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Correlation of Phenylalanine ammonia lyase (PAL) and Tyrosine ammonia lyase (TAL) activities to phenolics and curcuminoid content in ginger and its wild congener, *Zingiber zerumbet* following *Pythium myriotylum* infection

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Abstract The biochemical basis of resistance exhibited by a wild *Zingiber* species, *Zingiber zerumbet* (L.) Smith, towards the economically devastating soft-rot disease caused by necrotrophic *Pythium myriotylum* was investigated. Quantification of phenolic compounds revealed higher total phenolic (TP), total flavonoid (TF) and total tannin (TT) content in the uninfected susceptible ginger (*Z. officinale*) cultivar compared to the resistant taxon. However systemic induction in activities of rate-limiting enzymes of phenolic biosynthetic pathway, phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), were observed in the resistant wild taxon. In the ginger cultivar, even though the inherent PAL specific activity was observed to be higher (24.2 ± 1.9 U mg^{-1}) compared to the wild taxon (4.2 ± 0.8 U mg^{-1}), a subsequent gradual decrease in both PAL and TAL activities were observed following infection of rhizomes with *P. myriotylum*. This was in contrast to the gradual increase in PAL (13.1 ± 0.8 U mg^{-1}) and TAL (442.5 ± 35.1 U mg^{-1}) specific activity after 5 days post infection (dpi) in the wild taxon. Subsequent HPLC analysis of rhizomes showed an increase in total curcuminoid content in the wild taxon compared to the ginger cultivar. Results are indicative of phenylpropanoid pathway regulation in a manner such that the induced defense metabolites contribute to restrict pathogen invasion in the resistant wild taxon.

Keywords Total phenolics · *Pythium* · *Zingiber zerumbet* · PAL, TAL, Curcuminoid

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Introduction

Ginger (*Zingiber officinale* Rosc.), a major spice crop in tropical and subtropical countries, is affected by soft rot disease, caused by *Pythium myriotylum*, inflicting substantial loss in yield (Sarma 1994; Dake 1995; Dohroo 2005). Disease management strategies rely on chemical pesticides, especially mancozeb and metalaxyl (Rathaiah 1987), unmindful of the collateral damage they pose to environment and human health. Ginger is unknown in the wild state (Davidson and Jaine 2006) and so is its domestication and early history. Due to its obligatory vegetative mode of propagation, it is reasonable to assume that in consequence of continued domestication for selective traits over a long period, ginger has lost its natural dissemination by seed dispersal mechanisms. Plants are constrained by a trade-off between growth and defense so that agronomic selection for increased yield and growth rate result in reduction in plant defenses (Rosenthal and Dirzo 1997; Olsen and Gross 2008). On the contrary, the mode of propagation of wild *Zingiber* species ranges from asexual to sexual (Kavitha and Thomas 2008). Evolutionary success of

plants relies on sustenance of sexual reproduction (Jaenike 1977) which allows genetic reshuffling and maintains adaptive evolution of the plant innate immune system. Previous experiments evaluating soft-rot resistance in wild *Zingiber* germplasm has revealed *Z. zerumbet* to exhibit marked resistance to *P. aphanidermatum* (Kavitha and Thomas 2008).

Among the diverse defense mechanisms evolved by plants, the arsenal of low-molecular weight phenolics represents inbuilt constitutive chemical barriers to infection (Nicholson and Hammerschmidt 1992; Osbourn 1996; Hammerschmidt 2005; Mary 2006). Phenolics, implicated as resistance/incompatibility factors (Osbourn 1996; Hammerschmidt 2005) are synthesized via shikimate-phenylpropanoid-flavonoid pathway (Harborne 1999). Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and Tyrosine ammonia-lyase (TAL; EC 4.3.1.) are key regulatory enzymes involved in the phenylpropanoid pathway (Winkel-Shirley 2001; Koes et al. 2005; Vogt 2010). PAL converts L-phenylalanine to trans-cinnamic acid (Koukol and Conn 1961) where as TAL catalyzes an analogous reaction with L-tyrosine as substrate and generating ammonia and p-coumaric acid (Neish 1961). Subsequent metabolism results in generation of a wide variety of phenolic metabolites that include simple phenolics or salicylates, coumarins, lignins, tannins, flavonoids and anthocyanins (Jones 1984; Dixon and Paiva 1995; Dixon et al. 2002; Morrison and Buxton 1993). Systemic induction and accumulation of plant polyphenolics is observed in response to various diseases (Matern and Kneusel 1988; Picinelli et al. 1995; Wallis et al. 2008; Petkovsek et al. 2008). Besides experiments documenting phenolics as important markers for resistance to pathogens (Witzell and Martin 2008), significant fluctuations in PAL and TAL activities have also been reported in plant tissues subsequent to various physical and chemical stimuli (Jones 1984; Ju et al. 1995; Schmidt et al. 2004).

