

Endophytic Bacterium-Triggered Reactive Oxygen Species Directly Increase Oxygenous Sesquiterpenoid Content and Diversity in *Atractylodes lancea*

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Oxygenous terpenoids are active components of many medicinal plants. However, current studies that have focused on enzymatic oxidation reactions cannot comprehensively clarify the mechanisms of oxygenous terpenoid synthesis and diversity. This study shows that an endophytic bacterium can trigger the generation of reactive oxygen species (ROS) that directly increase oxygenous sesquiterpenoid content and diversity in *Atractylodes lancea*. *A. lancea* is a famous but endangered Chinese medicinal plant that contains abundant oxygenous sesquiterpenoids. Geo-authentic *A. lancea* produces a wider range and a greater abundance of oxygenous sesquiterpenoids than the cultivated herb. Our previous studies have shown the mechanisms behind endophytic promotion of the production of sesquiterpenoid hydrocarbon scaffolds; however, how endophytes promote the formation of oxygenous sesquiterpenoids and their diversity is unclear. After colonization by *Pseudomonas fluorescens* ALEB7B, oxidative burst and oxygenous sesquiterpenoid accumulation in *A. lancea* occur synchronously. Treatment with exogenous hydrogen peroxide (H₂O₂) or singlet oxygen induces oxidative burst and promotes oxygenous sesquiterpenoid accumulation *in planta*. Conversely, pretreatment of plantlets with the ROS scavenger ascorbic acid significantly inhibits the oxidative burst and oxygenous sesquiterpenoid accumulation induced by *P. fluorescens* ALEB7B. Further *in vitro* oxidation experiments show that several oxygenous sesquiterpenoids can be obtained from direct oxidation caused by H₂O₂ or singlet oxygen. In summary, this study demonstrates that endophytic bacterium-triggered ROS can directly oxidize oxygen-free sesquiterpenoids and increase the oxygenous sesquiterpenoid content and diversity in *A. lancea*, providing a novel explanation of the mechanisms of oxygenous terpenoid synthesis *in planta* and an essential complementarity to enzymatic oxidation reactions.

Atractylodes lancea is a traditional Chinese medicinal plant and is the main ingredient of many famous Chinese medicines. Oxygenous sesquiterpenoids, such as hinesol, β-eudesmol, atractylone, and caryophyllene oxide, are the main active components in *A. lancea* and have medicinal efficacy against rheumatic diseases, digestive disorders, night blindness, and influenza (1). The quality of *A. lancea* strongly depends on the area in which the herb is produced, and *A. lancea* grown in the Maoshan area of the Jiangsu Province is the geo-authentic herb (2), which is characterized by higher oxygenous sesquiterpenoid content and diversity than herbs grown in other areas (3). In recent years, the geo-authentic *A. lancea* has become endangered due to habitat destruction and overexploitation. Although cultivation ensures the production of medicinal materials, the oxygenous sesquiterpenoid content and diversity in cultivated *A. lancea* is considerably decreased compared to that of the wild herb. Currently, knowledge on the biosynthesis and diversity of oxygenous sesquiterpenoids is insufficient. Some studies have explained the mechanisms of oxygenous sesquiterpenoid synthesis by enzymatic reactions (4). However, this cannot explain the diversity seen among oxygenous sesquiterpenoids, as there are no corresponding enzymes to catalyze the synthesis of so many different sesquiterpenoids (5).

Endophytes are microorganisms that asymptotically colonize the internal tissues of almost all plants (6, 7). Our previous studies have demonstrated that the endophytes inside *A. lancea* grown in the Maoshan area are more abundant than those inside *A. lancea* grown in other areas (8–10), and several endophytes, such as *Acinetobacter* sp. strain ALEB16 and *Gilmaniella* sp. strain

AL12, can efficiently increase oxygenous sesquiterpenoid accumulation in *A. lancea* (11–13). These endophytes can activate signaling molecules, such as hydrogen peroxide (H₂O₂), salicylic acid, and jasmonic acid (14, 15), which increase the expression of genes encoding key enzymes involved in sesquiterpenoid biosynthesis pathways (13), promoting the formation of sesquiterpenoid hydrocarbon scaffolds in *A. lancea*. However, the mechanisms by which endophytes promote the oxidation of sesquiterpenoid hydrocarbon scaffolds and increase oxygenous sesquiterpenoid accumulation are unknown. The endophyte *Pseudomonas fluorescens* ALEB7B has been isolated from *A. lancea* grown in the Maoshan area and has been shown to establish a stable mutualistic relationship with *A. lancea* (10, 16). It is worth noting that *P. fluorescens* ALEB7B can increase oxygenous sesquiterpenoid accu-

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mulation in *A. lancea* more efficiently than other endophytes, such as *Acinetobacter* sp. strain ALEB16 (13).

Oxidative burst is one of the earliest plant responses to microbial infection (17). We propose that endophyte-triggered reactive oxygen species (ROS), such as H₂O₂, not only can act as signaling molecules but also can directly oxidize oxygen-free sesquiterpenoids to generate oxygenous sesquiterpenoids. The diverse array of oxygenous sesquiterpenoids cannot be obtained from enzymatic reactions alone, which oxidize sesquiterpenoids at specific sites. However, the oxidation induced by ROS can lead to the formation of diverse oxygenous sesquiterpenoids because of the random nature of chemical oxidation and differing degrees of oxidation. Therefore, the sesquiterpenoid diversity seen in *A. lancea* may be a result of direct oxidation caused by ROS.

The aims of this study were to investigate whether the endophytic colonization of *A. lancea* by *P. fluorescens* ALEB7B can cause an oxidative burst and to demonstrate the impact of ROS on oxygenous sesquiterpenoid composition in *A. lancea*. This study confirms that endophytic bacterium-triggered ROS can directly oxidize sesquiterpenoids and increase oxygenous sesquiterpenoid content and diversity in *A. lancea*.

