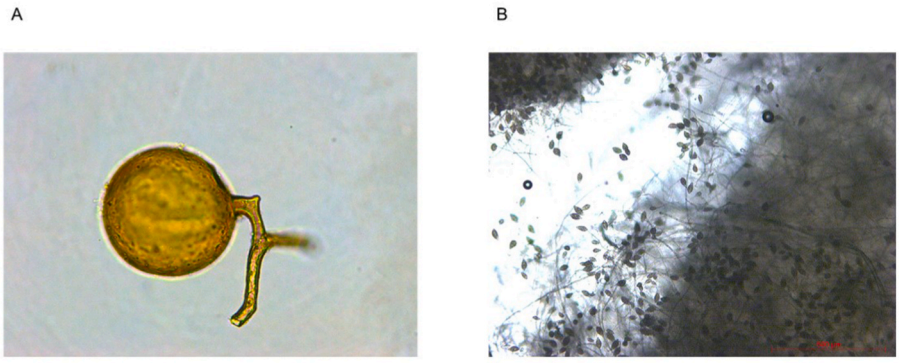

Fig. 1. A) AM spore used for the inoculation; B) The sporulated *P. capsici* sporangium were used for plant inoculation.

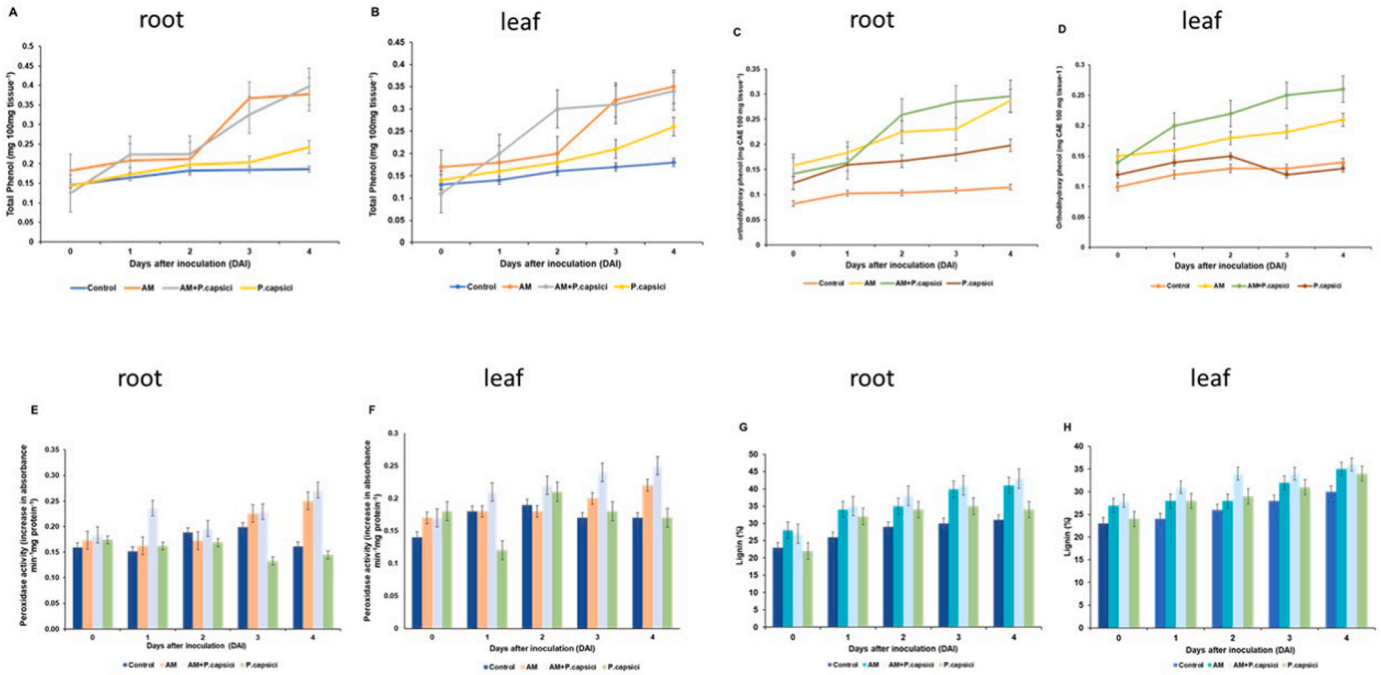

Fig. 2. Biochemical defense responses in roots and leaves of black pepper to mycorrhizal colonization and *P. capsici* infection.

with continuous shaking and filtered using Whatman no. 1 filter paper (Eynek et al., 2009). The reaction mixture consisted of 0.2 mL of sample and 0.5 mL of Folin-Ciocalteau reagent kept for incubation of 3 min. After that 1 mL of saturated Na_2CO_3 solution and 10 mL of water were added. After 1 h, absorbance was measured at 725 nm in a spectrophotometer and quantified against a gallic acid standard.

Methanol extracted sample (0.2 mL) was mixed with 1 mL of 0.1 M phosphate buffer (pH 6.5) and 2 mL of 5% $Na_2MoO_4.2H_2O$ solution and incubated for 15 min for estimation of Orthodihydroxy phenols (Gutfinger, 1981). The absorbance was measured at 350 nm in a spectrophotometer and quantified against the standard of caffeic acid.

For the estimation of acetyl bromide soluble lignin (ABSL) content, 10 mg of dried powdered samples and 2 mL of acetyl bromide in glacial acetic acid {1:3 v/v containing perchloric acid (70%, 0.08 mL) were added to a brown vial (Liyama and Wallis, 1990). The reaction mixture was incubated at 70 °C for 30 min and transferred to 50 mL volumetric flasks containing 5 mL of 2 M sodium hydroxide and 12 mL of acetic acid. Absorbance was measured at 280 nm and lignin content was determined using SAC (Specific Absorption Coefficient) of lignin, 20 g^{-1} cm^{-1}.