Although previous studies have documented an abundance of ESTs for phenylpropanoid pathway enzymes in ginger (Koo et al. 2013), there is paucity of information on phenolic profiling in *Zingiber* spp. Earlier experiments have documented absence of localized programmed cell death (PCD) or hypersensitive response (HR) in *Z. zerumbet* following soft rot infection (Kavitha and Thomas 2008). HR being characteristic of plant defense responses (Lam et al. 2001), the reported observations provided the rationale to undertake the present study to determine if phenolics afford plant protection against *Pythium* spp in the wild congener. Thus experiments were carried out to analyze the biochemical basis of defense by determining: (i) the role of inherent polyphenolic content in resistance of the wild taxon, and (ii) whether variations in PAL and TAL activities account for the differential response of the ginger cultivar and the wild taxon to soft rot infection.

Fresh rhizomes of *Z. zerumbet* (*Zz*) and *Z. officinale* cv. Varada (*Zo*) collected from Indian Institute of Spices Research (IISR), Calicut, Kerala were cleaned and surface sterilized twice with 70 % alcohol for 10 min followed by 0.1 % sodium hypochlorite for 5 min and rinsed twice with sterile distilled water for 10 min to remove traces of sterilants. Seven day old *P. myriotylum* strain (RGCBN14) maintained on potato dextrose agar (PDA) at 25 ± 2 °C was used for infection. Rhizomes (20–30 mm diameter) of both the ginger cultivar and the wild taxon were divided into two groups, one group as control (non- inoculated) comprised of rhizome with PDA discs placed over them while the other was inoculated with *P. myriotylum* mycelial disc (5 mm) and incubated at 25 °C for 4 days. Inoculated and uninoculated rhizomes were ground to a fine powder in liquid nitrogen and extracted using methanol (20 % *v/v*) containing ascorbic acid (0.02 % *w/v*). Extracts were incubated at 25 °C for 90 min under subdued light by wrapping with aluminium foil. Following incubation, the extracts were filtered and used for estimation of TP, TF and TT content. TP content was determined with Folin-Ciocalteu's reagent using gallic acid (25–250 μg/ml) as standard (Li et al. 2008) and expressed as Gallic acid equivalent (GAE) in milligram per 100 g dry weight. TF was estimated by colorimetric method according to Xu and Chang (2007). A standard curve was calibrated with (+)-catechin (10-100 μg/ml) and expressed as mg catechin equivalent (CE)/ 100 g dry weight. TT was quantified by Folin-Denis method (Schanderl 1970) and expressed as tannic acid equivalent (TAE).

Enzyme extracts were prepared from both inoculated and uninoculated rhizomes incubated at different time intervals of 0, 1, 2, 3, 5, 7 and 9 days post infection (dpi). For this, rhizomes (1 g) were homogenized in chilled sodium borate buffer (0.1 M; pH 8.8) containing β-mercaptoethanol (5 mM), PVP (10 % *w/v*) and ascorbic acid (0.2 % *w/v*). After centrifugation at 8000 rpm for 30 min at 4 °C, filtered supernatant was used for enzyme assays according to the method of Beaudoin-Eagan and Thorpe (1985). Briefly, the extract was incubated at 37 °C for 1 h in 0.1 M sodium borate buffer (pH 8.8) containing either 20 mM L-phenylalanine for PAL or

20 mM tyrosine for TAL assays. Reactions were stopped by adding 5 N HCl and amount of trans-cinnamic acid or p-coumaric acids were estimated in triplicate by measuring the absorbance at 290 nm and 333 nm for determination of PAL and TAL activities respectively. Enzyme activities were expressed in nmoles of trans-cinnamic acid or p-coumaric acids formed in mg protein^{-1} min^{-1}.

