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ORIGINAL PAPER

Evidence for Methylerythritol Pathway (MEP) Contributions to Zerumbone Biosynthesis as Revealed by Expression Analysis of Regulatory Genes and Metabolic Inhibitors Studies

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Abstract

Sesquiterpenoid Zerumbone, the principal secondary metabolite in *Zingiber zerumbet* Smith, has been identified as the putative molecule conferring resistance against soft-rot causative, *Pythium myriotylum*. Metabolic precursors for sesquiterpenoid biosynthesis namely, isopentenyl diphosphate (IPP) and DMAPP, are generated either from cytosolic mevalonic acid (MVA) and/or the plastidal methylerythritol phosphate (MEP) pathway. Evaluation of expression pattern of regulatory genes of MEP and MVA pathway following *P. myriotylum* infection revealed that while transcripts of MVA regulatory gene, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*), did not show significant changes, biphasic expression pattern was observed for MEP pathway regulatory genes, viz., 1-deoxy-D-xylulose-5-phosphate reductase (*DXR*) and 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) as well for *ZzTPS* (terpene synthase) and Farnesyl phosphate synthase (*FPS*) compared with uninfected control. Contribution of the two pathways in zerumbone biosynthesis was evaluated in axenic tissue culture raised *Z. zerumbet* plantlets using MVA and MEP pathway specific inhibitors, mevinolin (MEV) and fosmidomycin (FOS) respectively. Results generated by regulatory gene and metabolite analysis are informative with respect to the role of plastidial IPP pool generated via MEP pathway in zerumbone biosynthesis and the possible redirection of flux in the event of metabolic perturbations mediated by chemical inhibitors.

Keywords Zerumbone · MVA · MEP · Mevinolin · Fosmidomycin · *HMGR* · *DXR* · *DXS*

Key Message
- Biphasic expression observed for methylerythritol (MEP) pathway regulatory genes in *Zingiber zerumbet* following *Pythium myriotylum* infection.
- Metabolite analysis of in vitro plantlets treated with mevinolin (MEV) and fosmidomycin (FOS) reveals 54-fold decrease in zerumbone following FOS treatment.
- Identified plastidial IPP flux contributing more towards zerumbone biosynthesis.

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Introduction

Z. zerumbet (Family Zingiberaceae) also called pine cone or shampoo ginger is a perennial rhizomatous plant found either in cultivated, wild, or naturalized states (CABI 2014). Taxon has a Southeast Asian origin with center of diversity in the Indo-Malayan region and possibly spread to the Oceania (Yob et al. 2011) by humans. Rhizomes of *Z. zerumbet* find a wide range of ethnomedicinal uses (Sulaiman et al. 2010; Yob et al. 2011; Koga et al. 2016) as documented in Ayurvedic and Chinese Pharmacopoeias (Yob et al. 2011). Characterization of essential oil from *Z. zerumbet* rhizomes has identified zerumbone (37%), α-humulene (14.4%), and camphene (13.8%) as major metabolites (Ming et al. 2011). The principal bioactive sesquiterpenoid compound, zerumbone (1,2,6,9-humulatrien-8-one, or 2,6,9,9-tetramethyl-[2E,6E,10E]-cycloundeca-2,6,10-trien-1-one), has been widely investigated for its medicinal properties. Our earlier studies have identified zerumbone to play a crucial role in imparting *Pythium* resistance to the wild congener (Keerthi et al. 2014). *Z. zerumbet* being rich in terpenoids and phenylpropanoid

polyketides (Koga et al. 2016; Sulaiman et al. 2010; Baby et al. 2009), a significant part of the chemical diversity could be expected to protect the plant from invading phytopathogens. Pathogen attack has been reported to induce terpenoid biosynthesis (Vranová et al. 2013) via independently regulated cytosolic mevalonic acid (MVA) and the plastidal methylerythritol phosphate (MEP) pathways. Terpenoids are formed by consecutive condensation of C5 isoprenoid (IPP) units namely, isoprenyl diphosphate (IDP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Chen et al. 2011; Tholl 2015; Pazouki and Niinemets 2016), biosynthesized from the MVA and MEP pathway. Condensation of IDP and DMAPP precursors generates acyclic prenyl diphosphates, viz., geranyl diphosphate (GDP), farnesyl diphosphate (FDP), geranyl geranyl diphosphate (GGPP) and so on that give rise to monoterpenes, sesquiterpenes, and diterpenes respectively through reactions catalyzed by terpene synthases (*TPS*). Zerumbone biosynthesis from the sesquiterpenoid precursor FDP has been elucidated (Okamoto et al. 2011) and starts with cyclization of FDP to α-humulene by humulene synthase encoded by *ZSS*1 gene (Yu et al. 2008a, b) which is subsequently hydroxylated by cytochrome P450 enzyme encoded by *CYP*71*BA*1 to α-humulene-8-hydroxylase (Yu et al. 2011) that is acted upon by short-chain alcohol dehydrogenase enzyme to form zerumbone (Okamoto et al. 2011).

