

# The Endophytic Fungus *Phomopsis liquidambari* Increases Nodulation and N<sub>2</sub> Fixation in *Arachis hypogaea* by Enhancing Hydrogen Peroxide and Nitric Oxide Signalling

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Received: 22 September 2016 / Accepted: 22 January 2017 / Published online: 6 February 2017  
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**Abstract** The continuous cropping obstacles in monoculture fields are a major production constraint for peanuts. Application of the endophytic fungus *Phomopsis liquidambari* has increased peanut yields, and nodulation and N<sub>2</sub> fixation increases have been considered as important factors for *P. liquidambari* infection-improved peanut yield. However, the mechanisms involved in this process remain unknown. This work showed that compared with only *Bradyrhizobium* inoculation, co-inoculation with *P. liquidambari* significantly elevated endogenous H<sub>2</sub>O<sub>2</sub> and NO levels in peanut roots. Pre-treatment of seedlings with specific scavengers of H<sub>2</sub>O<sub>2</sub> (CAT) and NO (cPTIO) blocked *P. liquidambari*-induced nodulation and N<sub>2</sub> fixation. CAT not only suppressed the *P. liquidambari*-induced nodulation and N<sub>2</sub> fixation, but also suppressed the enhanced H<sub>2</sub>O<sub>2</sub> and NO generation. Nevertheless, the cPTIO did not significantly inhibit the induced H<sub>2</sub>O<sub>2</sub> biosynthesis, implying that H<sub>2</sub>O<sub>2</sub> acted upstream of NO production. These results were confirmed by observations that exogenous H<sub>2</sub>O<sub>2</sub> and sodium nitroprusside (SNP) reversed the inhibition of *P. liquidambari*-increased nodulation and N<sub>2</sub> fixation by the specific scavengers. The transcriptional activities of the symbiosis-related genes *SymRK* and *CCaMK* of peanut-*Bradyrhizobium* interactions also increased significantly in response to *P. liquidambari*, H<sub>2</sub>O<sub>2</sub> and SNP treatments. The pot experiment further confirmed that the *P. liquidambari* infection-enhanced H<sub>2</sub>O<sub>2</sub> and

NO signalling pathways were significantly related to the increase in peanut nodulation and N<sub>2</sub> fixation. This is the first report that endophytic fungus *P. liquidambari* can increase peanut-*Bradyrhizobium* interactions via enhanced H<sub>2</sub>O<sub>2</sub>/NO-dependent signalling crosstalk, which is conducive to the alleviation of continuous cropping obstacles via an increase in nodulation and N<sub>2</sub> fixation.

**Keywords** Peanut · Endophytic fungus *Phomopsis liquidambari* · *Bradyrhizobium* · Co-inoculation · Nodulation and N<sub>2</sub> fixation · H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk

## Introduction

The peanut (*Arachis hypogaea* L.) is an important oil and cash crop worldwide. China is a major producer of peanuts, with approximately 5 million hectares cultivated in each growing season, accounting for 30% of the total oilseed production [1, 2]. Due to increasing land scarcity and agro-industrialisation, peanuts are usually continuously monocropped on a large scale in the same field for many years without any crop rotation [1, 3]. However, there are various problems associated with such consecutive monoculture, including a continuous decline in yield and quality, which is described as ‘peanut continuous cropping obstacles’ [1–3]. Fan et al. [4] and Wang et al. [5] demonstrated that decreases in nodulation and N<sub>2</sub> fixation were important causes of peanut replant obstacles in the long-term monoculture field. Our previous studies showed that the application of an endophytic fungus, *Phomopsis liquidambari* [6], can effectively alleviate peanut continuous cropping obstacles [1, 5]. Further analysis demonstrated that the increase in nodulation and N<sub>2</sub> fixation may play an important role in *P. liquidambari* enhancement of peanut yield [7, 8]. However, the detailed mechanisms by

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which *P. liquidambari* improve nodulation and N<sub>2</sub> fixation in peanut are still unclear.