Samples were homogenized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 12,000 g for 10 min. The supernatants were used to determine the peroxidase activity. The guaiacol peroxidase activity was measured as oxidation of guiacol in the presence of hydrogen peroxide at 470 nm according to the method by Putter (1974). The activity was expressed as the amount of enzyme required to increase the absorbance per min per mg protein.

2.3. Gene expression studies

The leaves and roots for RNA isolation were collected on the 0^{th}, 1st, 2nd and 3rd day after inoculation. The samples were washed thoroughly and wrapped in silver foil, labelled, and kept on ice immediately after collection. The total RNA isolation was performed in a clean and RNase free environment using Sigma Spectrum™ Plant Total RNA Kit following the manufacturer's instructions. This RNA was quantified using a nanodrop microvolume spectrophotometer. The RNA was stored at −80 °C for further analysis.

The cDNA synthesis was carried out by Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Cat #K1622). Initially, components of the kit were thawed, mixed, briefly centrifuged, and stored on ice. Template RNA random hexamer primer 1 μl and nuclease free water 12 μl were added to a sterile nuclease free tube. To this, 5X reaction buffer (4 μl), ribolock RNase inhibitor (1 μl), 10 mM dNTP mix (2 μl) and RevertAid M-MuLVRT (1 μl) were added and mixed gently and centrifuged briefly. The mixture was incubated in the PCR machine at 25 °C for 5 min followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min.

2.3.1. Quantitative PCR

To check the quality of RNA, stability of reference genes, and relative gene expression analysis, qPCR was carried out. The reaction was performed in QuantStudio 7 Flex (The Applied biosystems) with 72 well rotors using QuantiFast SYBR Green PCR kit (Qiagen). The pooled RNA from three biological replicates, treated with DNase and reverse transcribed was used as the template. All the samples to be analyzed for a particular gene were processed and run simultaneously for accurate relative gene expression analysis. The primers used for qPCR analysis are listed in Table 1. The reaction mixture consisted of 10 μl of 2X SYBR green, 1 μl each of 10 μM gene specific forward and reverse primer, 0.4 μl of cDNA, and made up to a final volume of 20 μl with sterile water. PCR amplification was done with initial denaturation for 5 min at 95 °C followed by 35 cycles for 10 s at 95 °C and for 30 s at 60 °C. A melt curve program was done by increasing the temperature stepwise by 1 °C in the range of 65–99 °C to check the specificity of PCR products. Mean Ct values of three technical replicates were exported after setting the threshold to 0.1 and correcting the slope and baseline. The GAPDH gene was used as reference gene. The relative gene expression was calculated using the 2-ΔCt method (Livak and Schmittgen, 2001) and transformed to a log2 scale. A log2-ratio of zero indicates the transcript has equal expression values, whereas the transcripts are up-regulated if the ratio is above zero and down-regulated if the ratio is below zero (Bergemann and Wilson, 2011). A log2 ratio of 1 represents a 2-fold change and the transcripts with fold change $\geq 1/\leq -1$ were defined as significantly up-/down-regulated (Luo et al., 2005). The PCR products were run using agarose gel (1.2%) and sequenced to confirm their specificity.

Table 1
List of primers used for qPCR assay.

Gene	Primers	Sequence	Reference
NPR1	BP-NPR1-FP	5′-GAGGTTGACAAGGGCAAAGGA-3′	Asha and Soniya, 2016
	BP-NPR1-RP	5′-GTCTCAGCCAGTGGATTTCTTC-3′	
PAL-1	BP-PAL-FP	5′-GCCGAAGCAGGACGAAGCCG-3′	Hao et al. (2016)
	BP-PAL-RP	5′- CTTTCCACCATTAGAGTTGC-3′	
DEF-1	BP-DEF-FP	5′-GTTTCAACTTCCCTCCCACA-3′	Kumar et al. (2016)
	BP-DEF-RP	5′-ACTGAGCCACAGGTTCAAGG-3′	
β-1,3 glucanase	GLUC-BP-FP	5′- CCGGCCTACAGAATGACATC-3′	Vandana and Bhai (2018)
	GLUC-BP-RP	5′- GGGTAGACGTTGGCTAGGAG-3′	
Osmotin	OSM-BP-FP	5′- ACCGTGTTTAAGACCGACCA-3′	
	OSM-BP-RP	5′- ACCATTTCATGGGCAAAAGA-3′	
cAPX	cAPX-BP-FP	5′- GAACTCAGGCCGTCCTCAAC-3′	
	cAPX-BP-RP	5′- GACCTTCTCGCCGGAATTCA-3′	
GAPDH	GAPDH-BP-FP	5′-ATGAAGGATTGGCGAGGTGG-3′	
	GAPDH-BP-RP	5′-AGGCCATTCCAGTGAGCTTC-3′	

2.4. Metabolomic analysis of black pepper root exudates colonized with AM

Based on the treatments, on the second day after inoculation with *P. capsici*, the roots were subjected to metabolomic profiling as per the protocol of Meena et al. (2017). All dirt particles from the roots were removed and the roots were immersed with 50 mL of 0.01 mol L^{-1} KOH. After 5 min, the roots were washed with running tap water followed by distilled water and incubated in 50 mL sterile distilled water for 24 h. The solutions were by filter sterilized through 0.22 μm nitrocellulose membrane filters. The root exudates were stored at −20 °C until use. This ethyl acetate extract was dried and subsequently processed for GC-MS analysis.