For curcuminoid estimation, the methanolic extract was concentrated on a rotary evaporator (Basis Hei – VAP ML, Heidolph) under vacuum and filtered through ultra membrane filter (0.45 μm, Millipore). Quantitative analysis was carried out in an HPLC system (LC 2010CHT, Shimadzu, Japan) equipped with UV detector and Class LC solution software. A 20 μl aliquot was injected into an analytical reverse phase 2.5 μm C18 column (Phenomenex Luna, (100 × 3.0 mm i.d). The operating conditions were as follows: autosampler temperature of 15 °C, column temperature at 35 °C, eluent flow rate of 2.0 mL/min and a detection wavelength of 420 nm. Elution solvents were A (1 mM of phosphate buffer, pH 4.0) and B (100 % acetonitrile) with the following gradient: 0–0.01 min, solvent A: B (45:55, *v/v*); 0.01–4 min (40:60); isocratic from 6–15 min (45:55). Experiments were performed in triplicate with curcuminoid (Sigma) as internal standard. Antagonistic effect of curcuminoids on *P. myriotylum* was examined by radial diffusion assays. Mycelial disc (5 mm) from 7-day-old *P. myriotylum* culture grown in PDA was placed on Whatman No. 4 filter paper disc (10 mm) impregnated with increasing concentration of curcuminoid (600–1500 μg/ml) prepared in DMSO (dimethyl sulfoxide) containing polyethylene glycol (PEG) (0.5 %) in centre of PDA plates. Control experiments consisted of PDA discs placed on filter paper impregnated with DMSO containing PEG (0.5 %). Plates were incubated at 25 °C and radial growth measured in triplicate as mean ± SD of the inhibition zone calculated using the formula: $[I\% = (C - T)C^{-1}] \times 100$ where I% is the relative inhibition, C is the hyphal growth diameter measured in the control, experiment and T is the hyphal growth diameter in curcuminoid-treated plates. All measurements were done in triplicate and results expressed as mean ± standard deviation. Statistical evaluations were done using MiniTab statistical package version 14.0 (MiniTab Software Inc., USA). Two-way analysis of variance (ANOVA) was followed by Tukey's multiple comparison to identify significant difference among the means with hypothesis testing at $p < 0.05$.

Phenolic biosynthesis and their polymerization in the cell wall constitute an effective defense mechanism (Moerschbacher et al. 1990; Rengel et al. 1994; Massei and Hartley 2000; Espinosa-Alonso et al. 2006) against necrotrophic fungal pathogens (Hammerschmidt 2005; Osbourn 1996). To evaluate correlation between total phenolic content and soft rot resistance, inoculated and uninoculated rhizomes of susceptible *Z. officinale* cv. Varada and resistant *Z. zerumbet* were estimated for TP, TF and TT content. Results indicated higher TP, TF and TT content in the uninfected susceptible cultivar compared to the resistant taxon (Fig. 1). However *P. myriotylum* infection caused a 1.6-fold increase of TP in the wild taxon compared to 1.1-fold observed in the cultivar. The TF content were increased by 1.55-fold in both the taxa while 1.2-fold enhancement of TT content was observed in the wild taxon compared to 1.7-fold in the cultivar. In our study, despite an abundance of total phenolics, complete susceptibility to soft-rot in ginger could be attributed to the process of continuous breeding and selection of ginger varieties for higher phenolic content like gingerols, shogaols and zingiberene that resulted in decreasing potential of the species to overcome biotic challenges. Earlier studies have revealed genetic monomorphism in ginger cultivars in both neutral (Kavitha and Thomas 2008) and functional (Aswati and Thomas 2007; 2012) loci as a consequence of its obligate asexuality combined with selective breeding. Breeding objectives for ginger improvement focus on superior vegetative growth, low fibre content and higher content