A recent study by Zhang et al. [8] demonstrated that *P. liquidambari* co-inoculated with *Bradyrhizobium* significantly increased root flavonoid compound (peanut rhizobial *nod* gene inducer) secretion and lateral root (peanut rhizobial infection site) formation, which contributed to the induction of peanut-specific rhizobia enrichment and increased the efficiency of rhizobial infection. Further follow-up revealed that *P. liquidambari* infection indeed increased bradyrhizobial colonisation, the peanut nodulation and N<sub>2</sub> fixation [8]. It is well known that during legume–rhizobium interactions, rhizobial infection is dominant and host-specific, and it can occur via two different modes of entry into the roots (root ‘hair curling invasion’ and ‘cortex crack-entry’), followed by initiation of the nodule primordium and finally development of an effective nodule [9, 10]. During the early stages of the legume–rhizobium interaction, regardless of whether the initial rhizobial entry is through an infection thread or epidermal cracks, a subtle and complicated molecular dialogue between the two partners is required to coordinate this symbiotic process [9–12]. Therefore, in our previous study, although the inoculation of endophytic fungus *P. liquidambari* significantly increased the *Bradyrhizobium* infection efficiency and accelerated nodule initiation, the molecular regulations involved in enhancing peanut–*Bradyrhizobium* interaction in response to *P. liquidambari* infection remain unknown.

Many studies have demonstrated that the development of legume–rhizobium symbiosis involves the activities of a variety of endogenous signalling molecules in host plants [13, 14], which are important for controlling or mediating symbiotic responses. Among these signalling molecules, particularly hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitric oxide (NO) have been widely accepted as the signalling response to biotic and abiotic stress processes, and there is currently compelling evidence that H<sub>2</sub>O<sub>2</sub> and NO play important role in signalling processes during the establishment of legume–*Rhizobium* symbioses [15–20]. Previous studies have shown that high levels of H<sub>2</sub>O<sub>2</sub> and NO can be detected during the early stages of the legume–rhizobium symbiotic interaction and those changes in the levels of H<sub>2</sub>O<sub>2</sub> and NO or modifications of the H<sub>2</sub>O<sub>2</sub> and NO generation-related genes significantly impact legume–rhizobium symbiosis, nodule initiation and development [15–20]. Therefore, during the process of legume–*Rhizobium* symbiosis, H<sub>2</sub>O<sub>2</sub> and NO appear to play a key signalling role in the establishment and function of this symbiotic interaction.

Endophytic fungi, as a large and novel microbial resource, can be found in healthy plant tissues without causing any apparent symptoms of disease. Many studies have suggested that endophytic fungi can exert multifunctional ecological functions for promoting host growth [21]. For

the horizontal transmission of endophytic fungi, specific or non-specific infection forming a mutualistic symbiosis with various hosts can be randomly established at the proper moment in natural environments. During this symbiotic process, endophytic fungal infection can trigger host defence reactions, in which H<sub>2</sub>O<sub>2</sub> and NO are also important early events in the induction of plant systematic acquired resistance in response to this external infection stressor [22–24], finally establishing a subtle antagonistic balance between hosts and endophytic fungi via the complicated signalling molecule dialogue [22, 24, 25]. In addition, our previous studies have also provided evidence that endophytic fungal infection-induced H<sub>2</sub>O<sub>2</sub> and NO biosynthesis promotes *Atractylodes lancea* volatile oil accumulation [22, 23, 26]. It is believed that some signalling molecules, such as H<sub>2</sub>O<sub>2</sub> and NO, are shared during simultaneous rhizobial and specific endophytic fungal infection of their shared hosts. Therefore, in this study, we hypothesised that the H<sub>2</sub>O<sub>2</sub> and NO biosynthesis induced by *P. liquidambari* infection are important for promoting symbiotic peanut–*Bradyrhizobium* interactions, nodulation and N<sub>2</sub> fixation, which is responsible for alleviating continuous cropping obstacles associated with peanut.