2.5. Data analysis

The treatments were replicated in five times and the data were subjected to significant differences by using analysis of variance (ANOVA) with the SAS package (Version 9.3). The significant difference between means of treatments was determined using the least significant difference (LSD), $p < 0.05$ and $p < 0.01$. Multivariate analyses such as principal component analysis (PCA), Variable Important Projection (VIP) score, and heat map of root exudates compounds were performed using Metaboanalyst version 5.0 (Pang et al., 2021).

3. Results

3.1. Expression of defense-related genes in black pepper in response to mycorrhizal colonization and P. capsici infection

3.1.1. Cytosolic ascorbate peroxidase (cAPX) gene (PR7)

In roots, cAPX gene expression increased abruptly in AM + *P. capsici* inoculated plants on 2 DAI and 3 DAI compared with *P. capsici* alone inoculated plants. This indicated the up-regulation of gene expression on 2 DAI of AM primed plants-initiated host defense response upon subsequent pathogen attack (Fig. 3A). In leaves, cAPX gene expression was higher in both AM + *P. capsici* and *P. capsici* alone inoculated samples on 2 DAI (Fig. 3B).

3.1.2. Osmotin gene (PR5)

In roots, the osmotin gene expression increased up to 2 DAI and was then downregulated on 3DAI. Particularly, in AM + *P. capsici* inoculated plants the osmotin gene was strongly expressed on 2 DAI (Fig. 3C). In leaves, a stable increase in the relative expression of the osmotin gene

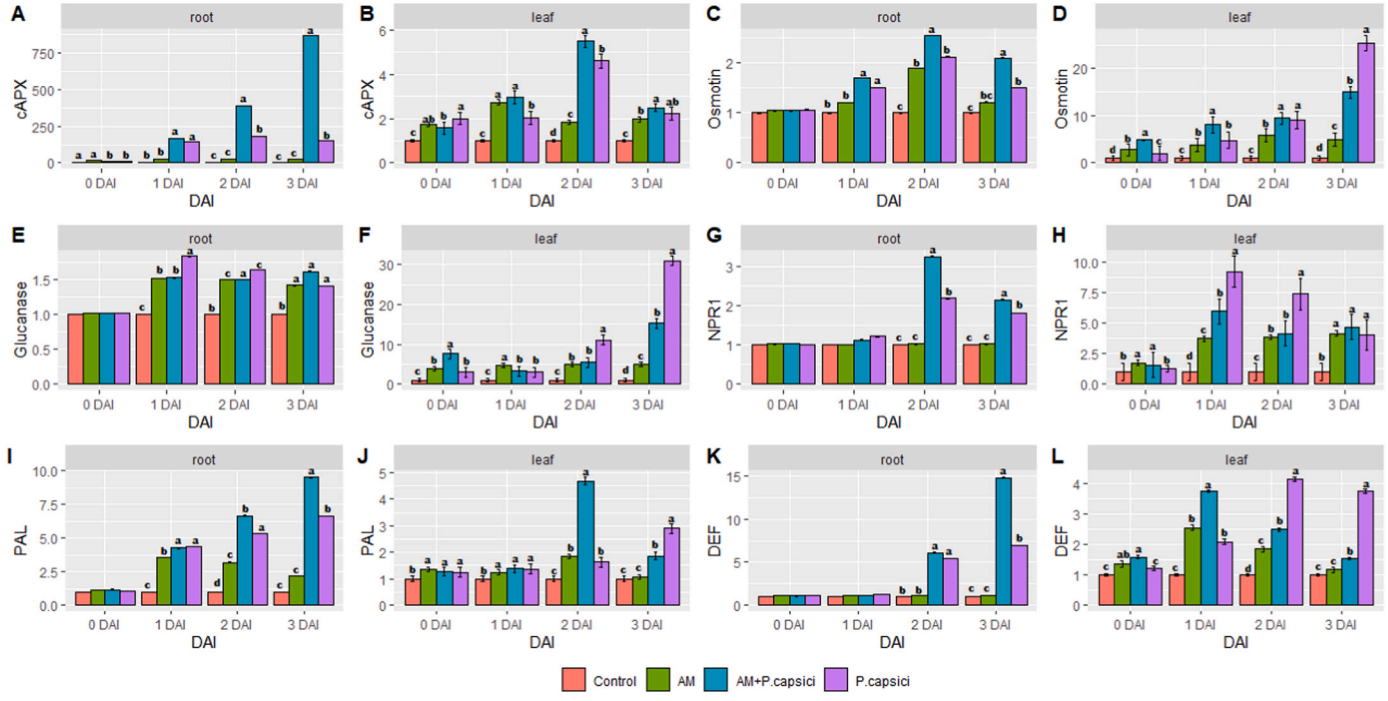

Fig. 3. Differential expression of Cytosolic ascorbate peroxidase (cAPX) Osmotin gene (PR5), Glucanase gene (PR2), NPR1gene, PAL gene, DEF gene in roots and leaves.

was evident in AM + *P. capsici* and *P. capsici* treatments (Fig. 3D). The osmotin gene expression was significantly high on 3DAI in *P. capsici* alone inoculated plants than AM + *P. capsici* inoculated plants, whereas they were on par on 2 DAI.

3.1.3. Glucanase gene (PR2)

In roots, the glucanase gene expression was high on 1 DAI in *P. capsici* alone inoculated plants, whereas, there was not much variation in glucanase gene expression levels in all other treatments (Fig. 3E). In leaves, 2 DAI onwards higher expression of glucanase gene was observed in *P. capsici* alone inoculated plants (Fig. 3F) which was significantly high compared to AM + *P. capsici* inoculated plants.

3.1.4. NPR1 gene

In roots, the NPR1 gene expression was significantly high on 2 DAI in AM + *P. capsici* inoculated plants and then the expression was reduced at 3DAI (Fig. 3G). Whereas, in leaves, the NPR1 gene expression was high on 1 DAI in *P. capsici* inoculated plants and it gradually decreased on 2 and 3 DAI (Fig. 3H).