MATERIALS AND METHODS

***A. lancea* plantlets and growth conditions.** *A. lancea* aseptic tissue culture plantlets were established as previously described (13, 18). Buds were collected from cultivated *A. lancea* and washed under running water, after which all procedures were conducted aseptically. Buds were surface sterilized by immersing in 75% (vol/vol) ethanol for 30 s, soaking in 1% (wt/vol) mercury chloride for 10 min, and thoroughly rinsing 5 times in sterile distilled water. Several buds were randomly selected, homogenized, and inoculated on potato dextrose agar to confirm the absence of endophytes. Intact, surface-sterilized buds then were transferred to 50 ml of Murashige-Skoog medium supplemented with 0.3 mg liter⁻¹ naphthaleneacetic acid (NAA) and 2.0 mg liter⁻¹ 6-benzyladenine in sealed 100-ml Erlenmeyer flasks.

When sufficient buds had germinated, they were separated and transplanted into 50 ml of Murashige-Skoog rooting medium supplemented with 0.1 mg liter⁻¹ NAA in sealed 100-ml Erlenmeyer flasks to develop into plantlets. All aseptic tissue culture plantlets were kept in a growth chamber with a photoperiod of 12 h, a light density of 3,400 lm m⁻², and a temperature cycle of 25/18°C day/night. Plantlets used in this study were 4 weeks old.

Endophytic bacterium and inoculation. *P. fluorescens* ALEB7B was isolated from the geo-authentic *A. lancea* grown in the Maoshan area (10) and stored at the China Center for Type Culture Collection (CCTCC AB 2013331). The molecular identification of *P. fluorescens* ALEB7B and its colonization inside *A. lancea* were confirmed in our previous study (16). Bacterium was grown in Luria-Bertani (LB) broth at 30°C with agitation (220 rpm) for 24 h, and bacterial cells were collected and resuspended in sterile double-distilled water with the concentration adjusted to 10⁶ cells ml⁻¹.

A. lancea aseptic tissue culture plantlets were sprayed with 200 μl of the bacterial suspension. The bacterial suspension flowed from leaf surfaces to the roots. Other plantlets were sprayed with 200 μl of sterile double-distilled water as a control. All bacterium-inoculated and control plantlets were randomized in the growth chamber.

Enumeration of *P. fluorescens* ALEB7B inside *A. lancea*. Plantlets were sampled and surface sterilized (10). The amount of *P. fluorescens* ALEB7B inside *A. lancea* was estimated using bacterial cell culture and quantitative PCR (qPCR).

Surface-sterilized plantlets were homogenized, serially diluted in sterile double-distilled water, and inoculated onto LB agar plates. The number of colonies on each plate was counted after incubation at 30°C for 3

days, and the number of CFU per gram of plant tissue was calculated. The 16S rRNA gene sequence of the reisolated bacterial strain had the highest similarity (100%) to the 16S rRNA gene sequence of the original bacterial strain (KF460526), confirming the reisolation of *P. fluorescens* ALEB7B from *A. lancea*.

qPCR was performed as described by Wang et al. (19). Primers were designed to amplify a fragment of the *gyrB* gene in *P. fluorescens*, which encodes DNA gyrase subunit B (*gyrB*-qPCR-F, TTGGCGACAGCGA AACCACC; *gyrB*-qPCR-R, GCCACCTCGTACTTGAACAGC) (16).

Chemicals and treatments. There are two main sesquiterpenoid biosynthesis pathways in *A. lancea*, including the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol phosphate (MEP) pathway (Fig. 1). Mevinolin (MEV) and fosmidomycin (FOS) are specific inhibitors of these two pathways. Ascorbic acid (AsA) was chosen as an ROS scavenger, as it can quench four main forms of ROS at the same time (20), including hydrogen peroxide (H₂O₂), singlet oxygen, superoxide anions, and hydroxyl radicals (21).

All inhibitors, oxidants, and ROS scavenger, including MEV (30 μM), FOS (200 μM), H₂O₂ (2, 5, or 10 mM), sodium hypochlorite (NaClO; 0.2, 0.5, or 1 M), and AsA (1,000 ppm), were dissolved or diluted in double-distilled water (13). MEV was previously converted to a water-soluble sodium salt as described by Hagen and Grunewald (22). FOS was purchased from Toronto Research Chemicals Inc. (North York, Canada), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were sterilized by filtration through 0.22-μm sterile filters.

The inhibitors or ROS scavenger were sprayed on plant leaves until dripping wet to allow a 1-day infiltration period before bacterial inoculation or oxidant application (13). The oxidant solutions (200 μl) were sprayed on plantlets, which flowed from leaf surfaces to plant roots. Chemicals and their dosages used here were based on the results of preliminary experiments. An equal volume of sterile double-distilled water was applied to plantlets as controls.

β-Caryophyllene and caryophyllene oxide standards were purchased from Sigma-Aldrich, and zingiberene and β-sesquiphellandrene were synthesized by WuXi AppTec Co., Ltd. (Tianjing, China). Their purities were higher than 99%. Hinesol, β-eudesmol, and atracylone were separated and purified in our laboratory to >95% purity.

Photosynthesis measurement. Leaf chlorophyll content was assayed using a spectrophotometric method according to Zhu et al. (23). The net photosynthetic rate was monitored using a Li-Cor 6400 portable photosynthesis system (Lincoln, NE) according to Li and Wang (24). Compressed air containing 350 μmol mol⁻¹ CO₂ was used as a gas source, and the photosynthetic photon flux density was 200 μmol m⁻² s⁻¹. Measurements were conducted from 9:00 a.m. to 10:00 a.m.

Soluble carbohydrate, acetyl coenzyme A, pyruvate, and malondialdehyde measurements in whole *A. lancea* plantlets. Soluble carbohydrates were measured according to the method of Wang et al. (18). Acetyl coenzyme A was extracted according to Tumaney et al. (25) and measured using the enzyme-linked immunosorbent assay kit (Shanghai Fankel Biological Technology Co., Ltd., China). Pyruvate content was measured using the 2,4-dinitrophenylhydrazine method, while malondialdehyde content was measured using the thiobarbituric acid method according to Zeng et al. (26).