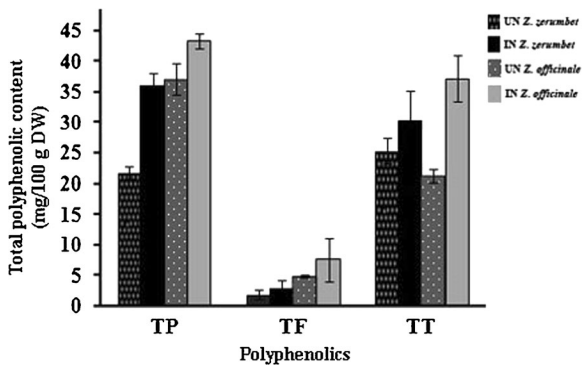

Fig. 1 Estimation of polyphenolics (total phenolics: *TP*, total flavonoid: *TF* and total tannin: *TT*) in methanolic extracts of healthy (UN) and *P. myriotylum* infected (IN) rhizomes of *Zingiber zerumbet* and *Zingiber officinale* cv Varada. TP, TF and TT content expressed respectively as mg GAE, mg CE and mg TAE equivalent per 100 g dry weight (DW). Values are mean ± SEM of 3–5 separate determinations. Bars represent ± standard error among three replicates

of oleoresins and includes the pungent phenolic metabolites like gingerols, shogaols and related compounds derived via phenylpropanoid pathway. The reduced effectiveness of phenolics in affording resistance in ginger could be due to the deployment of defense mediated by a single class of metabolites. Earlier studies on pigeon pea resistance to sterility mosaic disease (Rathi et al. 1986) and wheat resistance to karnal bunt (Gogoi et al. 2001) have also documented the lack of involvement of phenolics in defense. In contrast, the low polyphenolic content in soft-rot resistant wild *Z. zerumbet*, which is subject to systemic induction, is indicative of the role of other defense- related metabolites in imparting defense, as reported for many plant taxa (Baldwin 1989; Koricheva 2002; Moreira et al. 2012). Secondary metabolite biosynthesis in plants being dynamic, a single metabolite or class of metabolites does not comprise the only defense mechanism (Bennett and Wallsgroves 1994). Concomitant actions of various metabolites are known to contribute to the continuous chemical warfare between plants and pathogens (Bennett and Wallsgroves 1994; Neilson et al. 2013) and constitute an important part of the plant innate immune system (La Camera et al. 2004).

Earlier studies on analysis of rhizome-specific transcripts in ginger have identified an abundance of phenylpropanoid-pathway ESTs (Koo et al. 2013) but with low chalcone synthase (CHS) expression that prevents flavonoid accumulation (Ma and Gang 2006). PAL and/or TAL enzyme activities constitute important regulatory enzymes in phenylpropanoid biosynthesis (Winkel-Shirley 2001; Koes et al. 2005; Vogt 2010; Kong 2015) and abundance of PAL transcripts is often correlated with increased phenolics, which in turn corresponds to disease resistance (Jones 1984; Ju et al. 1995). Hence, in the present study further experiments were carried out to evaluate whether the variations in phenolics following soft-rot infection can be correlated to PAL and/or TAL activities. Quantification of PAL and TAL activities in uninfected rhizomes revealed TAL activity to be significantly higher in *Z. zerumbet* compared to the ginger cultivar, Varada ($p < 0.05$) (Table 1). However, higher PAL activity was observed in the uninoculated ginger cultivar (24.2 ± 1.9 U mg^{-1}) compared to the wild taxon (4.2 ± 0.8 U mg^{-1}) (Table 1). Higher PAL (40.1 ± 1.6 U mg^{-1}) and TAL (346.3 ± 21.6 U mg^{-1}) activities observed at 2 dpi in the ginger cultivar were observed to decrease at 9 dpi. This was in contrast to the low activities observed up to 3 dpi in the resistant taxon with gradual increases observed at 5 dpi for PAL (13.1 ± 0.8 U mg^{-1}) and TAL (442.5

Table 1 Variations in PAL and TAL specific activities in *Pythium myriotylum* infected (IN) and uninfected (UN) rhizomes of *Zingiber zerumbet* and *Zingiber officinale* cv. Varada (Zo)