Though the zerumbone biosynthetic pathway from FDP has been elucidated (Glazebrook 2005), the biosynthetic origin of IPP precursor for FDP generation and subsequent channelization towards zerumbone biosynthesis is yet to be discerned. Early research on various plant taxa indicated that the plastidial IPP pool generated by MEP pathway is directed for monoterpene, diterpene, and tetraterpene biosynthesis (Yang et al. 2012; Rodríguez-Concepción and Boronat 2002) whereas the cytosolic IPP was directed towards biosynthesis of sesquiterpenes and triterpenes (Rodríguez-Concepción and Boronat 2002; Bick and Lange 2003). However later studies have documented considerable cross-talk between the MVA and MEP pathways (Rodríguez-Concepción and Boronat 2002; Bick and Lange 2003; Laule et al. 2003). Some studies have reported fully bidirectional exchange of IPP (Hemmerlin et al. 2003; De-Eknamkul and Potduang 2003) whereas others have documented an efficient IPP transport from plastids to cytosol (Wu et al. 2006; Yang et al. 2012). This cross-talk between the two IPP biosynthetic pathways has been reported to increase under stress conditions with specific inhibition or decrease in IPP synthesis by either of the two pathways (Hemmerlin et al. 2003; De-Eknamkul and Potduang 2003). Nevertheless, the relative contribution of each pathway to the biosynthesis of terpenoids remains uncertain in many less studied tropical plants with a rich terpenoid profile.

Rate-limiting enzyme of the cytosolic MVA pathway is 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) (Pazouki and Niinemets 2016; Rodríguez-Concepción and Boronat 2002) while for plastidial MEP pathway, 1-deoxy-Dxylulose 5-phosphate synthase (*DXS*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) constitute the regulatory enzymes (Pazouki and Niinemets 2016; Rodríguez-Concepción and Boronat 2002). Activity of regulatory enzymes catalyzing the committed steps of MVA and MEP pathways can be blocked with specific inhibitors, viz., mevinolin (MEV) that inhibits *HMGR* enzyme and fosmidomycin (FOS) inhibiting *DXR* enzyme (Kanchiswamy et al. 2015; Palazón et al. 2003; Zhi et al. 2005; Dudareva et al. 2005). MEV and FOS have been used in many studies to evaluate the interaction between cytosolic and plastidial pathways such as taxane accumulation in *Taxus* spp. (Wang et al. 2003; Cusido et al. 2007), artemisinin biosynthesis in *Artemisia annua* (Towler and Weathers 2007), carotenoid biosynthesis in tomato fruit (Rodriguez-Concepcion and Gruissem 1999), and tetrahydrocannabinol in *Cannabis sativa* (Mansouri and Salari 2014) (De-Eknamkul and Potduang 2003; Palazón et al. 2003). Against this backdrop, present study was undertaken to elucidate metabolic changes in *Z. zerumbet* after (i) infection with *Pythium myriotylum* to study variations in expression of regulatory genes of MEP and MVA pathways and (ii) MEV and FOS treatment to identify the terpenoid biosynthesis pathway contributing to IPP precursor pool for zerumbone biosynthesis.

Materials and Methods

Pythium Infection, Total RNA Isolation, and Quantitative Real-Time PCR

Healthy *Z. zerumbet* rhizome pieces (1 g; 20–30 mm diameter) pre-treated with 70% ethanol (v/v in 0.1% DEPC water) were inoculated with 7-day-old *P. myriotylum* mycelial disc (5 mm diameter) grown in potato dextrose agar (PDA) medium (pH 6.5). The infected rhizomes were incubated for time periods: 2, 4, 6, 8, 16, and 24 h. Control experiments consisted of rhizome pieces inoculated with PDA discs. All experiments were carried out in triplicate. RNA was isolated according to Salzman et al. (1999) and the total RNA was treated with RNase-free DNaseI (Promega). cDNA was synthesized from 1 μg of DNase I-treated total RNA primed with 12-18 mer oligo dT and first strand cDNA synthesized using 200 U of MMLV-RT (Promega) according to manufacturer's instructions.