Measurement and histochemical staining of endogenous reactive oxygen species. Endogenous H₂O₂ and hydroxyl radical content were measured with H₂O₂ and hydroxyl radical assay kits (Nanjing Jiancheng Bio-Engineering Institute, China), respectively (14). Singlet oxygen levels were determined using a spectrophotometric method according to Yang et al. (27). Superoxide anion levels were determined using the nitroblue tetrazolium (NBT) colorimetric method according to Gao et al. (28).

Histochemical staining of H₂O₂ was conducted using 3,3-diaminobenzidine (DAB) as a substrate according to Wang et al. (29). Staining of superoxide anions was conducted using the NBT staining method according to Montiel et al. (30). Stained leaves and roots were observed and photographed.

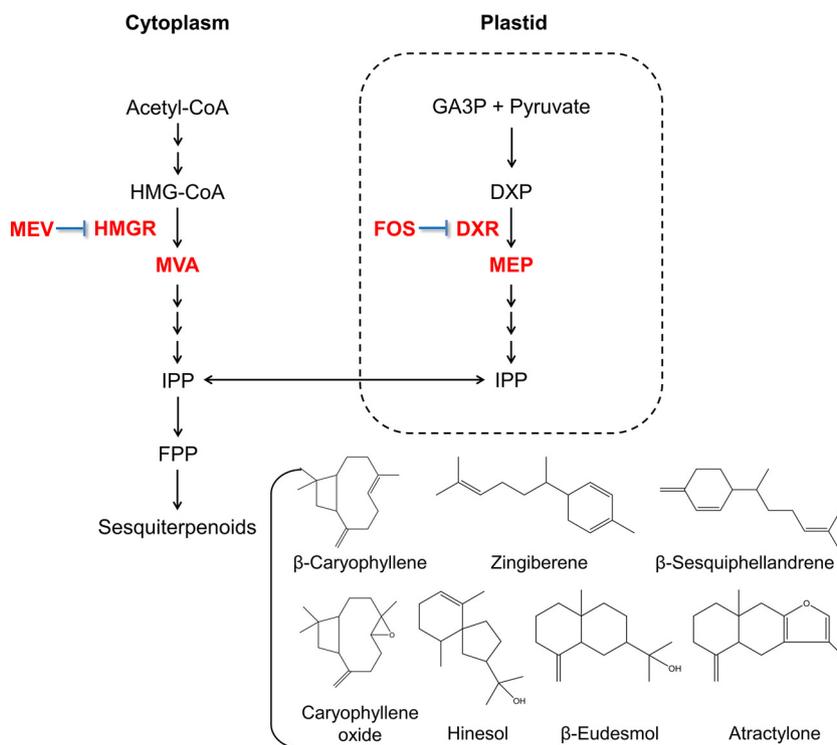


FIG 1 Proposed sesquiterpenoid biosynthesis pathway in *Atractylodes lancea* adapted from Wang et al. (13). Lines ending with a bar indicate the repression of enzymatic activities by inhibitors (mevinolin and fosmidomycin). Acetyl-CoA, acetyl coenzyme A; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; FOS, fosmidomycin; FPP, farnesyl pyrophosphate; GA-3P, glyceraldehyde 3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase gene; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol phosphate; MEV, mevinolin; MVA, 3R-mevalonic acid.

Sesquiterpenoid extraction and gas chromatography analysis. Harvested plantlets were dried at 36°C to a constant weight, and total sesquiterpenoids were extracted according to the method of Wang et al. (13). One gram of dried plantlets was ground and extracted in 4 ml of cyclohexane for 10 h. After sonication and centrifugation, total sesquiterpenoid extracts were dried over anhydrous sodium sulfate and filtered through 0.22- μm sterile filters before gas chromatography (GC) analysis.

GC analysis was performed on an Agilent 7890A GC equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA) as previously described (13). An Agilent DB-1HT capillary chromatographic column (30 m; 0.32-mm inside diameter, 0.1- μm film) was used with the following temperature program: initial temperature of 100°C for 4 min, increased to 140°C at 10°C min^{-1} for 10 min, increased to 220°C at 10°C min^{-1} for 10 min, and increased to 260°C at 10°C min^{-1} for 2 min. The injector and detector temperatures were 240°C and 350°C, respectively. High-purity nitrogen was used as the carrier gas with a flow rate of 0.8 ml min^{-1} . The injection volume was 1 μl , and the split ratio was 5:1.

Qualitative analyses of seven main sesquiterpenoids (β -caryophyllene, zingiberene, β -sesquiphellandrene, caryophyllene oxide, hinesol, β -eudesmol, and atractylone) were performed according to retention times of authentic standards (15). Quantitative measurements were made by comparing results to standard curves that were constructed according to concentrations and peak areas of the sesquiterpenoid standards in the gas chromatograms.

Sesquiterpenoids oxidized by reactive oxygen species *in vitro* and identification of oxidized products. Hydroxyl radicals were generated from the reductive cleavage of H_2O_2 (21), and singlet oxygen radicals were generated by reacting H_2O_2 with NaClO (31). Sesquiterpenoids were extracted from 1 g of dried *A. lancea* plantlets with 4 ml of anhydrous alcohol and were incubated with 4 ml of 5 mM H_2O_2 , 5 mM H_2O_2 plus 5 U ml^{-1} peroxidase (Sigma-Aldrich), or 5 mM H_2O_2 plus 0.2 M NaClO . The re-

action was allowed to proceed for 4 h, and oxidized products subsequently were extracted using 4 ml of cyclohexane. Sesquiterpenoids reacted with double-distilled water were used as controls.

Oxidized products were identified using an Agilent 7890A series GC connected to a 5975 mass selective detector (scanned range m/z 50 to 500) fitted with a DB-1ms capillary chromatographic column (30 m; 0.25-mm inside diameter, 0.25- μm film). The electron impact mass spectral data (70 eV) of each peak in the total ion chromatogram was obtained and compared to the National Institute of Standards and Technology (NIST) database to identify the corresponding compound (32).

