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RESEARCH PAPER

Effects of Exogenous Elicitors on Triterpenoids Accumulation and Expression of Farnesyl Diphosphate Synthase Gene in *Inonotus obliquus*

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Abstract Exogenous elicitors can influence accumulation of triterpenoids by regulating FPS (farnesyl pyrophosphate synthase) in *Inonotus obliquus*, such as birch bark (BB), birch rhizosphere soil (BS), *Frankia alni* (FA), and *Rhizobium indigoferae* (RI). Among them, the highest yield of biomass (15.6 mg/mL) could be detected by treatment with RI at 2 μg/mL for 10 d, about 2.1-fold of the control. Results showed the significant effect of elicitors on accumulation of triterpenoids by regulating FPS expression level. RI stimulated mycelium to achieve the highest accumulation of triterpenoids about 48.2 mg/g, which was about over 4-fold of the control (9.5 mg/g). Its effect on FPS expression level was greater than that of others, which was about over 10-fold of the control. However, accumulation of triterpenoids and FPS expression level were clearly down-regulated under treatment with BS. In addition, two endogenous factors (H_2O_2 and NO) in *I. obliquus* could affect accumulation of triterpenoids by regulating FPS expression level. Effect of RI on H_2O_2 and NO contents were higher than that of others, about 3.5-fold of the control. The minimum value of H_2O_2 content was detected by treatment with BS for 10 d, which was about 0.5-fold of the control. RI that assist with CAT (catalase) and SNP (sodium nitroprusside dehydrate) could stimulate triterpenoid contents to achieve highest accumulation, which was about 5.5-fold of the control. Fluctuation of H_2O_2 and NO content seems to play a pivotal role in accumulation of triterpenoids by regulating FPS expression level.

Keywords: exogenous elicitors, *Inonotus obliquus*, FPS expression, triterpenoids accumulation

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1. Introduction

Medicinal fungi have an established history of being used in nutritionally functional food as well as traditional oriental therapies [1]. Among them, *Inonotus obliquus* is a rare medicinal mushroom belonging to the family of Hymenochaetaceae Donk, which grew as parasitism on trunks of living birch in the colder northern climates [2]. *I. obliquus* can form an irregular shape of sclerotial conk, not fruiting body. Its sclerotia with no side effects, have been confirmed to have various health-promoting activities such as anticancer effects [3], immune-stimulating activity [4], and dual-effect on tyrosinase [5]. Among bioactive metabolites in *I. obliquus*, triterpenoids are the best-known secondary metabolites, which is one of the most important classes of natural products. And they also exhibited a wide range of structural diversity and biological activity, such as allelopathy [6], anti-fungi [7], anti-insect activities [8], and so on.

More studies focused on the relationship between biological activity and structure of triterpenoids in *I. obliquus*, which

also ignore the fact that low yield of triterpenoids. Due to slow growth and rarity in nature, the sclerotia of *I. obliquus* are not a reliable source for a large-scale production of bioactive metabolites. But these bioactive metabolites can be produced by submerged culture of mycelium, efficiently. Nevertheless, triterpenoids accumulation is determined by exogenous factors, such as UV, temperature, and coordination with other microbes [9]. In synthesis pathway of triterpenoids, lanosterol is the precursor that can be transformed to its derivatives in the presence of proper enzymes. Among various enzymes, farnesyl pyrophosphate synthase (FPS) (EC 2.5.1.10) is a key branch point and major chain elongation enzyme, which belongs to the E-family of the prenyltransferases [10]. Its activity is also greatly influenced by exogenous factors, which can lead to fluctuation of triterpenoids accumulation. Taken together, submerged culture of mycelium with proper exogenous factors is effective fermentation strategy for high yield of triterpenoids.

Recently, the addition of exogenous elicitors has great potential to increase metabolites accumulation by using submerged fermentation. Unlike pathogens, elicitors were unable to cause strong hypersensitive reactions in the host. Long-term colonization of elicitors could induce to accrue various kinds of metabolites in hosts. The fact that elicitors-host interactions affect the accumulation of metabolites is an intriguing issue, which has also drawn great interests of researchers. Methyl jasmonate (MJ) is the best-known exogenous elicitor, which is one of the most important signaling molecules [11,12]. Xu *et al.* described that the mycelia of *I. obliquus* cultured with additional MJ can increase yield of inotodiol and betulin about 3.0 and 21.8-fold increments, respectively [13]. Besides MJ, there is an increasing interest in the search for other exogenous elicitors to increase fungal polysaccharide accumulation, such as oils and fatty acids.

Currently, very little information is available regarding the stimulatory effect of elicitors on accumulation of triterpenoids. Few reports are available describing the effect of FPS expression on accumulation of triterpenoids. Therefore, the aim of this work is to demonstrate the feasibility of enhancing FPS expression on accumulation of triterpenoids in *I. obliquus* by adding elicitors (MJ as positive group) in shake flask cultures. Two of five elicitors are birch bark and birch rhizosphere soil, which were obtained from *I. obliquus*'s host *Betula platyphylla*. Other elicitors were extractions from azotobacter (*Frankia alni* [14] and *Rhizobium indigoferae* [15]). Both of azotobacter are the most widespread mutualistic symbionts among the symbionts between plants and microbes. In addition, the effects of H_2O_2 and NO as endogenous factors in *I. obliquus* on accumulation of triterpenoids and FPS expression level were comparatively evaluated.