Primers designed to conserved region of regulatory genes of MVA and MEP pathway, viz., *HMGR* (3-hydroxy-3-methylglutaryl-coenzyme A reductase), *DXS* (1-deoxyxylulose 5-phosphate synthase), *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), and *FPS* (farnesyl pyrophosphate synthase), were obtained from literature (Cheng et al. 2013) (Table 1). TPS primers were designed based on sequence information of *TPS* gene designated *ZzTPS*, cloned and characterized from *Z. zerumbet* rhizomes (Peter et al. 2019).The qRT-PCR was set up in a final volume of 20 μl containing

10 µl SYBR Green PCR core reagent (Applied Biosystems), 2 µl diluted cDNA, and 300 nM each of the gene-specific primers (Table 1). PCR conditions consisted of following steps: 50 °C for 2 min initially followed by 95 °C for 7 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s in a real-time PCR machine (ABI 7700). Housekeeping gene actin (*ActF*1 and *ActR*1) (Table 1) was used as endogenous control. Two biological replicates were analyzed at each time point with three technical replicates for each experimental sample. Data analysis was performed with Step-One Analysis Software package 1.7 (Applied Biosystems). The software uses comparative C_T method for expression analysis that mathematically transforms threshold cycle to relative expression values after normalization with reference genes.

Preparation of Explants

Mature rhizomes of *Z. zerumbet* were collected and placed in moist cotton for sprouting. After 8–10 days, axillary buds that sprouted from the rhizomes were used as explants. Buds were thoroughly washed under running tap water for 30 min, treated with 20% sterilization solution (2.5% sodium hypochlorite and 0.01% Tween 20) for 10 min under continuous agitation followed by rinsing with sterile distilled water. Subsequently the buds were sterilized with 0.1% (v/v) mercuric chloride for 3 min and washed with 70% ethanol for 2 min followed by three rinses with sterile distilled water for 15 min each to remove all traces of sterilants.

Inoculation and Establishment of Explants

Sterilized buds were inoculated in standard Murashige Skoog (MS) medium, supplemented with $CaCl_2$ (0.1 mM), sucrose (5%), and 0.8% agar. Cultures were incubated at 25 ± 2 °C with a 16-h photoperiod and irradiance of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Sub-culturing was carried out after every 14 days to fresh MS media. Healthy in vitro plantlets were used for further studies using metabolic pathway inhibitors.

Inhibitor Treatment

Mevinolin (MEV) that inhibits mevalonate (MVA) pathway by targeting *HMGR* and fosmidomycin (FOS) that binds *DXR* thereby inhibiting methylerythritol phosphate (MEP) pathway were used for the study. Stock solutions of MEV (2.18 µM) and FOS (12.35 mM) were prepared in absolute ethanol and sterile distilled water respectively. From the stock solutions, MEV and FOS were added to sterile MS media to a final concentration of 1 µM and 200 µM respectively. Healthy plantlets were then transferred to sterile MS media containing inhibitors and incubated for 10 days. In the control experiments, in vitro *Z. zerumbet* plantlets were cultured in MS medium wherein inhibitors were replaced with equal volume of absolute ethanol or sterile water. All experiments were carried out in triplicate.

GC-MS Analysis

The proliferated root mass of in vitro *Z. zerumbet* plantlets from inhibitor treated and untreated experiments were ground under sterile conditions in liquid nitrogen and suspended in absolute methanol containing 0.02% (w/v) ascorbic acid. The mixture was incubated at 25 °C for 90 min at 100 rpm. After centrifugation of homogenate at 150*g* at 25 °C, the metabolite(s) containing solvent phase was transferred to fresh tube.