The aims of this study were to investigate whether infection caused by the endophytic fungus *P. liquidambari* could induce an increase in endogenous H<sub>2</sub>O<sub>2</sub> and NO during the early stage of the symbiotic peanut–*Bradyrhizobium* interaction and to further investigate the cross-communication between H<sub>2</sub>O<sub>2</sub> and NO signalling to determine whether the enhancement of this signalling pathway in response to *P. liquidambari* infection promotes the peanut–*Bradyrhizobium* interaction, nodulation and N<sub>2</sub> fixation.

## Materials and Methods

### Experimental Strains and Soil

The endophytic fungus *P. liquidambari* was previously isolated from the inner bark of the stem of *Bischofia polycarpa* [6]. It was labelled with green fluorescent protein (GFP) by an expression vector, pCT74 [8, 27], and incubated in PDB medium (200 g L<sup>-1</sup> potato extract, 20 g L<sup>-1</sup> glucose, pH 7.0) for 48 h at 28 °C on a shaker at 180 rpm. Fungal mycelia were collected by filtering, washed three times with sterile distilled water and re-suspended in sterile distilled water to serve as the fungal inoculum. *Bradyrhizobium yuanmingense* was obtained from China Agricultural University; it had been previously isolated from the surface-sterilized nodules of peanut, cultured in YMB medium (D-Mannitol, 10 g L<sup>-1</sup>; yeast extract, 3 g L<sup>-1</sup>; MgSO<sub>4</sub>, 0.1 g L<sup>-1</sup>; NaCl, 0.1 g L<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub>, 0.25 g L<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub>, 0.25 g L<sup>-1</sup>) in an orbital shaker at

180 rpm and 28 °C for 6 days. The bacterial cells were centrifuged at 4000×g for 10 min, re-suspended in sterile distilled water and then diluted to an OD<sub>600</sub> of 0.8, corresponding to a concentration of 10<sup>8</sup> cells mL<sup>-1</sup>, to generate the *Bradyrhizobium* inoculum.

The soil was collected from an agricultural field at the Ecological Experimental Station of Red Soil, Chinese Academy of Sciences (Yingtian, Jiangxi Province, China, 28°13' N, 116°55' E). The field had continuously cropped peanuts for 5 years, and the selected soil was classified as Udic Ferrosol [FAO (1998) classification]. After removal of visible coarse plant material, the collected surface layer soil (0–20 cm) was passed through a sieve (2 mm) and stored for the bioassay. The basic soil physicochemical properties were as follows: organic matter 9.5 g kg<sup>-1</sup>, total N 0.6 g kg<sup>-1</sup>, total P 0.3 g kg<sup>-1</sup>, total K 5.7 g kg<sup>-1</sup>, available P 10.3 mg kg<sup>-1</sup>, available K 113.8 mg kg<sup>-1</sup>, and pH (1:2.5, w/v) 5.7.

### Plant Material, Pre-germination and In Vitro Inoculation

The peanut cultivar Guanhua-5 is a common planting variety that is grown in the red soil regions of Jiangxi Province [1], China, and was used in this study. The seeds were soaked for 5 min in 70% ethanol, followed by five washes with sterile distilled water, and further surface disinfection with 1% (v/v) sodium hypochlorite (NaOCl) for 3 min followed by another wash with sterile distilled water. The surface-sterilized seeds were pre-germinated in the dark at 28 °C until the radicles reached 2–3 cm. Germinated seeds exposed to similar growth conditions were transplanted into pots (12 cm in diameter, 15 cm in height) containing sterile vermiculite; each pot contained one peanut seedling. All pots were cultivated in a growth chamber at 28 °C with 16 h of light and at 25 °C with 8 h of dark, with 70% relative humidity. After 3 days of cultivation, the peanut seedling was subjected to four treatments: (1) non-inoculated control; (2) seedling inoculated with only *B. yuanmingense* (leading to an inoculation level of 10<sup>9</sup> cells/seedling) (B treatment); (3) seedling inoculated with only *P. liquidambari* (9.4 mg mycelial dry weight/seedling) (P treatment); and (4) seedling co-inoculation with *P. liquidambari* and *B. yuanmingense* (P + B treatment). The non-inoculated seedlings received sterilized water. Hoagland's nutrient solution was added every 5 days, and sterilized distilled water was added when needed. Three replicates were evaluated for each treatment, and three plants (from three pots) were pooled as one replicate for each sampling point. All seedlings were randomly distributed in the growth chamber, and the culture conditions were consistent with those described above.