2. Materials and Methods

2.1. Strains and reagents

F. alni DSMZ 21154^T and *R. indigoferae* DSMZ 8683^T were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). Birch bark and birch rhizosphere soil were collected from Changbai Mountain (127°56' E, 41°35' N). Methyl jasmonate and standard compounds (99.9%) was purchased from Spring Autumn Biological Engineering Co., Ltd (Nanjing, China). Nitric oxide (NO) and H_2O_2 were purchased from Sinopharm Co., Ltd (Beijing, China). Reagents that used as donators or scavengers were purchased from Sigma (Darmstadt, Germany), including sodium nitroprusside dehydrate (NO donator, SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (NO scavenger, cPTIO), and catalase (H_2O_2 scavenger, CAT).

2.2. Elicitors preparation

Birch bark (*B. platyphylla*), rhizosphere soil of *B. platyphylla*, *F. alni*, *R. indigoferae*, and methyl jasmonate were selected as exogenous elicitors. 100 g birch bark and rhizosphere soil were boiled with 1 L of water for 30 min, and then filtered to obtain supernatant solution. Ethanol extractions of *F. alni* and *R. indigoferae* was obtained by method described by Li *et al.* [16]. Above extractions were frozen as dry powder. Above powders and methyl jasmonate were dissolved in sterile H_2O at 2 g/mL, named BB, BS, FA, RI, and MJ (positive group), respectively. Above solutions were sterilized through a 0.22 μm diameter microporous membranes and stored at 4°C in darkness.

2.3. Culture conditions of *I. obliquus* and treatments with elicitors

A disc (5 mm) of *I. obliquus* was cut from the margins of the PDA plate and transferred into the PDB broth to obtain mycelium pellets after incubation at 28°C for 3 d. The flask culture experiments were performed in a 500 mL flask containing 200 mL liquid medium supplemented with mycelium pellets (0.3 g/L) and various elicitors at 28°C and 180 rpm/min. Final concentration of elicitors was 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg/mL. Samples (20 mL) were collected every 2 day and then mycelium pellets were harvested.

2.4. Effect of elicitors on biomass, triterpenoids, and FPS expression level

Mycelia biomass was determined by the method that described by Zheng *et al.* [17]. The yield of triterpenoids was measured by UV-Vis spectrophotometry methods [18,19]. For analysis of FPS expression level, total RNA was extracted from 50 mg mycelium by using total RNA

kit according to manufacturer's instructions (Sigma, USA). First-strand cDNA was synthesized by using PrimeScript RT Reagent Kit (Takara, Japan). RT-PCR was performed in an optical 96-well plate with StepOnePlus™ RT PCR System (Life, USA) and method was described by Hu *et al.* [20]. Data were exported to LinReg software to determine the PCR amplification efficiency for each primer pair [21]. Relative transcript levels were normalized on the basis of expression of an invariant control orthologous to 18S rDNA using the equation (1), where ΔC_t is the difference between control and target products.

$$\Delta C_t = Ct_{gene} - Ct_{act} \quad (1)$$

Thus, the calculated relative expression values were normalized to the control expression level $2^{-\Delta\Delta Ct}$ [22,23], where

$$\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control} \quad (2)$$

The expression of the gene in the control group was set to "1". The primers followed as FPS, S1: 5'-CATCCTATT ATTTCTTCCACG-3', A1: 5'-AGGAAATCAACAACA GGAAG-3' and 18S, S2: 5'-GCTCAAATCCAACTC TCAAA-3', A2: 5'-TTCCATCTTTCACACTTTCC-3'. All treatments were performed in triplicate.

2.5. Analysis of triterpenoids and FPS expression level involvement of NO and H_2O_2

I. obliquus were incubated by elicitors at optimum concentration with/without donators/scavengers and were harvested after 10 d for determination of triterpenoids, FPS expression level, NO and H_2O_2. All donators/scavengers were sterilized by 0.22 μm diameter microporous membranes with final concentration of donators and scavengers at 150 μmol/L SNP, 3 mmol/L cPTIO, and 5.25 mKat/L CAT [24,25]. The total triterpenoids and FPS expression level were determined by methods in above section. The NO level was determined using a NO detection kit (Enzo, USA) according to the manufacturer's instructions. One unit of NO was defined as the absorbance variation caused by the internal standard of 1 μM NO per gram fresh weight [26]. The generation of H_2O_2 by *I. obliquus* was measured by chemiluminescence in a ferricyanide-catalyzed oxidation of luminol. One unit of H_2O_2 was defined as the chemiluminescence caused by the internal standard of 1 μM H_2O_2 per gram fresh weight [27]. All treatments were performed in triplicate.