3.1.5. PAL gene

In roots, the PAL gene expression was consistently increased in the AM + *P. capsici* inoculated plants and *P. capsici* alone inoculated plants (Fig. 3I). In leaves, this defense gene expression was significantly induced on 2 DAI and was later down-regulated in AM + *P. capsici* inoculated treatments. However, the PAL gene expression was increased progressively in *P. capsici* alone inoculated plants (Fig. 3J).

3.1.6. 2.6 DEF gene

The DEF gene expression was strongly induced in roots of AM + *P. capsici* inoculated and *P. capsici* alone inoculated treatments at 2 DAI and 3 DAI. (Fig. 3K). In leaves, the DEF gene expression was increased gradually in *P. capsici* alone inoculated plants (Fig. 3L).

Altogether, the expression of NPR1 and PR protein genes was high in AM + *P. capsici* and *P. capsici* inoculated plants at 2 DAI which indicated the induction of salicylic acid (SA) pathway genes. Hence, the root exudates were collected on the 2nd day irrespective of the treatments.

3.2. Metabolite profiling of mycorrhizal root exudates of black pepper

The exudates of mycorrhizal black pepper roots infected with or without *Phytophthora* infection were analyzed using GC-MS. The analysis detected 28 compounds among treatments (Table 2). The compounds detected mainly fall in the category of alkanes, aliphatic hydrocarbons, fatty acids, fatty acyls, fatty alcohols, phenolics, and a few ketones and epoxide group of compounds. Supplementary Fig. 1 Shows the TIC (Total Ion Chromatogram) of four treatments studied. The predominant compounds detected across all the treatments were trans-9-hexadecen-1-ol, hecadecane, E−15-heptadecenal, octadecane, 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, dibutyl phthalate, 1-nonadecene, eicosane, 1-docosene and nonacos-1-ene albeit variation among treatment groups. AM alone group showed a total of 16 compounds and AM + *P. capsici* and control treatments showed 19 compounds each and a maximum number of compounds (21) were detected in *Phytophthora* inoculated treatment. Amongst, octadecane, eicosane, and 3-ethyl-5-(2-ethylbutyl) octadecane were found significantly higher ($p < 0.01$) in AM alone treatment. The following compounds were detected in higher amounts ($p < 0.01$) in AM + *P. capsici* treatment: dodecyl acrylate, tetradecyloxirane, and tetradecyloxirane. *Phytophthora* treatment showed significant ($p < 0.01$) alteration in its exudate compounds as compared to control and other treatments. Few compounds like 2-undecanone, 1,2,4,5-tetraethylcyclohexane, octylcyclohexane, 4-cyclohexylundecane, decylcyclohexane, 10-nonadecanone, 17-pentatriacontene and benzophenone were found exclusively in this treatment. Among these, fatty acyl compounds with cyclohexane moiety differentiated this treatment group from others. The compounds like 2,6,10-trimethyldodecane, hexadecane, E−15-heptadecenal, 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, n-hexadecanoic acid, 1-nonadecene, octadecanoic acid, 1-docosene and nonacos-1-ene were found higher ($p < 0.01$) in control treatment.

Table 2
Metabolites identified in the root exudates of black pepper in response to mycorrhizal colonization and *P. capsici* infection.

Compounds	RT	Peak Area (%)			
		AM	AM + *P. capsici*	*P. capsici*	Control
2,6,10-trimethyldodecane	9.13	0.00^{B}	0.00^{B}	0.00^{B}	0.50^{A}
2-Undecanone	9.32	0.00^{B}	0.00^{B}	0.49^{A}	0.00^{B}
1,2,4,5-tetraethylcyclohexane	10.48	0.00^{B}	0.00^{B}	0.86^{A}	0.00^{B}
(Z)-3-Tetradecene	10.81	0.00^{D}	2.17^{B}	4.84^{A}	0.75^{C}
Octylcyclohexane	11.97	0.00^{B}	0.00^{B}	1.04^{A}	0.00^{B}
2,4-Di-tert-butylphenol	13.28	0.00^{B}	0.64^{A}	0.61^{A}	0.00^{B}
4-cyclohexylundecane	13.61	0.00^{B}	0.00^{B}	0.90^{A}	0.00^{B}
4-hexyl-, 2,3-dicyano-4-(pentyloxy) phenyl ester, cyclohexanecarboxylic acid	13.89	0.00^{B}	0.00^{B}	0.58^{A}	0.00^{B}
trans-9-Hexadecen-1-ol	14.92	2.32^{C}	9.62^{A}	10.40^{A}	8.72^{B}
Hexadecane	15.06	0.99^{B}	1.45^{A}	1.50^{A}	1.61^{A}
Benzophenone	15.90	0.00^{B}	0.00^{B}	0.62^{A}	0.00^{B}
decylcyclohexane	16.31	0.00^{B}	0.00^{B}	0.87^{A}	0.00^{B}
Dodecyl acrylate	17.05	1.19^{B}	1.62^{A}	0.00^{C}	1.02^{B}
Tetradecanoic acid	18.45	0.00^{B}	0.42^{A}	0.00^{B}	0.37^{A}
E−15-Heptadecenal	19.12	10.04^{B}	13.20^{A}	9.64^{B}	14.10^{A}
Octadecane	19.25	2.63^{A}	1.30^{BC}	0.87^{C}	1.63^{B}
tetradecyloxirane	19.82	1.33^{A}	1.67^{A}	0.00^{B}	1.32^{A}
7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	21.75	1.61^{B}	1.30^{C}	1.74^{B}	2.21^{A}
n-Hexadecanoic acid	22.45	2.81^{A}	2.95^{A}	0.00^{B}	3.16^{A}
Dibutyl phthalate	22.54	2.57^{B}	2.00^{B}	4.66^{A}	2.64^{B}
1-Nonadecene	23.06	9.58^{B}	11.87^{AB}	6.04^{C}	13.27^{A}
Eicosane	23.18	2.96^{A}	0.87^{BC}	0.59^{C}	1.26^{B}
10-Nonadecanone	24.61	0.00^{B}	0.00^{B}	2.04^{A}	0.00^{B}
Octadecanoic acid	26.18	2.43^{A}	2.96^{A}	0.00^{B}	3.51^{A}
17-Pentatriacontene	26.26	0.00^{B}	0.00^{B}	0.71^{A}	0.00^{B}
1-Docosene	26.85	1.26^{D}	7.86^{B}	2.85^{C}	9.24^{A}
tetradecyloxirane	27.57	1.64^{B}	2.17^{A}	0.00^{D}	1.23^{C}
Nonacos-1-ene	29.74	3.35^{B}	4.86^{A}	1.25^{C}	5.63^{A}
3-ethyl-5-(2-ethylbutyl) octadecane	29.80	0.83^{A}	0.50^{B}	0.00^{C}	0.46^{B}