### Determination of Endophytic Fungal Infection in Peanut Roots

The root samples were collected after 3 and 7 days of inoculation and processed for microscopy [27]. The fungal structures were observed under a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany). These images were then collected and analysed using a Sensicam QE cooled digital camera system (Cooke Corporation, Germany) with a MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA, USA). Meanwhile, the collected peanut roots were carefully washed with sterile distilled water, and the root total genomic DNA was further extracted from freeze-dried powdered material using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Bio-sciences, Union City, CA, USA) following the manufacturer's instruction. A fragment of the *P. liquidambari*-specific ITS locus was amplified with previously designed primers Bf1 (5'-CTGGCCCCCTCGGGGTCCTGG-3') and Br1 (5'-TTTCAGGGCCTGCCCTTTTACAGGC-3') to further confirm endophytic fungal colonisation [28]. The PCR mixture and thermal cycling conditions were similar to those reported by Wang et al. [28]. In addition, the amounts of the endophytic fungus *P. liquidambari* in the infected roots were also estimated by quantitative PCR (qPCR) according to the method described by Yang et al. [27] and Wang et al. [28].

### Chemicals and Treatments

Catalase (CAT) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) were used as specific scavengers of H<sub>2</sub>O<sub>2</sub> and NO, respectively. H<sub>2</sub>O<sub>2</sub> solution and sodium nitroprusside (SNP) were used as donors of H<sub>2</sub>O<sub>2</sub> and NO, respectively. CAT, cPTIO, H<sub>2</sub>O<sub>2</sub>, and SNP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All scavengers and exogenous donors were dissolved in sterile distilled water and filtered through 0.22-µm diameter sterile filters before use. The reagents and their dosages were chosen based on our preliminary study. Scavengers were sprayed on seedling leaves and roots 1 day before the application of exogenous donors or microbial inoculation. An equal volume of sterile distilled water was used as the control. Three replicates were assessed for each treatment.

### Peanut Nodulation and N<sub>2</sub> Fixation Parameter Analysis

The peanut seedlings were collected after 21 and 42 days of inoculation. The roots (21 days) were carefully cleaned with sterile distilled water, and the number of nodule initials (primordia) was counted using a staining procedure [8]. In addition, the active nodules (pink colour) were excised from the roots (42 days) with scalpels to measure the nodule

number, individual nodule dry weight, individual nodule size and total nodule dry weight [29]. Peanut growth parameters such as shoot (leaf and stem) dry weight and root dry weight were also measured at 42 days of inoculation. The nodules, shoots and roots were dried in an oven at 80 °C to a constant weight before weighing. The harvested shoot samples were ground to a fine powder, and the N concentration was determined using the Kjeldahl method. The amount of fixed N<sub>2</sub> was estimated by the N difference method, in which the N yield of the shoot of the uninoculated peanut was subtracted from that of the inoculated peanut, and the difference was assumed to be derived from N<sub>2</sub> fixation.

### Extraction and Measurement of H<sub>2</sub>O<sub>2</sub> and NO

The production of H<sub>2</sub>O<sub>2</sub> and NO was measured using the H<sub>2</sub>O<sub>2</sub> and NO detection kit (Nanjing Jiancheng Bio-engineering Institute, China) according to the manufacturer's instructions [22, 26]. Approximately 1 g of fresh root sample was ground in 5 mL of sterile distilled water to evaluate H<sub>2</sub>O<sub>2</sub> and 5 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) to assess NO. The homogenate was centrifuged at 14,000×g for 10 min. The supernatant was used to measure H<sub>2</sub>O<sub>2</sub> and NO, respectively.