Table 1 Primers used for qRT-PCR experiments

Primer name	Direction	Nucleotide sequence (5′→3′)	Reference
TPSrtFor1 TPSrtRev1	Forward Reverse	gagaactacttgtggaccgtg atagacatcataatacacatcatcgat	Peter et al. 2019
HMGRrtFor	Forward	aagtagcgaagcccaagtac	Cheng et al. 2013
HMGRrtRev	Reverse	aatcgtctgggtgctgaatg	
MEPrtFor	Forward	acacattctgctccgctttc	Cheng et al. 2013
MEPrtRev	Reverse	gggcgtttttattggtgag	
DXRrtFor	Forward	tcggctacttggacatcatc	Cheng et al. 2013
DXRrtRev	Reverse	acagcagtgagattgatggc	
FPSrtFor	Forward	gagaaaaagatgaagccaag	Cheng et al. 2013
FPSrtRev	Reverse	ccgtagttttccttgatgag	
DXSrtFor	Forward	agagcgactacgactgctttgg	Cheng et al. 2013
DXSrtRev	Reverse	caggtagccagcattgttcatt	
ActF1	Forward	atctggcaccacaccttctacaatgagctgcg	Kadowaki et al. 2001
ActR1	Reverse	cgtcatactcctgcttgctgatccacatctgc	