2.6. Physicochemical properties and compositions of elicitors

Elicitors were dried at 70°C for a constant weight. Moisture content, ash, and crude fiber were determined by official analytical chemists methods [28]. Crude protein (N × 6.25) was calculated as nitrogen contents (N) [29]. Polysaccharide content was determined by the phenol-sulfuric acid method [30]. Color reactions were investigated, including α-naphthol reaction, iodination reaction, Fehling's test, carbazole reaction, $FeCl_3$ reaction, and coomassie brilliant blue reaction using methods described by Yan *et al.* [31].

10 mg elicitors were suspended in 10 mL methanol, sonicated and filtered through a 0.45 μm diameter microporous membranes. Component of elicitors were determined by HPLC. The chromatographic system consisted of a column Inertsil ODS-SP (250 × 4.6 mm i.d., 5 μm) (LC-20AT, Shimadzu, Japan) with the photodiode array detector (PAD). A linear gradient elution of a 0.5% acetic acid (A) and acetonitrile (B) was used for separation. The elution program was optimized and conducted as follows: a linear gradient of 0% B (0-5 min), 2% B (5-6 min), 3% B (6-7 min), 4% B (7-10 min), 3% B (10-15 min), 2% B (15-20 min), 1% B (20-25 min), and 0% B (25-30 min). The peaks were recorded using PAD absorbance at 254 nm and the solvent flow rate was 0.5 mL/min and the oven temperature was set at 30°C. The injection volume was 10 μL for standard and samples. All treatments were performed in triplicate.

3. Results

3.1. Effect of elicitors on accumulation of biomass and triterpenoids

Overall, the effect of elicitors on biomass was presented in Fig. 1. Among elicitors, BB and BS were able to inhibit biomass. And BS had greater inhibition effect than that of BB. Results showed that increasing effect of FA, RI, and MJ on biomass. After treatment with 3 μg/mL FA, maximum value of biomass was 11.4 mg/mL, about 2-fold of control. Fig. 1D showed that increasing effect of RI on biomass was greater than that of other elicitors. The maximum value of biomass was 15.6 mg/mL by 2 μg/mL RI, which was 1.95-fold of control. Maximum value of biomass was 13.4 mg/mL by 2.0 μg/mL MJ.

The effect of elicitors on accumulation of triterpenoids was presented in Fig. 2. BS, FA, RI, and MJ showed increasing effect on accumulation of triterpenoids. The highest accumulation of triterpenoids was detected by treatment with RI at 2.0 μg/mL for 10 d. Its maximum value was 48.2 mg/g, which was 1.76-times greater than that of control. And then the highest accumulation of triterpenoids was 43.7 mg/g by 1.5 μg/mL BB for 10 d. After treatment with 2 μg/mL FA, accumulation of triterpenoids reached maximum value, about 41.2 mg/g. For positive group, the highest accumulation of triterpenoids was 47.6 mg/g by 2 μg/mL MJ for 10 d, which was 1.45-