*Values with the different alphabet in each column differed significantly from each other ($P \leq 0.05$).

For better understanding, the dataset was subjected to multivariate analysis which provided remarkable information from the present study. In PCA analysis, cumulative variances of 68.3% and 20.2% for PC1 and PC2, respectively were observed. The treatment AM alone with the 95% confidence region was well-separated from other treatments (Fig. 4A). The biplots linked the score plot of different treatments with each variable. Compounds like tetradecanoic acid, 1-docosene, E−15-heptadecenal, n-hexadecanoic acid, octadecene, octadecanoic acid, and Nonacos-1-ene were detected at a similar level in control and AM + *P. capsici* and these treatments were placed closely in the lower left quadrant of PCA plot (Fig. 4B). The AM treatment was placed in the upper left quadrant and *P. capsici* treatment was placed in the upper right quadrant of the PCA plot due to the smaller number of common compounds with a similar percentage of occurrence in these treatments.

Variable important projection (VIP) score of root exudate compounds indicated that the compounds *viz*., 3-octadecane, tetradecyloxirane, dodecyl acrylate, trans-9-hexadecen1-ol, and n-hexadecanoic acid had maximum variation among the treatments. Except for hexadecen-1-ol, all the content of other compounds mentioned above was higher in AM treatment and lower in *P. capsici* treatment (Fig. 5A). The heat map of the relative abundance of volatile compounds in treatments is

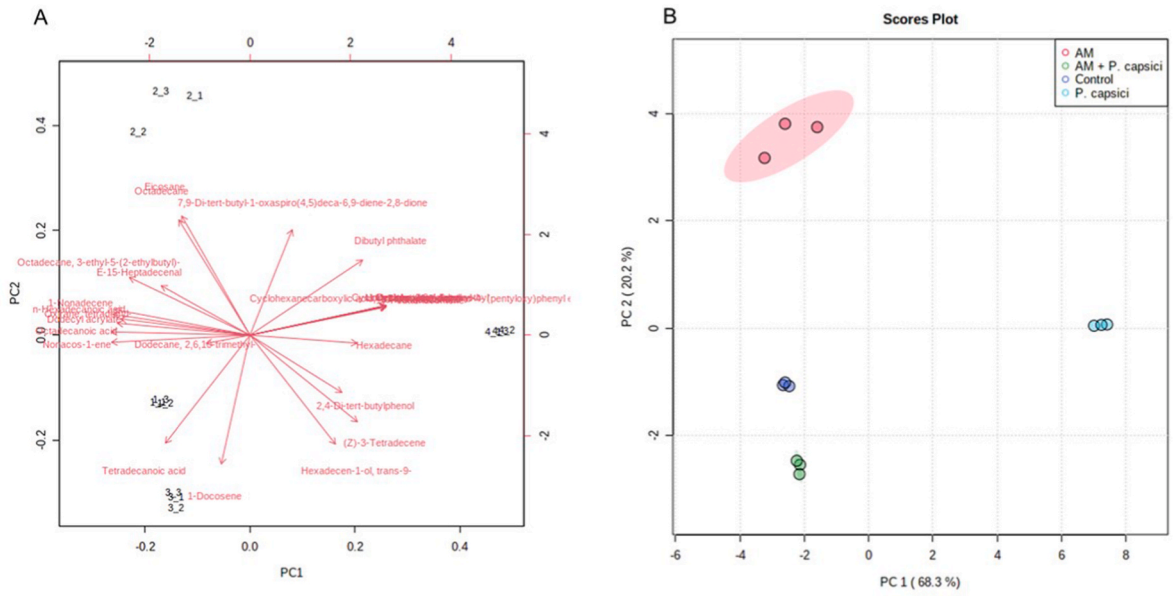

Fig. 4. A) Principal component analysis of biplot showing the influences of variables B) Score plots showing the volatile compounds secretion patterns released by pepper plants when inoculated with AM (Red) *P. capsici* alone (in blue); the AM + *P. capsici* (in green), and control (in violet). Metabolites significantly differed (p-value <0.01).