Enzyme	Days of infection (doi)	*Z. zerumbet* (U mg^{-1})		*Z. officinale* cv. Varada (U mg^{-1})	
		UN	IN	UN	IN
PAL	0	4.2 ± 0.8	–	24.2 ± 1.9	–
	1	15.6 ± 1.6	10.5 ± 0.8	23.4 ± 1.1	25.5 ± 1.3
	2	6.8 ± 1.5	8.4 ± 0.6	33.6 ± 1.9	40.1 ± 1.6
	3	2.6 ± 0.8	8.3 ± 0.9	28.5 ± 1.0	24.2 ± 0.9
	5	10.4 ± 0.9	13.1 ± 0.8	17.2 ± 1.0	21.9 ± 0.8
	7	17.4 ± 0.7	15.5 ± 0.9	17.8 ± 1.8	27.6 ± 1.7
	9	8.6 ± 0.5	17.6 ± 0.9	10.4 ± 0.8	9.4 ± 1.2
TAL	0	537.8 ± 8.4[a]	–	162.3 ± 17.0[b]	–
	1	499.3 ± 11.8 [a]	345.1 ± 26.0	213.2 ± 5.9 [b]	276.5 ± 27.0
	2	314.1 ± 16.5	337.9 ± 21.5	316.5 ± 33.5 [b]	346.3 ± 21.6
	3	339.4 ± 27.8 [a]	251.2 ± 31.5	278.3 ± 16.6 [b]	260.1 ± 22.4
	5	407.4 ± 27.7 [a]	442.5 ± 35.1	167.9 ± 8.6 [b]	213.7 ± 10.3
	7	618.8 ± 37.9 [a]	518.8 ± 32.7	174.5 ± 6.9 [b]	207.6 ± 7.9
	9	620.2 ± 37.5 [a]	573.3 ± 29.0	110.4 ± 8.6 [b]	149.4 ± 5.3

Each value is the mean ± SE for $n = 5$. Significance level: [a] $p < 0.001$ compared to control (UN) *Zingiber officinale* cv. Varada; [b] $p < 0.01$ compared to (IN) *Zingiber officinale* cv. Varada