Statistical analysis. All experiments were performed in triplicate with three biological replicates in each repeat. As similar results were shown in each repeat, only one typical example was shown for each experiment. The means and standard deviations (SD) were calculated using SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA), and the final experimental data were represented as the means \pm SD. When an analysis consisted of only a control and an experimental group, an independent *t* test was performed using SPSS Statistics 17.0 software, and when three or more groups were compared, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple-comparison test ($P < 0.05$) (33).

RESULTS

Colonization dynamics of *P. fluorescens* inside *A. lancea*. Bacterial cell culture and qPCR were conducted to monitor the colonization dynamics of *P. fluorescens* ALEB7B inside *A. lancea*. The number of cultivable bacteria peaked 5 days after inoculation ($36,433.3 \pm 6,785.4$ CFU g^{-1} , fresh weight) and subsequently decreased and became stable after 15 days (Fig. 2). The bacterial DNA concentration also peaked 5 days after inoculation (173.5 ± 18.1 copies ng^{-1} total DNA) and followed a pattern

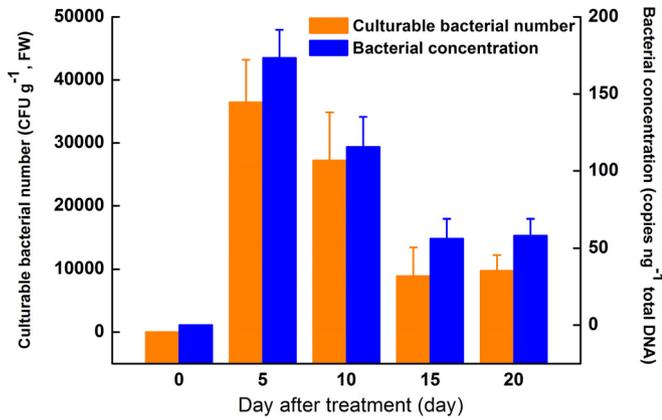


FIG 2 Culturable bacterial number and bacterial DNA concentration of *Pseudomonas fluorescens* ALEB7B inside *Atractylodes lancea*. The culturable bacterial number is represented as the number of CFU per gram of plant tissue. The bacterial DNA concentration is represented as the number of *gyrB* gene copies per nanogram of total DNA extracted from *A. lancea* inoculated with *P. fluorescens* ALEB7B. FW, fresh weight.

similar to that of the cultivable bacteria, reaching a stable level after 15 days. Results of both bacterial cell culture and qPCR showed similar bacterial colonization dynamics. The high bacterial load at the early stages of colonization might induce plant defense responses, which might limit the bacterial growth *in planta*. The stable colonization of *P. fluorescens* ALEB7B at later stages indicated the adaptation of each symbiotic partner. Therefore, we detected the impact of bacterial colonization on the host plant to further elaborate the symbiotic interaction between *A. lancea* and *P. fluorescens*.

Endophytic *P. fluorescens* triggers oxidative burst and increases oxygenous sesquiterpenoid content in *A. lancea*. Histochemical staining and quantification of ROS in *A. lancea* were conducted. To identify any correlation between endophytic bacterium-triggered oxidative burst and the accumulation of sesquiterpenoids, the levels of seven main sesquiterpenoids in *A. lancea* were measured after inoculation with *P. fluorescens* ALEB7B.

Oxidized DAB (brownish insoluble polymer) was observed in both plant leaves and roots 10 days after bacterial inoculation, allowing us to visualize H₂O₂ accumulation *in situ* (Fig. 3A). Superoxide anion production *in situ* was visualized by the formation of formazan precipitates. Levels of all four forms of ROS in *A. lancea* increased significantly 5 days after bacterial inoculation. The H₂O₂ content peaked (1.3-fold higher than the control) 10 days after inoculation and then slowly decreased (Fig. 3B). The singlet oxygen level peaked (1.4-fold greater than the control) after 5 days and returned to the control level after 20 days (Fig. 3C). The level of superoxide anions peaked (1.3-fold greater than the control) after 10 days and then decreased rapidly (Fig. 3D). The hydroxyl radical content remained at a relatively high level (approximately 1.5-fold higher than the control) 10 to 15 days after bacterial inoculation (Fig. 3E).

Total sesquiterpenoid accumulation in *A. lancea* significantly increased 15 days after inoculation with *P. fluorescens* ALEB7B (Fig. 4A). Accumulation of three oxygen-free sesquiterpenoids (β -caryophyllene, zingiberene, and β -sesquiphellandrene) significantly increased after 15 days (Fig. 4B to D), while caryophyllene oxide content began increasing (1.3-fold higher than the control) after 10 days (Fig. 4E). Hinesol and β -eudesmol content peaked (1.5-fold and 2.1-fold higher than the control, respectively) after 5 days (Fig. 4F and G), and atractylone con-