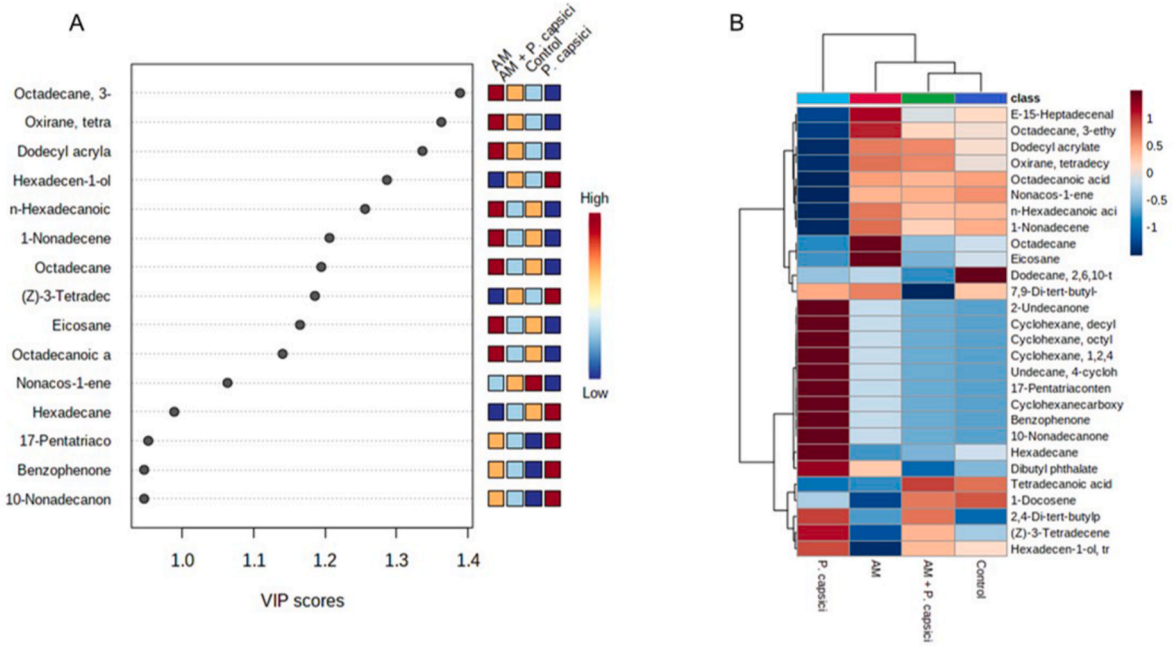

Fig. 5. A) Variable important projection (VIP) score of root exudate compounds; B) Heat map of relative abundance of volatile compounds.

depicted in Fig. 5B. At the treatment level, AM + *P. capsici* and control samples had a similar profile as evidenced in PCA analysis also. The *P. capsici* treatment had a distinct profile with very low similarity with other treatments. Though AM treatment clustered separately, it was closely related to control and AM + *P. capsici* treatment as compared to *P. capsici* treatment. As mentioned earlier, the cyclohexane group of compounds was the major differentiating factor in *P. capsici* treatment. On the whole, a complete mechanism of the AM-mediated alterations in gene expression and metabolome would help in deciphering the mechanisms underlying plant tolerance to pathogens.

4. Discussion

The AM and plant pathogens interactions have been studied widely and the majority of them are related to fungal root borne pathogens (Whipps, 2004; Ronsheim, 2016). AM colonization affects the metabolism of host plants which induces significant changes in enzymatic activities and physiological mechanisms leading to the accumulation of secondary metabolites, such as polyphenols and carotenoids (Toussaint et al., 2007). It also enhances the phenolic content in host plants, thereby protecting the plants against pathogens. An enhanced phenol in mycorrhizal colonized plants represents the priming of the defense system of the host plant against pathogens. In our study, we recorded the enhanced production of polyphenolic compounds in black pepper plants

colonized with AM. Similarly, in different AM-colonized plants like *Allium cepa, Vitis vinifera*, *Solanum lycopersicum*, *Leucaena leucocephala*, and *Arachis hypogaea* enhanced phenolics content was reported (Torres et al., 2016; Mada and Bagyaraj 1993). Increased phenolics and OD phenol activity in AM + *P. capsici* inoculated plants can be explained in terms of elicitation of defense response by plants to pathogen infection. OD phenols play a significant role in the commencement of defence mechanisms thereby increasing resistance against plant pathogens (Vandana et al., 2014).

Further, the higher lignin content in AM treated plants appears to be on account of higher production of phenolics that are known to exhibit a positive correlation with lignin content (Bhatt et al., 2012). The present study also showed that black pepper plants inoculated with AM had higher peroxidase activity in both leaves as well as roots compared to uninoculated plants under pathogen stress. There are reports on the stimulation of peroxidase activity of AMF inoculated roots as compared to non-AMF roots (Zhu et al., 2010; He et al., 2007; Jaleel et al., 2009).

Black pepper plant response to *Phytophthora* was proved by the accumulation of lignin like polymer in combination with expression of peroxidase activity (Bhai et al., 2021). Though many researchers proved that AM inoculation has induced disease resistance, its specific mechanism was not clearly deciphered. It is well established that lignification of host plant cell wall is a resistant response in plant-pathogen interaction. Previous reports mentioned that AM fungi could induce metabolic processes to improve the lignification of the plant cell wall, consequently forming physical barriers for pathogen invasions. In tomato, the distinct defense responses against *Phytophthora parasitica* has been shown by *Glomus mosseae* (Yanan et al., 2015). The present study demonstrated that the mycorrhizae inoculation altered the lignin contents in black pepper roots and these changes enhanced the resistance of roots against the invading pathogens.

Mycorrhizal colonization affects plant gene expression and metabolic pathways activation leading to the production of pathogenesis-related proteins (Sanmartín et al., 2020; Gallou et al., 2011). RNA sequence analysis exposed that mycorrhizae priming led the transcriptional changes in signalling, hormone metabolism, and biotic and abiotic stresses (Cervantes-Gámez et al., 2015). Our study also confirmed that priming is an important mechanism involved in mycorrhiza for disease resistance in host plants.