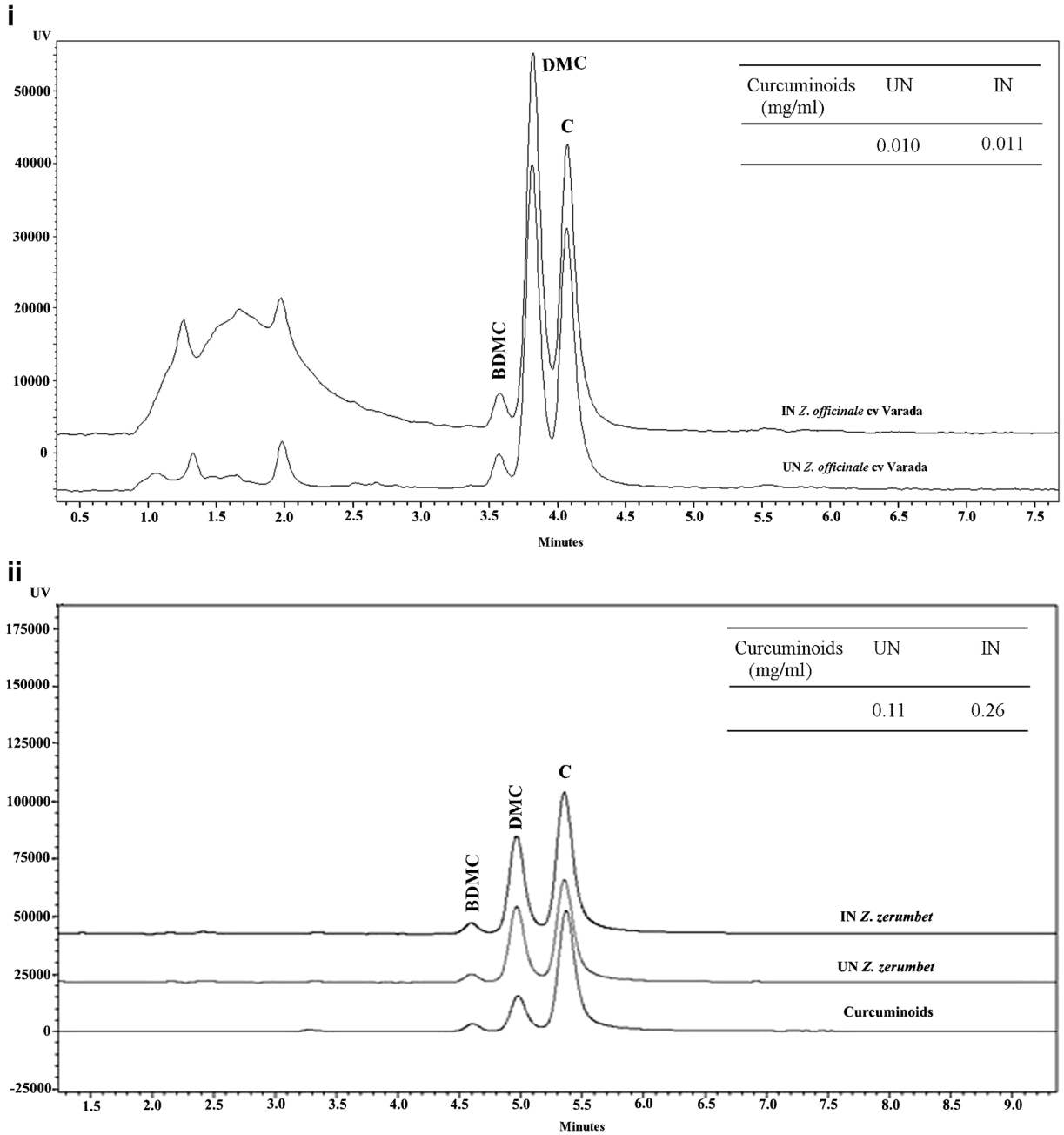

Fig. 2 HPLC chromatogram of curcuminoids in *Pythium myriotylum* infected (IN) and uninfected (UN) rhizomes of **(i)** *Zingiber officinale* cv. Varada) and **(ii)** *Zingiber zerumbet*. The curcuminoids detected in the two taxa are shown in inset and include curcumin (C) and its analogues, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) Experiments were carried out in triplicate with extracts made from rhizomes at 5 dpi and were analyzed at 420 nm by reverse phase HPLC on C18 column by gradient elution using mobile phase comprising of (A) 1 mM of phosphate buffer (pH 4.0) and (B) 100 % Acetonitrile

±35.1 U mg^{-1}). At 9 dpi both PAL and TAL specific activities were observed to be elevated in rhizomes of wild taxon. Higher specific activities of PAL and TAL in the wild taxon following infection could be correlated to systemic induction of PAL/TAL, as has been previously reported in various plant taxa infected with *Pythium* spp. and other necrotrophs (Inés Ponce de León and Montesano 2013).

Studies have indicated that the phenylpropanoid metabolic flux in ginger is driven towards biosynthesis of polyketides like curcuminoid and gingerol, than towards synthesis of other phenylpropanoid pathway-derived metabolites (Ramirez-Ahumada et al. 2006). PAL being an important regulatory enzyme in curcuminoid biosynthesis (Ramirez-Ahumada et al. 2006; Katsuyama et al. 2009) has been used in metabolic engineering for heterologous production of curcuminoids in *Escherichia coli* (Katsuyama et al. 2008; Wang et al. 2013). Curcuminoids are known to display a broad spectrum of biological activities (Joe et al. 2004) that includes inhibitory activities against various phytopathogens (Kim et al. 2003). Towards determining if the systemic induction of PAL/TAL observed in wild taxon in the present study is regulating curcuminoid biosynthesis, HPLC analyses of methanolic extracts of rhizomes of the ginger cultivar and wild taxon were carried out following *P. myriotylum* infection (Fig 2(i) and (ii)). HPLC chromatograms of extracted curcuminoids from both taxa detected curcumin (C) and its analogues, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). There was a 10-fold difference in total curcuminoid content in the uninfected rhizomes of the wild taxon (0.11 mg/ml) compared to the ginger cultivar (0.01 mg/ml) (Fig. 2(i) inset). In the wild taxon, following *P. myriotylum* infection, a 2-fold increase in total curcuminoids was observed compared to uninfected rhizomes (Fig. 2(ii) inset). In the cultivar, no significant increase in total curcuminoids was observed following infection with *P. myriotylum*. Towards determining whether the induced curcuminoids contribute to resistance in the wild taxon, further experiments evaluating the antagonistic effect of curcuminoids on *P. myriotylum* growth were carried out. Results obtained did not reveal any significant inhibitory effect (Fig. 3) indicating that curcuminoids alone are not contributing to defense. Similar observations have been reported earlier wherein curcuminoids did not display antimicrobial activity whereas turmeric extracts showed significant inhibitory effects (Chopra et al. 1941; Apisariyakul et al. 1995). These observations can be accounted to the poor solubility of curcuminoids that result in its incomplete absorption and hence reduced bioavailability (Ravindranath and Chandrasekhara 1980). In the present study, dissolution of curcuminoid in DMSO was improved by adding PEG 4000 which is used to improve the solubility of many water insoluble drugs (Thong et al. 2014; Nguyen et al. 2015).