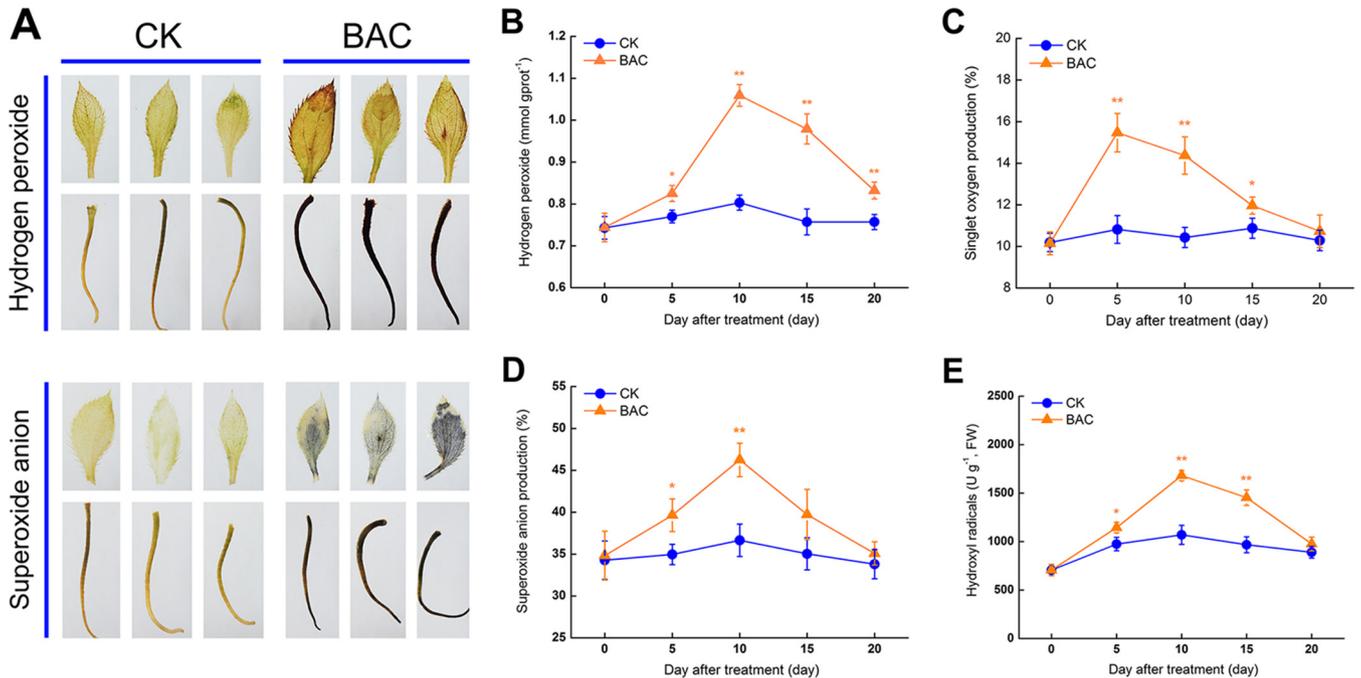


FIG 3 Histochemical staining and level of reactive oxygen species in *Atractylodes lancea* inoculated with *Pseudomonas fluorescens* ALEB7B. (A) Histochemical staining of hydrogen peroxide (H₂O₂) and superoxide anions in plant leaves and roots after 10 days. (B to E) Level of H₂O₂, singlet oxygen, superoxide anions, and hydroxyl radicals in *A. lancea* over time. Results are means from three biological replicates. Error bars indicate standard deviations. *, significant differences at *P* < 0.05; **, significant differences at *P* < 0.01. CK, control; BAC, *P. fluorescens* ALEB7B.

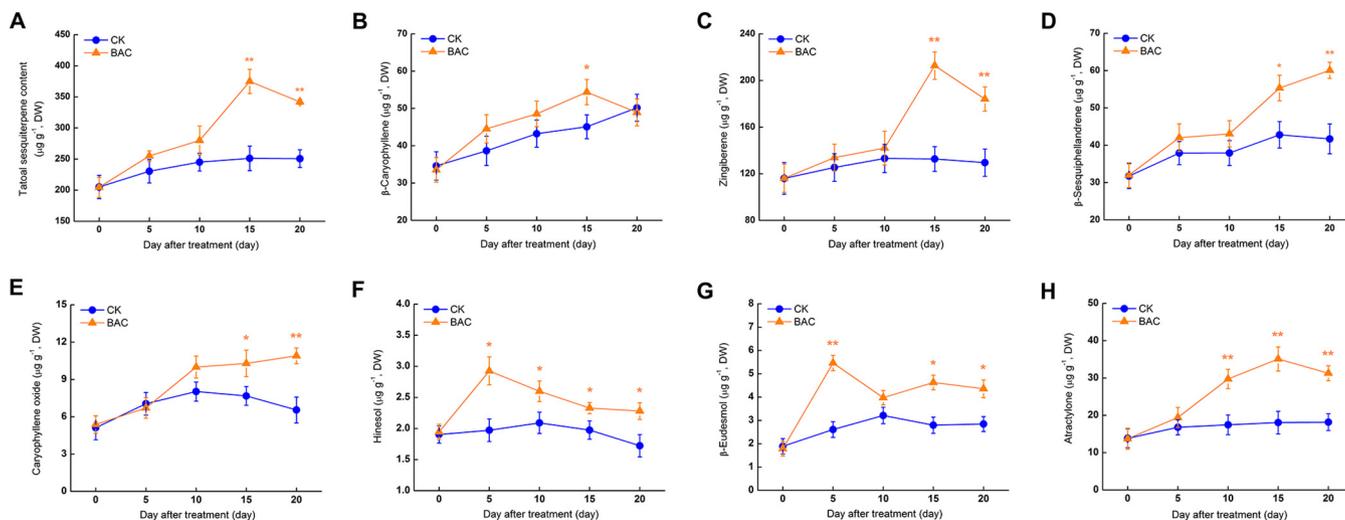


FIG 4 Accumulation of total sesquiterpenoid and seven main sesquiterpenoids in *Atractylodes lancea* inoculated with *Pseudomonas fluorescens* ALEB7B over time. (A) Total sesquiterpenoid. (B) β -Caryophyllene. (C) Zingiberene. (D) β -Sesquiphellandrene. (E) Caryophyllene oxide. (F) Hinesol. (G) β -Eudesmol. (H) Atractylone. Results are means from three biological replicates. Error bars indicate standard deviations. *, significant differences at $P < 0.05$; **, significant differences at $P < 0.01$. CK, control; BAC, *P. fluorescens* ALEB7B.

tent significantly increased (1.7-fold above the control) after 10 days (Fig. 4H). The endophytic bacterium-triggered oxidative burst and oxygenous sesquiterpenoid accumulation in *A. lancea* occurred synchronously, indicating that the endophytic bacterium-triggered ROS directly oxidizes oxygen-free sesquiterpenoids. Therefore, we subsequently investigated the impacts of exogenous hydrogen peroxide and singlet oxygen on oxygenous sesquiterpenoid accumulation *in planta*. Increased oxygen-free sesquiterpenoid content *in planta* at later stages might have been the result of increased primary metabolites, as acetyl coenzyme A and pyruvate are precursors of sesquiterpenoids (see Fig. S1 in the supplemental material).