Reports also stated higher expression of pathogenesis-related (PR) proteins when mycorrhizae colonized plants are infected with pathogens (Zhang et al., 2018). Peroxidase is involved in the crosslinking reactions between proteins and polysaccharides in the interface between the arbuscule and the plant cell plasma membrane (Song et al., 2015). In our study, the increased *cAPX* activity of plants occurred not only in mycorrhizal plants but also during pathogen challenge inoculation. The mycorrhizal plants had higher peroxidase enzyme activity compared to the uninoculated ones.

Our findings suggested a priming phase of plant defense with stimulation of PR genes in the treatment. The priming phase is the biological process of adaptive mechanism, which takes place from the initial activation through exposure to various stress conditions (Mauch-Mani et al., 2017; Alaux et al., 2020). Stimulation of pathogenesis-related (PR) proteins as an indicator for the induced systemic responses in plants. Upregulation of β-1,3-glucanase and osmotin has been connected with induced systemic resistance in tomato against *Fusarium oxysporum* (Pozo et al., 2002) and *Alternaria solani* (Lawrence et al., 1996). PR genes have been commonly used as marker for systemic acquired resistance in various cultivated plants (Mitsuhara et al., 2008). Our study showed that AM inoculation systemically induced both enzyme activities and gene expression of peroxidase in the leaves of black pepper. Pozo et al. (2002) found that *F. mosseae* colonization in tomato plants reduced both local and systemic disease symptoms incited due to *Phytophthora parasitica* infection, as well as upregulation of β-1,3-glucanase and osmotin protein expression.

Marquez et al. (2018) reported that AM + *Macrophomina phaseolina* inoculated soybean plants displayed a lower number of up-regulated genes as compared with AM + *M. phaseolina* which could be possibly due to the counteraction or balancing cost of *M. phaseolina* infection. In another study, Alaux et al. (2020) observed the elicitation of the Jasmonic acid/ethylene signaling pathway dependent defense gene in healthy potato plants compared to AM inoculated potato plants infected by *P. infestans*. Other responses in mycorrhizal roots included changes in the accumulation of enzymes involved in cell wall modification. In the present study, we noticed the accumulation of transcripts of osmotin, *β-1,3*-glucanase, NPR1, PAL and DEF genes in *P. capsici* alone inoculated plants than in AM + *P. capsici* inoculated plants except for the peroxidase gene which was higher in the roots of AM + *P. capsici* inoculated plants. Peroxidase is involved in cross linking of cell walls which strengthens the root system and thus acts as a physical barrier to *P. capsici* infection. Whereas in *P. capsici* alone inoculated plants, the defense related genes were induced, but later, the pathogen might have overcome this leading to infection.

In the present study, the peroxidase gene was induced at a higher level in roots and leaves of AM + *P. capsici* inoculated plants and this gene was also induced in leaves of *P. capsici* alone inoculated plants. In AM + *P. capsici* inoculated plants, the osmotin gene expression increased up to 2 DAI than it was downregulated in roots. Where as in *P. capsici* alone inoculated plants, the osmotin gene was strongly expressed on 2 DAI. In previous studies in black pepper, similar results of higher expression of peroxidase and osmotin genes upon interaction with *P. capsici* and *F. oxysporum* was reported (Kumar et al., 2016; Hao et al., 2016). In this study, increased accumulation of β-1,3-glucanase transcripts was found especially in leaves at 2 and 3 DAI. Similar induction of β-1,3-glucanase transcripts was observed by Kumar et al. (2016) and Vandana and Bhai (2018) during *P. capsici* and black pepper interaction. In our study pathogen inoculation was done after one month of AM inoculation. Hence the defence gene upregulation was higher in AM + *P. capsici* plants than AM alone plants. Similarly, AM pre-colonized tomato plants results did not show changes in the defence genes except slightly induced transcripts of PR1 and PR2 genes. However, AM pre-inoculation strongly upregulated defense genes upon *Alternaria solani* attack (Song et al., 2015).

NPR1 is a key regulator of salicylic acid-dependent induction of gene expression during systemic acquired resistance (SAR) (Dong, 2004; Maier et al., 2011). Asha and Soniya (2016) found down-regulation of NPR1 transcripts expression in both leaves and roots of *P. capsici* inoculated black pepper plants. In the present study also, the NPR1 transcripts were reduced gradually during the 2nd and 3rd DAI in leaves and roots. Induction of phenylalanine ammonia-lyase (PAL) activity is a reliable indicator of plant resistance mechanism (Kim and Hwang, 2014). PAL accumulated in mycorrhizal roots is the first step of the phenylpropanoid pathway in the biosynthesis of phytoalexins. An increase in PAL activity in mycorrhizal colonized roots indicates that enhanced accumulation of phenolic compounds is triggered upon pathogen attack. PAL gene induction was also observed in AM-colonized potato plants challenge inoculated by *P. infestans* (Gallou et al., 2011) and during *P. capsici* infection in black pepper (Hao et al., 2016). Similarly, in our study also the expression of the PAL gene was higher at 2 DAI in both AM + *P. capsici* and *P. capsici* alone inoculated black pepper plants. Defensin genes expression was reported in *Medicago truncatula* in a variety of tissues and response to various abiotic and biotic stimuli (Uhe et al., 2018). During *P. capsici* infection in black pepper, the DEF gene expression was high at 2nd DAI in both roots and leaves particularly in *P. capsici* alone inoculated plants.