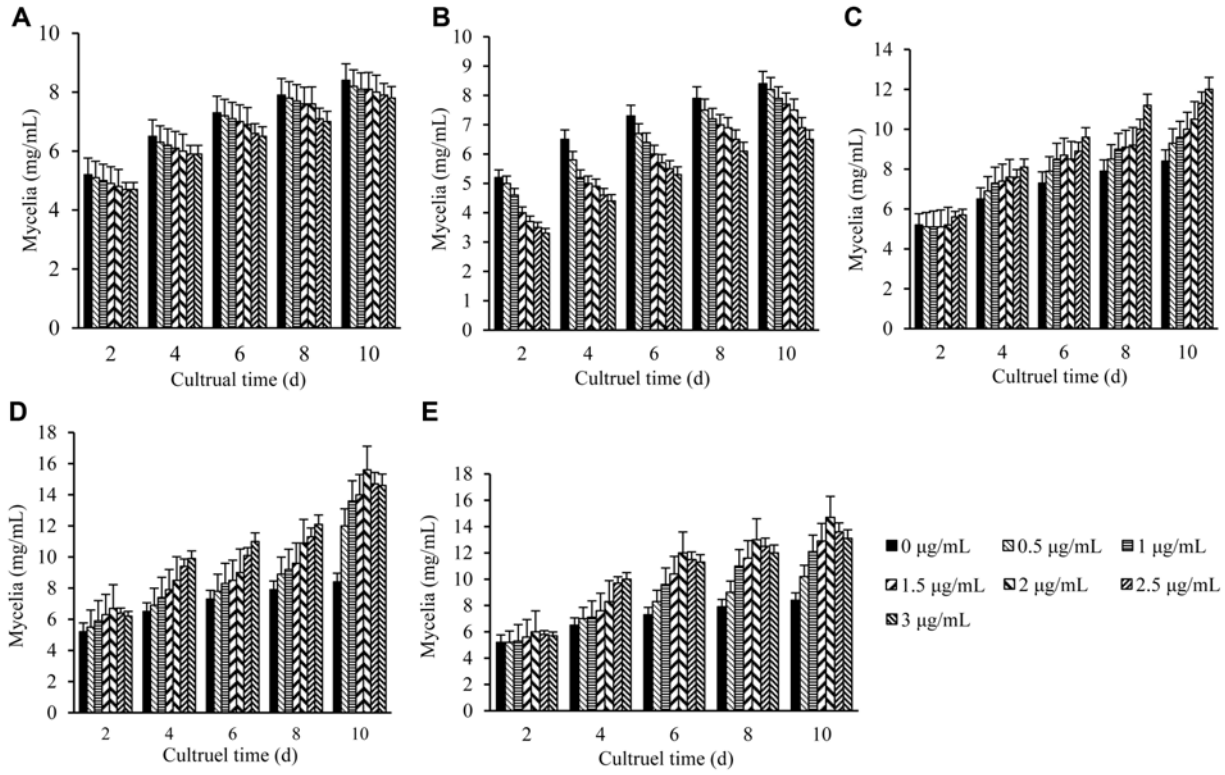

Fig. 1. Effect of elicitors on biomass of *Inonotus obliquus*. Concentrations of elicitors were 0, 0.5, 1, 1.5, 2, 2.5, and 3 μg/mL. (A): birch bark (BB), (B): birch rhizosphere soil (BS), (C): *Frankia alni* (FA), (D): *Rhizobium indigoferae* (RI), (E): methyl jasmonate (MJ).

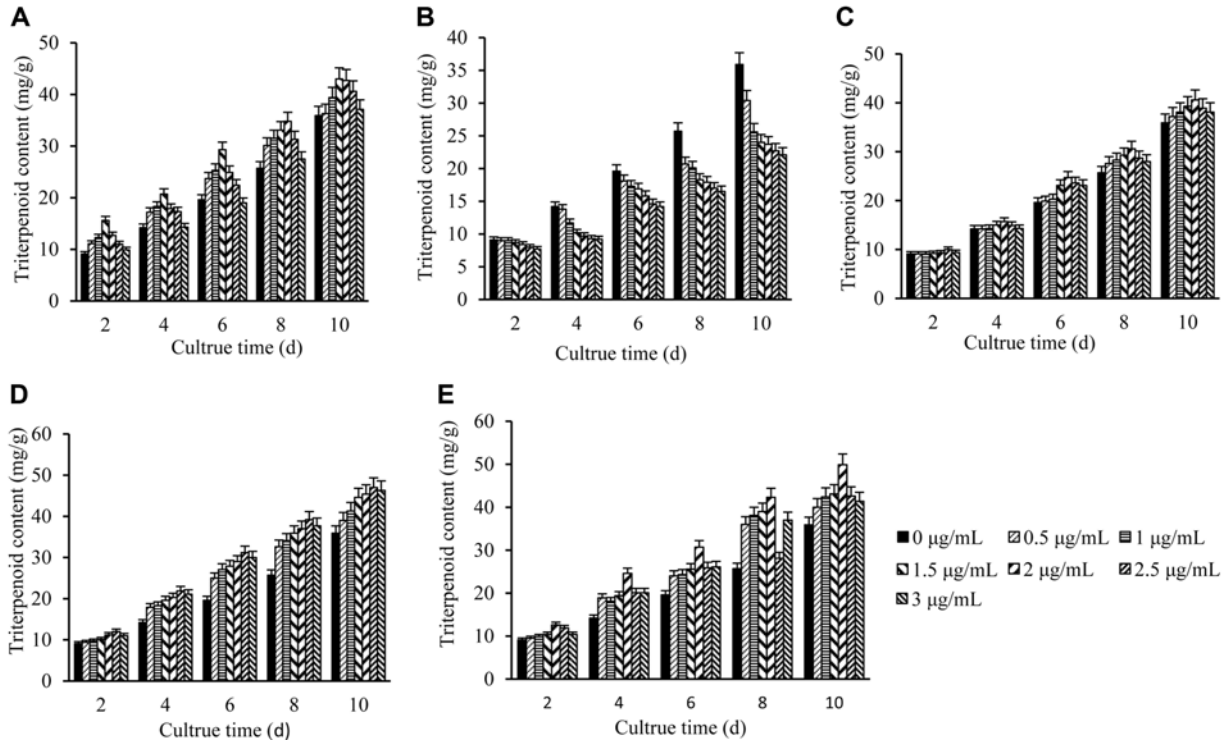

Fig. 2. Effect of elicitors on accumulation of triterpenoids in *Inonotus obliquus*. Concentrations of elicitors were 0, 0.5, 1, 1.5, 2, 2.5, and 3 μg/mL. (A): birch bark (BB), (B): birch rhizosphere soil (BS), (C): *Frankia alni* (FA), (D): *Rhizobium indigoferae* (RI), (E): methyl jasmonate (MJ).

times greater than that of control. By contrast, BS showed significant inhibition effect on accumulation of triterpenoids at dose and time dependent manner. The lowest accumulation of triterpenoids was 22.3 mg/g by 3 μg/mL MJ for 10 d.