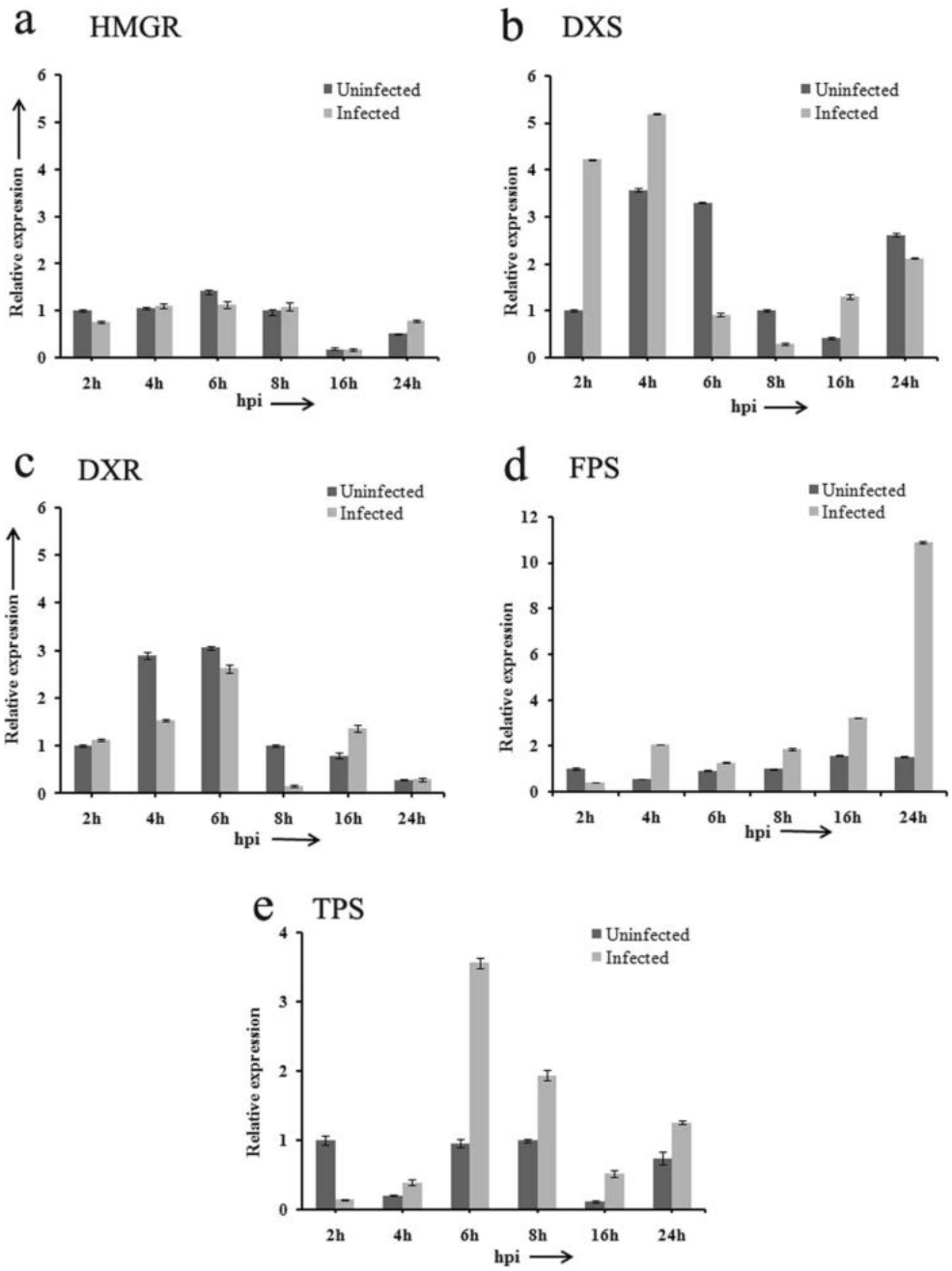

Fig. 1 Relative expression levels of regulatory genes of mevalonate (MVA) and methylerythritol phosphate (MEP) pathways. Time course expression analyses from 2 to 24 h post infection (hpi) of **a** *HMGR*, **b** *DXS*, **c** *DXR*, **d** *FPS*, and **e** ZzTPS were determined by qRT-PCR after *P. myriotylum* infection. Relative expression is the mean of three technical replicates and bars indicate the standard errors

The solvent extract was concentrated in a water bath at 42 °C for 1 h, and the resultant concentrated extract was subjected to GC-MS analysis. Analysis was done using high-resolution Agilent GC 7890A (injector temperature 250 °C) coupled to Agilent 5975C mass detector in the split mode of 50:1. Separation was carried out in a DB 5 MS column with dimensions 30 m × 0.25 mm × 0.25 µm. Carrier gas used was helium with a flow rate of 1.0 ml min^{-1}. Initial temperature of the oven was 40 °C with an increase of 5 °C min^{-1} to 280 °C. Peaks obtained were identified by matching the mass spectra in Wiley and National Institute of Standards and Technology (NIST) Mass Spectral Library.

Results and Discussion

Z. zerumbet–P. myriotylum Interactions and Modulation of MEP and MVA Pathway Regulatory Genes

Genes involved in terpenoid production have been well studied in model plants with the cytosolic MVA and plastidial MEP pathways being two main pathways contributing the IPP precursor (Nagegowda 2010; Pazouki and Niinemets 2016). Transcript levels of regulatory genes of MEP and MVA pathways were investigated after *P. myriotylum*