Exogenous hydrogen peroxide and singlet oxygen increase oxygenous sesquiterpenoid accumulation in *A. lancea*. Based on our preliminary experiments, 5 mM H_2O_2 and 0.2 M NaClO plus 5 mM H_2O_2 were applied to *A. lancea* plantlets, as they increased endogenous H_2O_2 (see Fig. S2A in the supplemental material) and singlet oxygen levels (see Fig. S3A) to those seen when the plants were inoculated with *P. fluorescens* ALEB7B (Fig. 3B and C). Levels of three oxygen-free sesquiterpenoids (β -caryophyllene, zingiberene, and β -sesquiphellandrene) significantly increased 10 or 15 days after treatment with 5 mM H_2O_2 and then decreased rapidly (see Fig. S2B to D). Levels of four oxygenous sesquiterpenoids (caryophyllene oxide, hinesol, β -eudesmol, and atractylone) all peaked after 5 days and then reduced to a level that was the same as or lower than that of the control (see Fig. S2E to H).

The β -caryophyllene content significantly decreased 5 days after treatment with 0.2 M NaClO plus 5 mM H_2O_2 and remained at a level lower than that of the control (see Fig. S3B in the supplemental material). Levels of zingiberene and β -sesquiphellandrene both significantly decreased from 5 to 10 days after the treatment and increased after 15 days (see Fig. S3C and D). Meanwhile, the levels of three oxygenous sesquiterpenoids (caryophyllene oxide, hinesol, and β -eudesmol) all significantly increased from 5 to 15 days after the treatment (see Fig. S3E to G), but atractylone content remained relatively unchanged across the whole experimental

period (see Fig. S3H). Oxygenous sesquiterpenoid accumulation in *A. lancea* plantlets stimulated by exogenous H_2O_2 or singlet oxygen occurred earlier than the accumulation of oxygen-free sesquiterpenoids. These results indicated that oxygen-free sesquiterpenoids were directly oxidized by H_2O_2 or singlet oxygen at an early stage posttreatment. Furthermore, increased oxygen-free sesquiterpenoid accumulation at a later stage after the treatment reflected the signaling function of H_2O_2 or singlet oxygen.

The ROS scavenger AsA reverses oxygenous sesquiterpenoid accumulation caused by endophytic *P. fluorescens*. To further confirm the direct oxidation of ROS to oxygen-free sesquiterpenoids, we investigated whether the ROS scavenger AsA could reverse oxygenous sesquiterpenoid accumulation in *A. lancea* caused by *P. fluorescens* ALEB7B. Pretreatment of plantlets with 1,000 ppm AsA reduced H_2O_2 and singlet oxygen levels in *A. lancea* inoculated with *P. fluorescens* ALEB7B to control levels (see Fig. S4A and B in the supplemental material). Compared with untreated plantlets, the content of both the oxygen-free (β -caryophyllene, zingiberene, and β -sesquiphellandrene) (see Fig. S4C to E) and oxygenous sesquiterpenoids (caryophyllene oxide, hinesol, β -eudesmol, and atractylone) (see Fig. S4F to I) in pretreated plantlets significantly decreased but still were higher than those in the control plantlets. A single treatment of *A. lancea* with AsA had no obvious impact on oxidative burst or sesquiterpenoid accumulation. These results indicated that AsA reversed oxygenous sesquiterpenoid accumulation caused by *P. fluorescens* ALEB7B by reducing ROS levels *in planta*, further demonstrating the direct oxidation of ROS to oxygen-free sesquiterpenoids. We next conducted *in vitro* oxidation experiments, which further confirmed the direct impact of ROS on oxygenous sesquiterpenoid formation.

***In vitro* sesquiterpenoid oxidation by hydrogen peroxide and singlet oxygen.** Total sesquiterpenoids extracted from cultivated *A. lancea* reacted with 5 mM H_2O_2 or 0.2 M NaClO plus 5 mM H_2O_2 . After incubation with H_2O_2 for 4 h, the contents of two compounds in the total extracted sesquiterpenoids were clearly

TABLE 1 Impacts of *P. fluorescens* ALEB7B on sesquiterpenoid content, plant dry weight, and malondialdehyde content in *Atractylodes lancea* organisms when two main sesquiterpenoid synthesis pathways are blocked by MEV and FOS

Parameter and day after treatment	Value ^a for treatment with:			
	CK	BAC	FOS+MEV	BAC+FOS+MEV
Total sesquiterpene content ($\mu\text{g g}^{-1}$, DW)				
0	206.3 \pm 17.8 a	208.3 \pm 17.8 a	207.3 \pm 19.6 a	202.9 \pm 17.2 a
5	214.7 \pm 9.9 a	221.4 \pm 14.4 a	223.1 \pm 7.9 a	187.9 \pm 14.4 a
10	200.2 \pm 13.5 b	245.8 \pm 19.2 b	240.3 \pm 16.7 b	162.1 \pm 19.8 a
15	227.3 \pm 15.9 b	327.4 \pm 15.6 c	158.4 \pm 15.2 a	151.7 \pm 18.6 a
20	235.0 \pm 10.9 b	303.6 \pm 15.9 c	156.3 \pm 12.4 a	147.3 \pm 19.6 a
DW (g)				
0	0.06 \pm 0.01 a	0.06 \pm 0.01 a	0.06 \pm 0.01 a	0.06 \pm 0.01 a
5	0.05 \pm 0.01 a	0.07 \pm 0.01 a	0.23 \pm 0.05 b	0.04 \pm 0.00 a
10	0.05 \pm 0.01 b	0.07 \pm 0.01 c	0.14 \pm 0.01 d	0.03 \pm 0.01 a
15	0.07 \pm 0.01 b	0.08 \pm 0.01 b	0.11 \pm 0.01 c	0.04 \pm 0.01 a
20	0.08 \pm 0.01 b	0.09 \pm 0.01 b	0.10 \pm 0.01 b	0.04 \pm 0.01 a
Malondialdehyde (nmol mg protein ⁻¹)				
0	5.4 \pm 0.4 a	5.5 \pm 0.5 a	5.4 \pm 0.4 a	5.3 \pm 0.4 a
5	6.2 \pm 0.5 a	5.9 \pm 0.6 a	7.7 \pm 0.5 a	10.3 \pm 0.6 b
10	6.3 \pm 0.5 a	5.3 \pm 0.7 a	9.3 \pm 0.7 b	11.5 \pm 0.9 b
15	8.5 \pm 0.5 a	6.9 \pm 0.4 a	9.3 \pm 0.5 a	11.0 \pm 0.4 b
20	6.1 \pm 0.5 a	4.3 \pm 0.5 a	11.7 \pm 0.8 b	16.6 \pm 0.9 c