3.2. Effects of elicitors on FPS expression level

To determine whether elicitors is involved in the FPS expression in *I. obliquus*, FPS expression level was performed by RT-PCR analysis (Fig. 3). Results showed that FPS expression level responding to BB, FA, RI, and MJ was clearly unregulated, compared with that of control. The highest level of FPS expression was detected by treatment with 2.5 μg/mL RI for 4 d, which was 10-fold of control. With BB and FA dose-dependent manner, FPS expression was initially increased and then decreased, such as FPS expression level by 2.0 μg/mL BB for 4 d was 4.5-fold of control, FPS expression level by 2.0 μg/mL FA for 10 d was 6.0-fold of control. MJ also increased FPS expression level, as well as the ability of MJ to stimulate accumulation of triterpenoids. The highest level of expression was 7.5-fold of control by 2 μg/mL MJ for 6 d. However, FPS expression level was decreased soon under treatment with BS at dose dependent manner. The lowest level of FPS expression was 0.4-fold of control by 3 μg/mL MJ for 10 d.

3.3. Analysis of triterpenoids and FPS expression level through NO and H_2O_2

Accumulation of triterpenoids and FPS expression level of *I. obliquus* was determined under treatment with or without donators/scavengers to investigate a possible relationship in NO and H_2O_2 content (Fig. 4). cPTIO and CAT could inhibit accumulation of triterpenoids and FPS expression level, showing that NO and H_2O_2 was an important response factor in accumulation of triterpenoids and FPS expression level. H_2O_2 could reverse suppression effect of cPTIO, but SNP could not reverse suppression effect of CAT. It implied that H_2O_2 could mediate growth of *I. obliquus* and NO played a role in accumulation of triterpenoids by H_2O_2-assisted. In addition, SNP could stimulate H_2O_2 production, but H_2O_2 showed no effect on NO production. BB with donators/scavengers could increase accumulation of triterpenoids, H_2O_2 and NO content, slightly. BS with cPITO could decrease accumulation of triterpenoids and NO content, its inhibition effect was greater than that of BS alone. It suggested that BS was able to inhibit synthesis of triterpenoids by decreasing NO content. In the presence of H_2O_2 or SNP, FA could increase accumulation of triterpenoids and H_2O_2 content, not on NO content, which was applied as potential H_2O_2 irritant. Similarly, RI was able to stimulate NO and H_2O_2 production, which led to

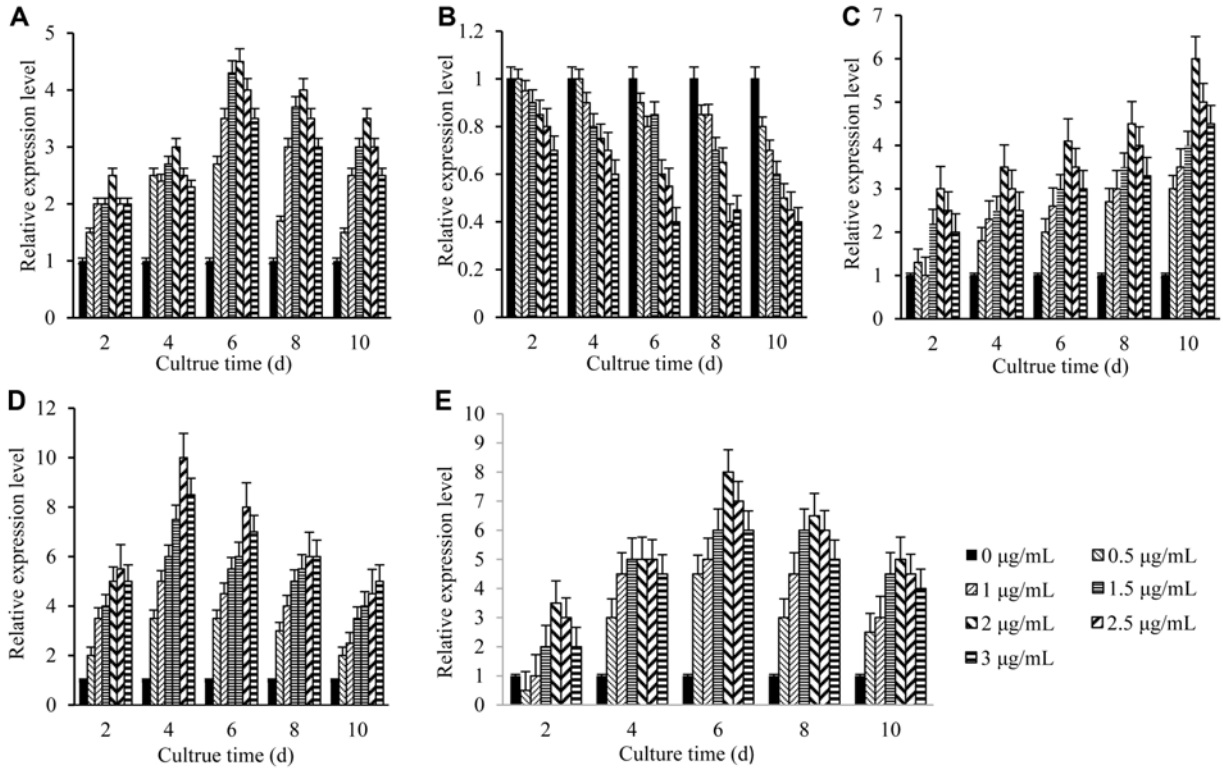

Fig. 3. Effect of elicitors on farnesyl pyrophosphate synthase (FPS) expression level in *Inonotus obliquus*. Concentrations of elicitors were 0, 0.5, 1, 1.5, 2, 2.5, and 3 μg/mL. (A): birch bark (BB), (B): birch rhizosphere soil (BS), (C): *Frankia alni* (FA), (D): *Rhizobium indigoferae* (RI), (E): methyl jasmonate (MJ). Data are expressed as a control group which was set at 1.