The root is the primary organ in plant systems which supports numerous activities like water and nutrient uptake, acting as anchor, etc. All plants synthesize and secrete a variety of plant growth regulating compounds into the soil environment which aids in the chemotaxis of a variety of microorganisms to its rhizosphere niche (Compant et al., 2010). Effective colonization of plant growth promoting microorganisms like AM is influenced by root exudates. The process of

mycorrhization also modifies the root exudate profile of plants by eliciting several metabolic pathways (Mythili and Ramalakshmi, 2022). Some of these metabolites play an essential role in defense mechanism against plant pathogenic microorganisms (Rapparini et al., 2007). Several studies have reported the impact of AM colonization on root exudate composition in host plants *viz*., *Solanum lycopersicum, Artemisia annua*, *Valeriana officinalis*, *Anadenanthera colubrina*, *Dioscorea esculenta*, *Cynara cardunculus*, *Ocimum basilicum*, *Angelica archangelica*, *Panax quinquefolius*, *Zingiber officinale* and minor millets (Nell et al., 2010; Kapoor et al., 2007; Ceccarelli et al., 2010; Lu et al., 2015; Zitterl-Eglseer et al., 2015; Toussaint et al., 2007; Smith et al., 2010; Silva et al., 2008; Mythili and Ramalakshmi, 2022).

To our knowledge extents, this is the first study that has evaluated the root exudation profiles of AM colonization and/or *P. capsici* infection in black pepper. The composition of plant root exudates varies according to different factors, such as cultivar type, growth phase, and infecting microbe. Earlier studies indicated that unsaturated fatty acids in the root exudates conferred resistance to different pathogens in tobacco, tomato, and eggplant (Xing and Chin, 2000; Wang et al., 1998; Cohen et al., 1991). By these results, in this study, *Phytophthora* infection decreased the level of fatty acids and fatty acyl compounds whereas AM inoculation in *Phytophthora* infected plants reversed this phenomenon and showed an increased level of these compounds thereby providing resistance to the pathogen infection. Further confirmation in this regard was reported by Zhang et al. (2020) in a study involving *P. nicotianae* in tobacco. They showed that compounds like octadecanoic acid and tetradecanoic acid inhibited the mycelial growth of *Phytophthora nicotianae* in dose dependent manner. The regulation of fatty acid biosynthesis may be reflective of the obligate symbiosis between AM fungi and their plant hosts (Trépanier et al., 2005; Dutta et al., 2013).

Moreover, this result has given the differentiating factor that, *Phytophthora* infection does not require these fatty acids/fatty acyls for infection as evidenced by the decrease or absence of some of them in *P. capsici* treatment alone and AM treatment overpowers this characteristic by establishing the contact with host plant by increasing their synthesis in the root exudates. Zhang et al. (2020) showed that in susceptible cultivars, the percentage of hydrocarbons in the root exudates was more in *Phytophthora nicotianae* infected plants compared to resistant plants of tobacco. In this study also, we observed that the percentage of alkane hydrocarbons in root exudates of *Phytophthora capsici* infected plants was more compared to AM inoculated and control plants. These results showed that the synthesis and release of root exudates are highly influenced by the category of microbes associated. Apart from this, we also showed that inoculation of *P. capsici* after AM inoculation normalized the root exudates profile and to some extent did not produce some of the alkane hydrocarbons detected in *P. capsici* applied treatment. This indicated that the root infected with AM alters the root metabolism and helps in managing biotic stress in plants (Luginbuehl et al., 2017).

We observed the presence of the antioxidant compound benzophenone only in *P. capsici* inoculated root exudates. This could be synthesized as a part of a plant defense mechanism against *P. capsici* infection (Fontana et al., 2009). More importantly, *P. capsici* challenge inoculated plants had significantly higher quantities of antifungal compounds *viz*., E−15-Heptadecenal, 1-Nonadecene, and 1-Docosene. The increase in abundance of the antioxidant and antifungal compounds in root exudates explains their enhanced resistance against pathogens (Kumar et al., 2011). The difference in root exudate compounds in control and AM plants could be explained by the fact that plants originally perceive mycorrhizae as putative pathogens, as a consequence of the temporary activation of plant defense responses during the early phases of root colonization (Garcia-Garrido and Ocampo, 2002).

The changed root exudation is also imitated in the systemic autoregulation of the mycorrhizal colonization by the host once roots are colonized by AM during which the plants regulate further colonization by both AM and pathogenic microorganisms through the secondary metabolites (Vierheilig et al., 2008). Mycorrhizal inoculation has been shown to reduce the damage caused by soil-borne pathogens (Azcon-Aguilar and Barea, 1996). *Phytophthora parasitica* infection was suppressed in *Glomus mosseae* colonized tomato root (Cordier et al., 1998). Mycorrhizal strawberry plants' root exudates reduced the sporulation of *P. fragariae* under *in vitro* conditions (Norman and Hooker, 2000).

5. Conclusion

Based on the results emanated from the present investigation, we conclude that AM symbiosis conferred resistance to *P. capsici* infection in black pepper. An increase in the biochemical defense parameters such as phenols, lignin, and peroxidase activity indicated the induction of systemic defense response against the pathogen. The protective effect of AM symbiosis accompanied by the systemic activation of genes involved in induced systemic resistance confers the plant immunity against the pathogen. The induction of NPR1 and PR genes in *P. capsici* inoculated plants indicated the induction of the SA pathway. In AM-plant symbiosis, root exudate compounds have shown to undergo quantitative changes responding to AM colonization and further infection by *P. capsici*. In this study, an increase in fatty acids with AM colonization and an increase in alkane compounds with *P. capsici* infection were observed. Overall, the present investigation offers to unravel the molecular and biochemical mechanisms involved in the mycorrhiza-mediated resistance to *P. capsici* infection in black pepper, which prompts to take AM application to field conditions to reap the benefit.

Author contributions

Conceived and designed the experiment: CS. Biochemical and root exudate analysis: RS, CS, RR, and BP. Molecular analysis: AJ, CS, PG, RR, BP, and CNB. Statistical analysis: CS, VS and SC. CS wrote the draft of the manuscript; RS, AJ, VS and SC revised the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100651.

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