^a Values are means from three biological replicates with the corresponding standard deviations. Values followed by different lowercase letters are significantly different according to Tukey's multiple-comparison test ($P < 0.05$). CK, control; BAC, *P. fluorescens* ALEB7B; FOS, fosmidomycin; MEV, mevinolin; DW, dry weight.

increased compared to those of the control (see Fig. S5A in the supplemental material). Gas chromatography-mass spectrometry (GC-MS) analysis showed that these two compounds were caryophyllene oxide and humulene epoxide II (see Fig. S5B).

When the sesquiterpenoids were reacted with NaClO plus H₂O₂, the contents of seven oxygenous sesquiterpenoids were much higher than the control levels. These oxygenous sesquiterpenoids included aromadendrene oxide, hinesol, diepicedrene-1-oxide, tricyclo[5.2.2.0 (1, 6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-, 7-epi-cis-sesquisabinene hydrate, and spathulenol (see Table S1 and Fig. S5C in the supplemental material). Among these, hinesol and spathulenol were previously reported as oxygenous sesquiterpenoids in *A. lancea* (see Fig. S5D) (34). These results were consistent with the results of *in vivo* experiments, and hinesol and caryophyllene oxide were found to be derived from direct oxidation both *in vivo* and *in vitro*. Many oxygenous sesquiterpenoids were generated from *in vitro* oxidation, which showed that the sesquiterpenoid diversity in *A. lancea* was at least partially dependent on the direct oxidation of ROS.

Functions of sesquiterpenoids in the balance of antagonism between *A. lancea* and *P. fluorescens*. We detected the functions of direct oxidation of oxygen-free sesquiterpenoids by ROS in symbiotic interactions between *A. lancea* and *P. fluorescens*. When two sesquiterpenoid synthesis pathways in *A. lancea* (the MVA pathway and MEP pathway) were blocked by MEV and FOS, the impact of endophytic *P. fluorescens* ALEB7B on *A. lancea* changed from benefit to harm. It was clear that sesquiterpenoid accumulation in *A. lancea* plantlets was significantly inhibited by MEV and FOS (Table 1). When sesquiterpenoid synthesis was blocked, the dry weight of plants was significantly decreased and the malondialdehyde content *in planta* was significantly increased 5 days after bacterial inoculation. Moreover, the aboveground parts of plantlets that had been pretreated with MEV and FOS before bacterial inoculation appeared chlorotic and withered (Fig. 5). A single treatment with MEV and FOS had no obvious adverse effects on any of the indices tested. These results indicated that sesquiterpenoids protected host plants from oxidative damage caused by endophytic bacterial colonization and that oxygen-free sesquiterpe-



FIG 5 Typical phenotype of *Atractylodes lancea* whose two main sesquiterpenoid synthesis pathways are blocked by mevinolin and fosmidomycin 20 days after the inoculation of *Pseudomonas fluorescens* ALEB7B. CK, control; BAC, *P. fluorescens* ALEB7B; MEV, mevinolin; FOS, fosmidomycin.

noids that directly consumed ROS played essential roles in maintaining the balance of antagonism between *A. lancea* and *P. fluorescens*.

DISCUSSION

Oxygenous terpenoids are the active components of many medicinal plants, such as menthol, artemisinin, paclitaxel, and glycyrrhizic acid (35). The oxygen-containing groups are essential for the medicinal activities of oxygenous terpenoids, as in the case of artemisinin, whose activity is dependent upon the presence of a peroxide bridge (36). Enzymatic oxygenous terpenoid synthesis has been well studied (37, 38). Enzymatic reactions can catalyze hydroxylation or ketonization at specific sites of terpenoid hydrocarbon scaffolds. Much work has been done to clarify all of the intermediates and corresponding enzymes involved in the synthesis of artemisinic acid, the direct precursor of artemisinin; however, the enzymes that oxidize artemisinic acid to artemisinin have not yet been identified (35). It is clear, therefore, that enzymatic oxidation cannot explain the synthetic mechanisms of all oxygenous terpenoids. *A. lancea* contains diverse oxygenous sesquiterpenoids, but their compositions in different individuals vary greatly (3, 39). Directional oxidation catalyzed by enzymes cannot explain the diversity of oxygenous terpenoids that is seen. However, ROS can oxidize sesquiterpenoids at multiple sites, which promotes oxygenous sesquiterpenoid synthesis and gives rise to the diversity of these compounds.