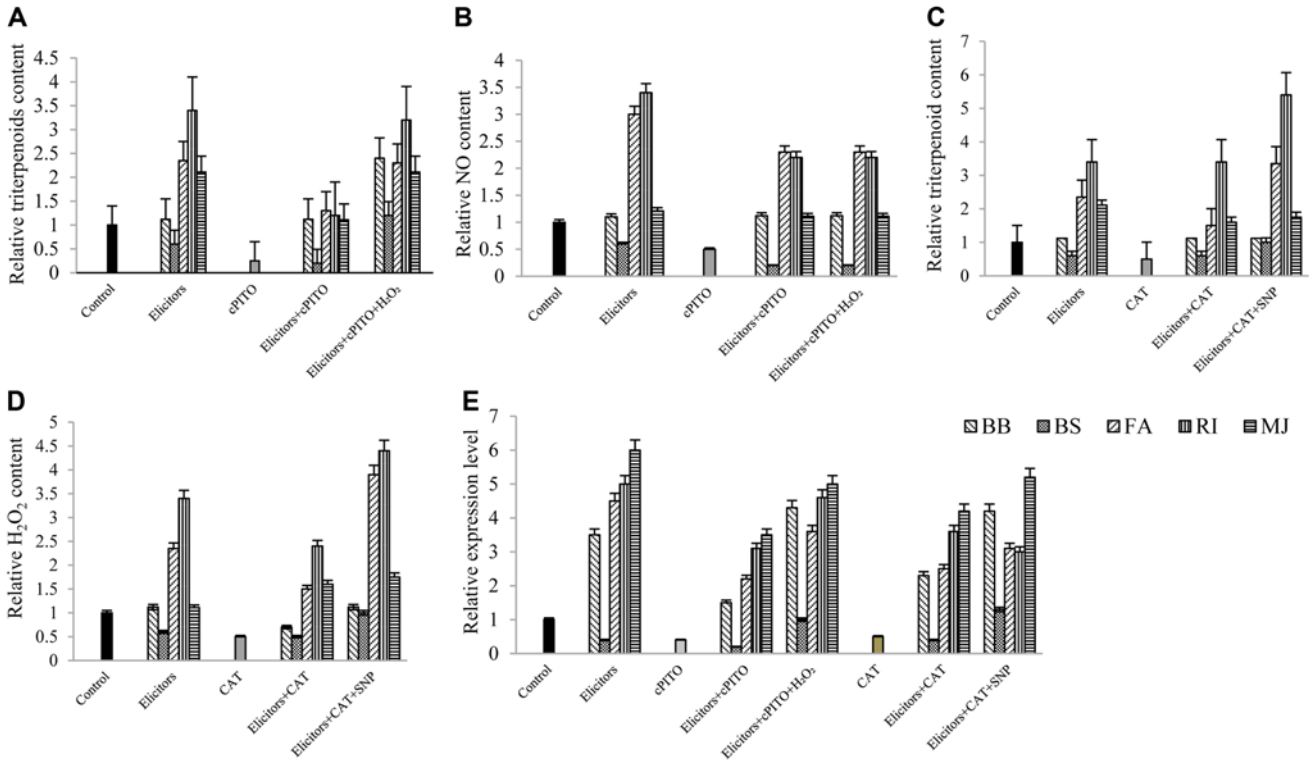

Fig. 4. Interactions between elicitors and NO or H_2O_2 on *Inonotus obliquus*. Elicitors (1.5 μg/mL birch bark (BB), 3 μg/mL birch rhizosphere soil (BS), 2 μg/mL *Frankia alni* (FA), 2.5 μg/mL *Rhizobium indigoferae* (RI), 2 μg/mL methyl jasmonate (MJ)) with or without donators and scavengers (150 μmol/L SNP, 3 mmol/L cPTIO, 5.25 mKat/L CAT) were harvested 10 d later for determination of triterpenoids, farnesyl pyrophosphate synthase (FPS) expression level, NO and H_2O_2 concent. Data are expressed as a control group which was set at 1.

increase accumulation of triterpenoids, which suggested that RI might be applied as potential H_2O_2 and NO irritant. MJ had significant effect on accumulation of triterpenoids and H_2O_2 content with donators/scavengers, which is similar with that of FA.