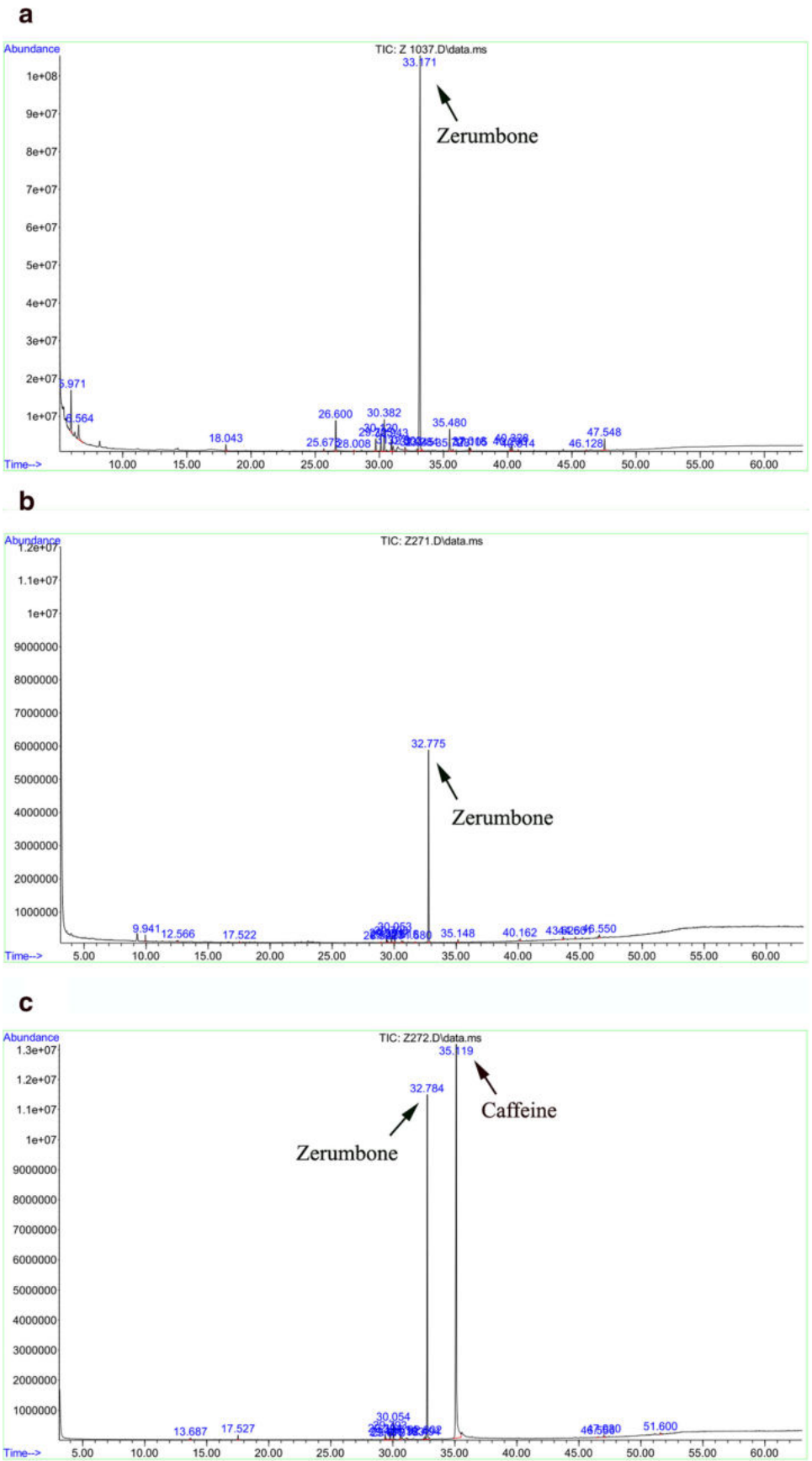

Fig. 2 GC-MS metabolite profile of *Z. zerumbet* in vitro plantlets after exposure to MVA and MEP pathway inhibitors, mevinolin (MEV) and fosmidomycin (FOS). Variations in metabolite profile were observed in **a** untreated, **b** MEV-treated, and **c** FOS-treated in vitro plantlets. The major metabolites detected are indicated with black arrowheads

Table 2 Phytoconstituents identified by GC-MS analysis in the untreated *Z. zerumbet* in vitro plantlet with the table indicating retention time and relative abundance (in %) of metabolites

S. no.	Compound name	Retention time	Relative abundance (%)
1	Benzene, [(methylsulfinyl)methyl]-	5.97	5.960
2	p-Dioxane-2,3-diol	6.564	2.927
3	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)-	18.043	0.794
4	Caryophyllene	25.676	0.315
5	α-Caryophyllene	26.600	3.535
6	Phenol, 2,5-bis(1,1-dimethylethyl)-	28.008	0.190
7	Caryophyllene oxide	29.725	1.546
8	Caryophyllene oxide	30.120	1.992
9	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R, 3E,7E,11R*)]-	30.382	3.576
10	Calarene epoxide	30.943	1.583
11	10-Methyl-8-tetradecen-1-ol acetate	32.945	0.398
12	*2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-*	*33.17*	*69.391*
13	Isoaromadendrene epoxide	35.480	3.048
14	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	35.728	0.280
15	Hexadecanoic acid, methyl ester	37.018	0.414
16	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	37.105	0.321
17	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	40.200	0.341
18	11-Octadecenoic acid, methyl ester	40.328	0.705
19	Octadecanoic acid, methyl ester	40.814	0.171
20	3-Benzyl-2-phenyl-2,3,4,5-tetrahydro-1H-benzo[d]azepine	46.128	0.128
21	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	47.548	1.416

Table content highlighted in italics indicate details of major metabolite with relative abundance at a specific retention time (tR)

infection in *Z. zerumbet* at different time intervals ranging from 2 to 24 hours post infection (hpi) by quantitative RT-PCR (Fig. 1). Transcript levels of MVA regulatory gene, *HMGR*, did not show significant changes during the examined time course (Fig. 1a). However, expression pattern of *DXR*, *DXS*, and *ZzTPS* (Fig. 1b, c, e) revealed biphasic upregulation with induction observed from 4 to 6 hpi compared with uninfected rhizomes, declining at 8–16 hpi and again being induced at 24 hpi. Expression profile of *FPS* which regulates the synthesis of IPP precursors towards sesquiterpenoid biosynthesis revealed a gradual induction in gene expression from 4 to 24 hpi (Fig. 1d) compared with uninfected sample. Much evidence has accumulated through studies in other plant taxa showing overexpression of *DXS* that catalyzes the rate-limiting step in MEP pathway to be accompanied by concomitant accumulation of corresponding SM as in *Mentha piperita* (Wildung and Croteau 2005) and *Vitis vinifera* (Savoi et al. 2016) as well as upregulation of taxadiene in transgenic *Arabidopsis thaliana* (Tholl and Lee 2011; Köksal et al. 2011). Results point to the plausible role of plastidial IPP generated from MEP pathway as contributing to biosynthesis of zerumbone that mediates terpenoid chemical defense in *Z. zerumbet*. Stress-mediated elicitation of principal secondary metabolites has been previously documented in various plant taxa (Srivastava et al. 2016; Ramakrishna and Ravishankar 2011). Our earlier experiments also made a similar observation with an 8-fold induction in zerumbone content following *P. myriotylum* infection (Keerthi et al. 2014). Hence, it could be presumed that the observed upregulation of MEP pathway regulatory genes could be contributing towards induction of zerumbone, following soft-rot infection.