The accumulation of active components in *A. lancea* strongly depends on its producing area (40). Higher oxygenous sesquiterpenoid diversity and content are characteristics of the geo-authentic *A. lancea* grown in the Maoshan area (3). Hinesol, β -eudesmol, atracylone, and caryophyllene oxide are characteristic sesquiterpenoid components in the geo-authentic *A. lancea* (14). The isolation rate of endophytes from the geo-authentic *A. lancea* is also much higher than that found when *A. lancea* is grown in other areas (8–10). Oxidative burst is one of the earliest plant responses to microbial infections (17). This study demonstrated that after colonization by *P. fluorescens* ALEB7B (Fig. 2), the endophytic bacterium-triggered oxidative burst and oxygenous sesquiterpenoid accumulation in *A. lancea* occurred at the same time (Fig. 3 and 4E to H). Furthermore, we showed that treatment of plants with exogenous H_2O_2 and singlet oxygen significantly increased the levels of endogenous H_2O_2 and singlet oxygen, respectively (see Fig. S2A and S3A in the supplemental material). Meanwhile, the oxygenous sesquiterpenoid content significantly increased (see Fig. S2E to H and S3E to H), but oxygen-free sesquiterpenoid content did not change or significantly decrease (see Fig. S2B to S2D and S3B to D). Conversely, when *A. lancea* plantlets were pretreated with the ROS scavenger AsA, *P. fluorescens* ALEB7B could not trigger the oxidative burst *in planta* (see Fig. S4A and B in the supplemental material), and the oxygenous sesquiterpenoid content in pretreated *A. lancea* plantlets was much lower than that in untreated plants (see Fig. S4F to I). These results indicate that endophytic bacterium-triggered ROS not only can act as signaling molecules (14) but also can directly oxidize oxygen-free sesquiterpenoids, increasing the content and diversity of oxygenous sesquiterpenoids *in planta*. Moreover, Yan et al. (41) recently reported that the fumitremorgin B endoperoxidase from *Aspergillus fumigatus* can catalyze the formation of the peroxide bridge in verruculogen, providing a basis for the identification of endophyte-derived oxidases that function in plant oxygenous terpe-

noid formation. Therefore, we speculate that endophytes promote oxygenous terpenoid formation *in planta* via three mechanisms. First, ROS induced by endophytic infection may directly oxidize oxygen-free terpenoids. Second, oxidases derived from endophytes may be secreted and function *in planta*. Third, signaling pathways activated by endophytic colonization increase the amount or activity of relevant oxidases.

In vitro oxidation experiments showed that H_2O_2 could oxidize total sesquiterpenoids extracted from cultivated *A. lancea* and generate caryophyllene oxide and humulene epoxide II (see Fig. S5A and B in the supplemental material). Singlet oxygen could oxidize extracted sesquiterpenoids and generate hinesol and spathulenol (see Fig. S5C and D). Caryophyllene oxide, humulene epoxide II, hinesol, and spathulenol all have been reported to be the main oxygenous sesquiterpenoids in wild *A. lancea* (3, 39). These results further demonstrate that endophyte-triggered ROS can lead directly to the formation of diverse oxygenous sesquiterpenoids in geo-authentic *A. lancea*. Some studies have suggested that the transformation of artemisinic acid to artemisinin is a non-enzymatic spontaneous oxidation and correlates with singlet oxygen levels *in planta* (27, 42). This supports our viewpoint, indicating that the ROS-induced direct oxidation of terpenoids have general biological significance. Steenackers et al. (31) have reported that H_2O_2 oxidizes terpenoids by elongating O—O bonds, and cleaving the OH moiety and singlet oxygen reacts with endocyclic double bonds of terpenoids (43). The process of terpenoids being directly oxidized by endophytic bacterium-triggered ROS is an essential complementarity to enzymatic terpenoid synthesis and modification *in planta*. As found in this study, peroxidase could protect some sesquiterpenoids, such as atracylone, from being peroxidized by H_2O_2 (see Fig. S5A). Therefore, we speculate that oxygenous terpenoids can be generated from two sources *in planta*. On the one hand, oxidases can catalyze oxidations at specific sites of terpenoid hydrocarbon scaffolds. On the other hand, ROS induced by stresses may directly and randomly oxidize terpenoids, and enzymatic reactions and ROS may function synergistically.

Direct oxidation of terpenoids by bacterium-triggered ROS also regulates interactions between endophytes and plants. The interactions between endophytes and plants can vary from mutualism to parasitism (44). The asymptomatic colonization of plants by endophytes is a delicate balance of antagonism between endophytes and host plants, during which plant secondary metabolites play essential roles (45). If host plants cannot effectively control endophyte-triggered ROS, oxidative damage to the plant will occur. Direct oxidation of terpenoids by ROS consumes endophyte-triggered ROS and maintains endogenous ROS levels at those which plants can endure. When two sesquiterpenoid synthesis pathways in *A. lancea* were blocked (Fig. 1), colonization by *P. fluorescens* ALEB7B caused chlorosis and withered symptoms in the aboveground parts of plantlets (Fig. 5). Meanwhile, the plant dry weight significantly decreased and the malondialdehyde content in plantlets significantly increased (Table 1). These results indicate that sesquiterpenoids act as antioxidants and participate in protecting plants from oxidative damage caused by environmental stresses. Furthermore, although bacterial colonization can enhance plant photosynthesis (see Fig. S1B and C in the supplemental material), it has little effect on plant biomass (see Fig. S1A). The increased photosynthates induced by endophytic bacterium are preferentially used by *A. lancea* to synthesize precursors of

sesquiterpenoids (see Fig. S1D to F). Furthermore, oxygenous sesquiterpenoids that have been oxidized from oxygen-free sesquiterpenoids have stronger antimicrobial activity (46). These processes restrict the overgrowth of endophytes *in planta* (47) and are essential strategies used by host plants to modulate the growth of endophytes *in vivo* and to maintain a mutualistic relationship with endophytes.

In summary, this study shows that endophytic bacterium-triggered ROS can directly oxidize sesquiterpenoids and increase oxygenous sesquiterpenoid content and diversity in *A. lancea*, which not only provides a novel viewpoint on the mechanisms of oxygenous terpenoid synthesis and diversity in multiple medicinal plants but also is essential to further understanding interactions between endophytes and plants.

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