### RNA Extraction and qPCR

Total RNA was extracted from peanut roots using TRIzol reagent, according to the manufacturer's recommendations (Sigma). The RNA samples were treated with RNase-free DNase (1 U μL<sup>-1</sup>) at 37 °C for 15 min and then at 65 °C for 10 min to remove genomic DNA contamination. The RNA integrity and concentration were measured by electrophoresis and NanoDrop spectrophotometry, respectively. First-strand cDNA was synthesized from 1 μg of total RNA (PrimeScript One Step RT Reagent Kit; Takara). qPCR was performed using the StepOne Real-time PCR system (Applied Biosystems) with SYBR Green I fluorescent dye (Takara, Dalian, China). Peanut *Actin* and *Ubiquitin* were used as internal controls as previously described [30, 31], with the gene-specific primers *AhActin*-Fw (5'-CTGGCATCATACCTTCTACAACG-3') and *AhActin*-Rv (5'-GAATGGCAACATACATAGCAGGG-3'), and *AhUbiquitin*-Fw (5'-AAGCCGAAGAAGATCAAGCAC-3') and *AhUbiquitin*-Rv (5'-GGTTAGCCATGAAGGTTCCA-3'), respectively. Moreover, the specific primer pairs used for quantitative measurement of the transcription of *SymRK* and *CCaMK*, respectively, were designed according to their partial mRNA sequences as follows: *AhSymRK*-Fw (5'-TGCTCTTACCCACTCTGA-3') and *AhSymRK*-Rv (5'-ATCTGTAGTTGGACCCTC-3') (GenBank Accession No. JQ780692.1); *AhCCaMK*-Fw (5'-TCTGTTGTCAGGAAAGGCATAA-3') and *AhCCaMK*-Rv (5'-CCGC

AATCTGGCGAATAA-3') (GenBank Accession No. EU395429.1). The qPCR reaction mixture contained 10 μL of SYBR Premix *Ex Taq* (dNTP, *Ex Taq* polymerase, SYBR Green I, and *Ex Taq* reaction buffer; Takara), 0.4 μL of each primer (10 μM) and 0.4 μL ROX Reference Dye (50×), 20 ng cDNA template, and sterile distilled water up to a final volume of 20 μL. The qPCR protocol was as follows: 94 °C for 1 min, followed by 45 cycles of 94 °C for 15 s, 60 °C for 45 s and 72 °C for 30 s. The specificity of the amplification was verified by a melting curve analysis and the presence of single PCR products in the electrophoretic gel. The relative abundance of *SymRK* and *CCaMK* transcription was normalized to the levels of the two reference genes and calculated using the  $\Delta\Delta C_t$  method. Each sample was amplified in triplicate in each experiment.

### Pot Experiment

The pot experiment was performed to further test the proposed hypothesis. One kilogramme of non-sterile soil was weighed and added to a plastic pot (15-cm diameter, 13-cm high). The germinated peanut seeds were transplanted into these pots, and each pot contained one seedling. After 5 days of cultivation, the peanut seedlings were inoculated with *P. liquidambari* (9.4 mg mycelial dry weight/seedling) for the P treatment. The specific scavengers CAT and cPTIO of H<sub>2</sub>O<sub>2</sub> and NO were applied to evaluate the role of H<sub>2</sub>O<sub>2</sub> and NO signalling in *P. liquidambari*-induced nodulation and N<sub>2</sub> fixation, serving as the P + 5 mKat L<sup>-1</sup> CAT and P + 2 mM cPTIO treatments, respectively. Exogenous H<sub>2</sub>O<sub>2</sub> and NO supplementation were set up as P + 5 mKat L<sup>-1</sup> CAT + H<sub>2</sub>O<sub>2</sub> and P + 2 mM cPTIO + SNP, respectively. Non-inoculated seedlings were used as controls. Experimental pots were randomly arranged in the growth chamber, and the culture conditions were consistent with the descriptions provided above. All treatments were performed in triplicate. After 5 days of inoculation, the partial plants were collected to detect endogenous H<sub>2</sub>O<sub>2</sub>, NO and the transcription levels of the *SymRK* and *CCaMK* genes. In addition, peanut nodulation and N<sub>2</sub> fixation parameters were also analysed after 40 days of inoculation.