Metabolite Profiling in Response to MEP and MVA Pathway Inhibitors

In various plant taxa, studies have observed lack of correlation between induced regulatory gene expression and concomitant metabolite content (Yu et al. 2008a, b). In case of *Z. zerumbet*, treatment with methyl jasmonate (MeJA), a phytohormone known to induce terpenoid biosynthesis (Martin et al. 2003; Li et al. 2012), induced ZSS1-encoded humulene synthase gene expression but the expected α-humulene accumulation was not observed (Yu et al. 2008b). Hence, validation of the fate of plastidial IPP pool generated via MEP pathway was determined by carrying out metabolomic studies in presence of pathway inhibitors, viz., MEV and FOS (Kuzuyama et al. 1998), that allows precise identification of IPP generating pathway(s) responsible for biosynthesis of the principal sesquiterpenoid metabolite. GC-MS profile of untreated in vitro *Z. zerumbet* plantlet detected zerumbone as major

Table 3 Phytoconstituents detected by GC-MS analysis in MEV-treated in vitro *Z. zerumbet* plantlet. Retention time and relative abundance (in percentage) of the detected metabolites are indicated. The major metabolite detected is set in italics

S no.	Compound name	Retention time	Relative abundance (%)
1	Bis[{1,2-bis(diisopropylphosphino)ethane}platinum(0)]-[μ-(η-2,η-2-cycloocta-1,5-diene)]	9.94	1.135
2	Lycoxanthin	12.56	0.441
3	Camphor	17.52	0.520
4	Dodecanoic acid	28.92	0.655
5	Caryophyllene oxide	29.39	1.461
6	Diethyl Phthalate	29.48	0.964
7	Caryophyllene oxide	29.79	2.324
8	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-	30.05	4.185
9	cis-Z-α-Bisabolene epoxide	30.61	1.873
10	*2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-*	*32.77*	*77.607*
11	1,2-Longidione	35.14	1.335
12	10-Octadecenoic acid, methyl ester	40.16	1.312
13	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	43.62	1.651
14	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	44.60	1.295
15	Oleic Acid	46.55	2.337

metabolite (Fig. 2a) with relative abundance of 69.39% at 33.17 retention time (Rt) (Table 2). When treated with MEV, a 12-fold increase in zerumbone accumulation (77.61%) was observed (Table 3; Fig. 2b) compared with untreated control (Table 2; Fig. 2a). However in FOS-treated in vitro plantlets, a pronounced decrease of 54-fold was observed with the relative abundance of zerumbone detected as 31.99% (Table 4; Fig. 2c), thus validating the role of plastidial MEP pathway in generating IPP precursor for zerumbone biosynthesis. Though MEV and FOS have been identified as highly efficient plant growth inhibitor (Yang et al. 2012; Zhi et al. 2005), significant growth inhibition was not observed in present study at the selected concentration of MEV (1 μM) and FOS (200 μM). Hence, reduction in plant biomass can be overruled as the factor responsible for decrease in zerumbone content but rather the reduction is due to inhibition of MEP pathway. Another interesting observation was the detection of the purine alkaloid, caffeine, as another major metabolite in FOS-treated plantlets with relative abundance of 62.17% (Table 4; Fig. 2c). This observation sheds light on the multiplicity of

Table 4 Phytoconstituents detected by GC-MS analysis in FOS-treated in vitro *Z. zerumbet* plantlet with retention time and relative abundance (in %). Major metabolites detected are indicated in italics