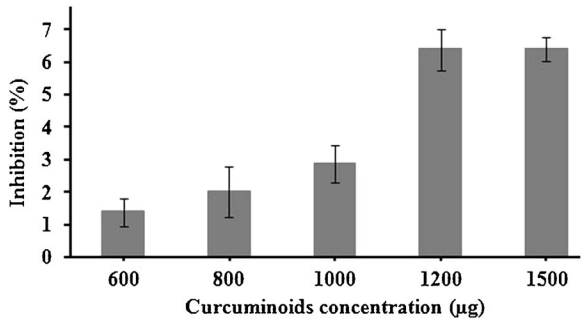

Fig. 3 Results of radial diffusion assays to determine antagonistic effect of curcuminoids on *Pythium myriotylum* growth at varying concentrations. *Bars* indicate ± standard error among three biological replicates

Plant defense being a plastic trait, the differential allocation of defense- related metabolites plays a crucial role in affording resistance to the invading pathogen (Meldau et al. 2012; Moreira et al. 2012). This is because the speed and duration of *de novo* phenolic biosynthesis is more important in plant resistance than relatively high constitutive concentrations (Bennett and Wallsgroves 1994). In earlier studies we had observed systemic induction of the sesquiterpenoid, zerumbone (Keerthi et al. 2014) in wild taxon. In the present study the observed increase in phenolic content, induction of regulatory enzymes of phenylpropanoid pathway besides the enhanced curcuminoid content in the wild taxon subsequent to infection suggests upregulation of flux towards curcuminoid biosynthesis. Upregulation of the phenylpropanoid pathway towards curcuminoid biosynthesis thus constitutes an additional inducible chemical defense strategy employed together with sesquiterpenoid-based defenses (Keerthi et al. 2014) and may be a cost amelioration strategy evolved in the wild taxon for rapid reinforcement of defense preventing *P. myriotylum* ingress. However phenolics and terpenoids being carbon-based, it is unlikely that plants would deploy high levels of both phenolics and terpenoids during defense as it will be a considerably costly trade-off (Goodger et al. 2012). This accounts for the detection of inherently lower phenolic levels observed during this study in uninfected wild taxon. Despite the high allocation cost of terpenoids compared to phenolics, effectiveness of terpenoid-based defense has resulted in regulation of the *Z. zerumbet* terpenoid biosynthetic pathway by evolving a single multi-product sesquiterpene synthase gene capable of producing six sesquiterpenes from a single substrate (Yu et al. 2008). Such deployment of terpene mixtures is possibly

a cost amelioration strategy evolved by the wild taxon and provides an ecological advantage to the species by ensuring durable resistance than an equivalent amount of a single metabolite or metabolite class. In this regard the present study has provided further valuable information on the biochemical basis of soft rot resistance exhibited by *Z. zerumbet*.

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Compliance with ethical standards We hereby certify that the communicated manuscript is not submitted to any other journal for simultaneous consideration, nor been published previously (partly or in full). Furthermore, authors declare that they have no conflict of interest concerning this article. The investigations reported in the present manuscript do not involve any clinical studies engaging human participants or animals.

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