### Statistical Analysis

All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Mean values and standard deviations (SD) were calculated, and the final experimental data are presented as the mean (±SD) of three replicates of each treatment. When an analysis consisted of only a control and an experimental group, an independent *t* test was performed using SPSS 13.0 software, and when three or more groups were compared, one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple-



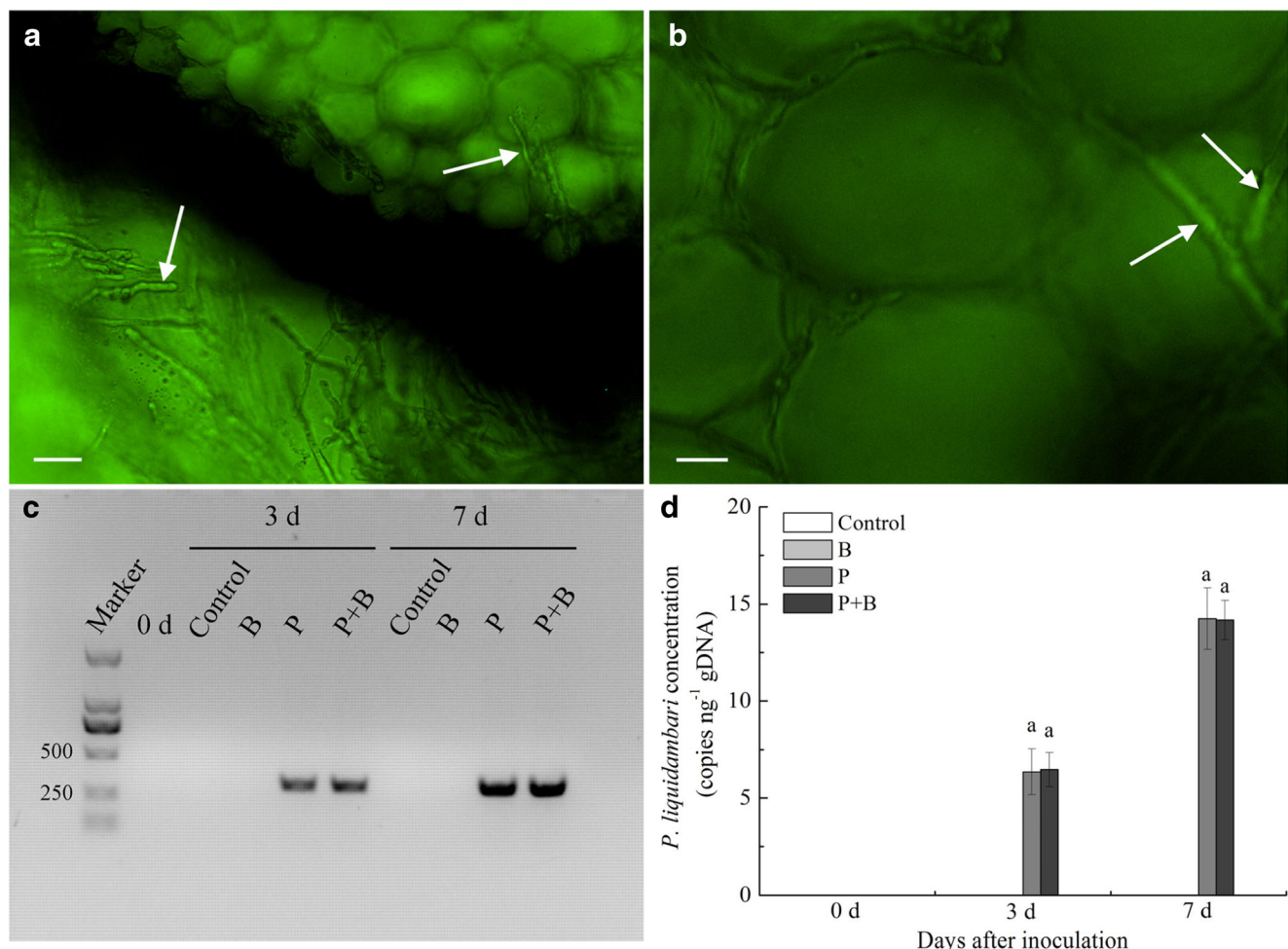
comparison test. Comparisons between different treatments were considered significant at  $p < 0.05$ .