Sl no.	Compound name	Retention time	Relative abundance (%)
1	Eucalyptol	13.687	0.170
2	Camphor	17.52	0.463
3	Caryophyllene oxide	29.39	0.506
4	Diethyl Phthalate	29.48	0.183
5	3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E)	29.67	0.088
6	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-	30.05	0.709
7	Alloaromadendrene oxide-(1)	30.61	1.571
8	2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	31.18	0.668
9	2H-Oxocin-2,8(5H)-dione, 3,4,6,7-tetramethyl-	32.49	0.114
10	10-Methyl-8-tetradecen-1-ol acetate	32.60	0.151
11	*2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-*	*32.78*	*31.990*
12	*Caffeine*	*35.11*	*62.171*
13	[1,1'-Bicyclopropyl]-2-octanoic acid, 2′-hexyl-, methyl ester	46.55	0.186
14	Methyl 11-docosenoate	47.3	0.438
15	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	51.60	0.222

Table content highlighted in italics indicate details of major metabolite with relative abundance at a specific retention time (tR)

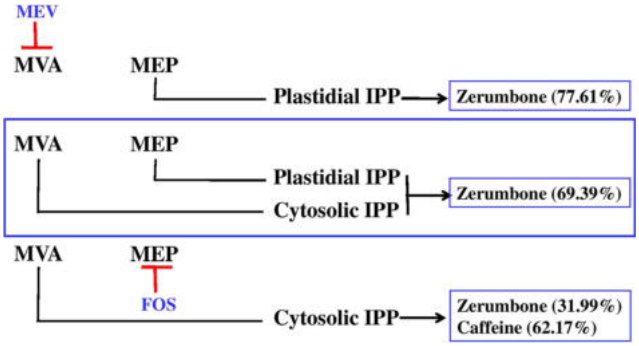

Fig. 3 Model depicting the IPP metabolite flux in *Z. zerumbet* in response to chemical inhibitors

adaptive pathways utilized following metabolic perturbations by the enzyme inhibitor, FOS. Phylogenetic studies have revealed convergent evolution of caffeine synthesis in plants with approximately 30 of the 30,000 angiosperms having the ability to synthesize the purine alkaloid (Huang et al. 2016). A possible explanation for detection of caffeine after FOS treatment in *Z. zerumbet* could involve redirection of primary metabolites towards de novo purine synthesis leading to caffeine production following the metabolic block in IPP generating MEP pathway. A model to understand the IPP metabolite flux in *Z. zerumbet* in response to chemical inhibitors is shown in Fig. 3.

Conclusion

Z. zerumbet has been previously documented as a putative soft rot resistance donor for ginger improvement (Kavitha et al. 2005). Wild plants are usually well adapted to extreme conditions and resist pathogen infection with multiple approaches that include extraordinary metabolite profile, presence of novel genes/pathways, and/or other biological factors, none of which is thoroughly understood in *Z. zerumbet*. The rich metabolic repertoire comprising of terpenoids and phenylpropanoid polyketides (Koga et al. 2016; Sulaiman et al. 2010; Yu et al. 2008a, b; Baby et al. 2009) and robust biotic resistance in *Z. zerumbet* makes it a good non-model candidate to study the metabolic pathways contributing to chemical defenses. Various studies have shown redirection of IPP metabolite flux following pathogen interactions, confirmed by marked changes in transcript levels of regulatory genes of MVA and MEP pathway (Strack and Fester 2006; Walter et al. 2010). There exists considerable variability in the plant kingdom with regard to the origin of IPP/DMAPP for sesquiterpenoid biosynthesis. Sesquiterpenes were previously thought to be synthesized exclusively in cytosol using IPP precursors generated from MVA pathway. Present study contradicts this observation with the plastidial IPP flux contributing more towards biosynthesis of sesquiterpenoid zerumbone (77.61%) than cytosolic IPP (31.99%). Similar observations on exclusive utilization of plastid-derived IPP/DMAPP in sesquiterpene biosynthesis have been made in wild tomato (Sallaud et al. 2009), grapes (May et al. 2013), *Stevia rebaudiana* (Wölwer-Rieck et al. 2014), and *Solidago canadensis* (Steliopoulos et al. 2002). The results therefore provide novel insights into zerumbone biosynthesis utilizing the plastidial IPP pool generated via MEP pathway and the possible redirection of flux in the event of metabolic perturbations mediated by chemical inhibitors.

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Author Contributions RAN conceived and designed the experiments. RAN and KD performed the experiment. RAN, KD, and PP analyzed the results and wrote the manuscript. All authors have read and approved the final manuscript.

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