Meanwhile, possible mediating role of elicitors on FPS expression level involvement of H_2O_2 and NO was investigated. Fig. 4 showed that cPITO and CAT was able to decrease FPS expression level. H_2O_2 could reverse its effect, but SNP did not reverse inhibition effect on FPS expression level, such as RI-treated with CAT+SNP. BB was able to increase FPS expression level under treatment with cPITO+H_2O_2 or CAT+SNP. In the presence of scavengers, FA and RI also increased FPS expression level with donators stimulation. SNP was unable to reverse FPS expression level in *I. obliquus* by RI with CAT, which suggested NO had no closely linked in FPS expression level.

3.4. Physicochemical properties and compositions of elicitors

Table 1 showed the main physicochemical properties of elicitors, such as α-naphthol reaction, iodination reaction, fehling's test, carbazole reaction, $FeCl_3$ reaction, and coomassie brilliant blue test. Compositions of elicitors were investigated by HPLC (Fig. 5). Main compositions of BB were catechin, epicatechi, and procyanidin B1. BS contained oxalic acid and citric acid, suggesting carboxyl groups might decrease triterpenoids synthesis rate by decreasing FPS expression level [32]. FA contained α

Table 1. Physicochemical properties of elicitors (+ positive, -negative)

Parameters	BB	BS	FA	RI
Moisture content (%)	4.1	3.4	4.1	3.5
Ash	0.45	0.31	0.56	0.71
Crude fiber	0.3	0	0	0
Protein content (%)	0	0	0.80	0.92
Polysaccharide content (%)	0.13	0.12	0.14	0.12
α-naphthol reaction	+	+	+	+
Iodination reaction	-	-	-	-
Fehling's test	-	-	+	+
Carbazole reaction	-	-	-	-
$FeCl_3$ reaction	+	-	+	+
Coomassie brilliant blue reaction	-	-	+	+

BB: birch bark
BS: birch rhizosphere soil
FA: *Frankia alni*
RI: *Rhizobium indigoferae*

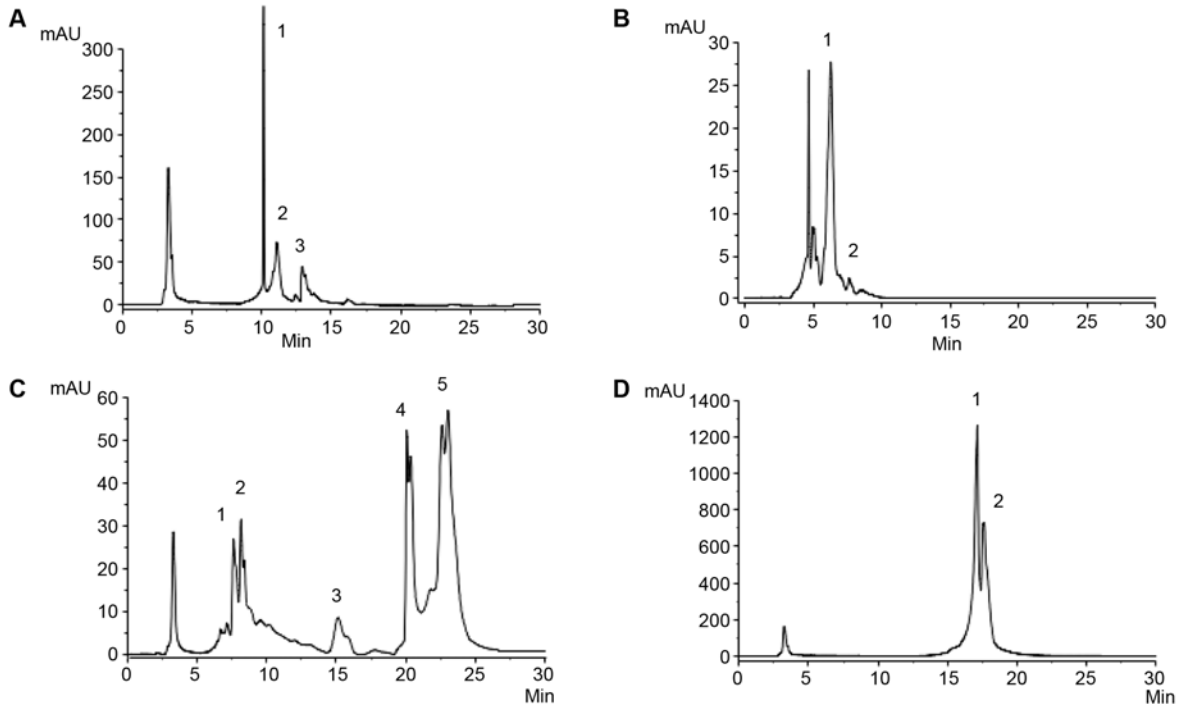

Fig. 5. HPLC analysis of elicitors. (A) birch bark (BB), Peak 1: catechin, Peak 2: epicatechi, Peak 3. procyanidin B1; (B) birch rhizosphere soil (BS), Peak 1: oxalic acid, Peak 2: citric acid; (C) *Frankia alni* (FA), Peak 1: α amino acid esters, Peak 2-3: unknown compound, Peak 4: ergosterol, Peak 5: ergosterol peroxide; (D) *Rhizobium indigoferae* (RI), Peak 1: ergosterol, Peak 2: ergone.

amino acid esters, ergosterol, ergosterol peroxide, and two unknown compounds. However, absorption peaks of two unknown compounds had bifurcated. The most possible reasons were deduced as follows: two unknown compounds might be isomers of ergosterol and ergosterol peroxide [33], which was decomposed in the analysis process [34]. RI contained ergosterol and ergone as main compounds. As mentioned above, ergosterol might act an important role in synthesis of triterpenoids. However, mechanism of the action of a class substance of ergosterol was not known clearly, which need to be investigated further.

4. Discussion

A wide variety of extractions derived from natural products were used as exogenous elicitors to regulate synthesis of metabolites [35]. Thus, this study examined effects of elicitors that extracted from host and microorganism on accumulation of triterpenoids and FPS expression level in *I. obliquus*. BB and BS that used as environmental elicitors were extracted from *I. obliquus*' host *B. platyphylla*. FA and RI as microbial elicitors were selected from mutualistic symbionts (*F. alni* and *R. indigoferae*). Mutualistic symbionts as actinomycete morphology provide nitrogen source for non-legume plants [14]. *R. indigoferae* was also azotobacter as bacterial morphology to provide nitrogen source for legume plants [36,37]. At last MJ was up-regulator of gene expression as a positive group, which was considered a key compound in many signaling pathways [11,38].

BB was able to increase accumulation of triterpenoids at some extent. However, BS had inhibition effect on accumulation of triterpenoids, which had significant difference effect on that of BB. According to growth environment of *I. obliquus* described by Lee *et al.* [39], one special compound might widespreadly exist in rhizosphere soil of *B. platyphylla*, which inhibited accumulation of triterpenoids. Above compound was widely distributed in root, which could be absorbed in the long-term growth process of *B. platyphylla*. Due to gravity, content of special compound in trunk of *B. platyphylla* was lower than that in root. It suggested that *I. obliquus* generally grow on the trunk rather than on root. Meanwhile, damaged bark might lead to terminate delivery of special compound, which was conducive to the growth of *I. obliquus* with less distribution of special compound (Fig. 6).

At present, this lack of information on metabolic controls and signaling pathways was a serious impediment to the development of strategies for bioengineering of triterpenoids [40]. Compared with control, FPS expression level in response to BB, FA, and RI was unregulated, which was able to increase accumulation of triterpenoids. By contrast, BS had inhibition effect on FPS expression level. In addition, this study also demonstrated that elicitors mediate accumulation

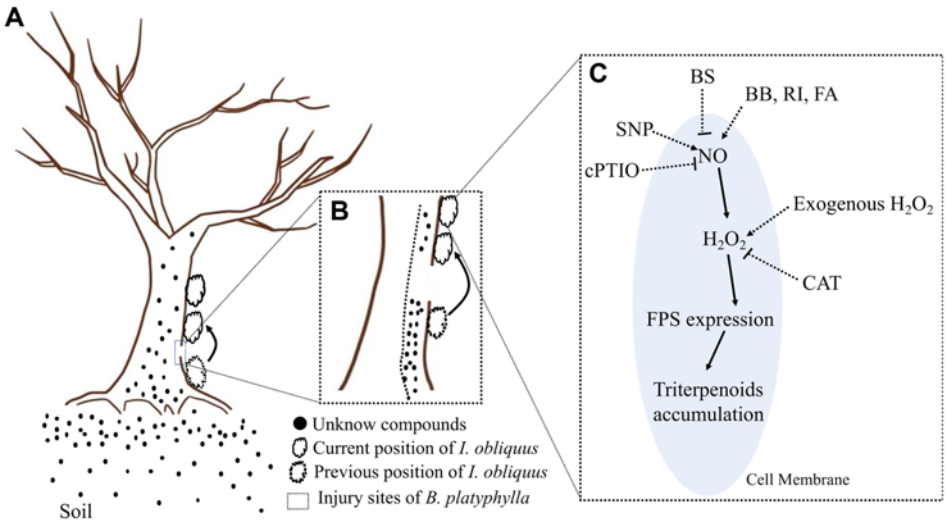

Fig. 6. One hypothesis for different effect of elicitors on *Inonotus obliquus*. BB: birch bark, BS: birch rhizosphere soil, FA: *Frankia alni*, RI: *Rhizobium indigoferae.*

of triterpenoids and FPS expression level involvement of NO and H_2O_2 (Fig. 6). Results showed that accumulation of triterpenoids and FPS expression level was directly mediated by NO and H_2O_2, which acted as a downstream signal molecule. FPS expression level was able to influence biosynthesis of triterpenoids, which suggested that accumulation of triterpenoids was increased by overexpression of FPS.

In this study, exogenous elicitors are able to influence accumulation of triterpenoids by regulating FPS expression level, which has a complementary interaction with endogenous factors NO and H_2O_2. It will help to better understand relationships between exogenous elicitors and accumulation of triterpenoids. Furthermore, it also provides strategies to improve the quality of medicinal fungi.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Ethical Statements

Neither ethical approval nor informed consent was required for this study.

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