## Results

### Infection and Quantification of *P. liquidambari* in Peanut Roots

In order to visualize the infection process of endophytic fungus *P. liquidambari* in peanut roots, the GFP-tagged *P. liquidambari* was used and observed by fluorescence microscopy after 3 and 7 days of inoculation. In the early stage of infection (3 days) (Fig. 1a), although autofluorescence was observed in the roots, a large number of *P. liquidambari* hyphae interweaved together and distributed on the root surface,

and only small amount of fungal hyphae could penetrate root epidermis and infect into the epidermal cell layer. After 7 days of inoculation (Fig. 1b), the GFP-tagged *P. liquidambari* was easily recognisable by its shape and size, and fungal hyphae can undergo intracellular infection and branched growth in the epidermal cell layer. There was no significant difference in fungal infection between *P. liquidambari* only inoculated (P) and co-inoculated (P + B) peanut roots, and *P. liquidambari* was not observed in non-inoculated roots. Figure 1c shows that the *P. liquidambari*-specific ITS fragment could be amplified from P and P + B treatments. In addition, the concentration of *P. liquidambari* within peanut roots was expressed as the number of *P. liquidambari*-specific ITS copies per ng total (root + fungal) genomic DNA in a qPCR analysis. As shown in Fig. 1d, the colonisation levels in roots between P and P + B treatments were similar at the same sampling time,



**Fig. 1** The analysis of endophytic fungal infection and colonisation in peanut roots. Fluorescence microscopy determination of endophytic fungus *P. liquidambari*-infected peanut roots after 3 days (**a**, scale bar = 20  $\mu$ m) and 7 days (**b**, scale bar = 10  $\mu$ m) of inoculation. Agarose gel electrophoresis (**c**) and qPCR (**d**) analysis of the

colonisation of peanut roots by endophytic fungus *P. liquidambari* after 3 and 7 days of inoculation. Values are the means of three independent experiments  $\pm$  SD. Data in columns marked by different letters are significantly different between the different treatments at the same sampling time ( $p < 0.05$ )

and colonisation concentration was 6.35 (P) and 6.47 (P + B) and 14.25 (P) and 14.17 (P + B) copies ng<sup>-1</sup> gDNA at 3 and 7 days of inoculation, respectively.

### *P. liquidambari* Improves Nodulation and N<sub>2</sub> Fixation Parameters

As shown in Table 1, compared to *Bradyrhizobium* infection treatment alone (B), co-inoculation with *P. liquidambari* and *Bradyrhizobium* (P + B) significantly increased the peanut nodule primordium and nodule numbers by 51.55 and 39.1%, respectively. Further nodule trait analysis indicated that P + B also significantly improved the individual nodule weight, size and total nodule weight by 8.24, 7.21 and 50.98%, respectively. Moreover, no difference was observed in the N content of shoots between control peanuts and ones infected with only *P. liquidambari*, but *Bradyrhizobium* infection alone significantly increased shoot N by 34.68%. However, the highest shoot N content was observed in response to P + B treatment, with an increase of 95.65% compared with the control. N<sub>2</sub> fixation analysis indicated that the co-inoculated peanuts fixed more nitrogen than the peanuts inoculated with only *Bradyrhizobium*, with an increase of 174.54%. In addition, compared with the control, the peanut shoot and root dry weight were enhanced by 31.03 and 56.52%, respectively, in the co-inoculation experiment. In contrast, no significant change was observed in the peanut biomass following inoculation with *Bradyrhizobium* alone, but infection with *P. liquidambari* alone significantly increased the root dry weight by 30.43%.

### Involvement of the Increase in H<sub>2</sub>O<sub>2</sub> in *P. liquidambari*-Induced Nodulation and N<sub>2</sub> Fixation Enhancement

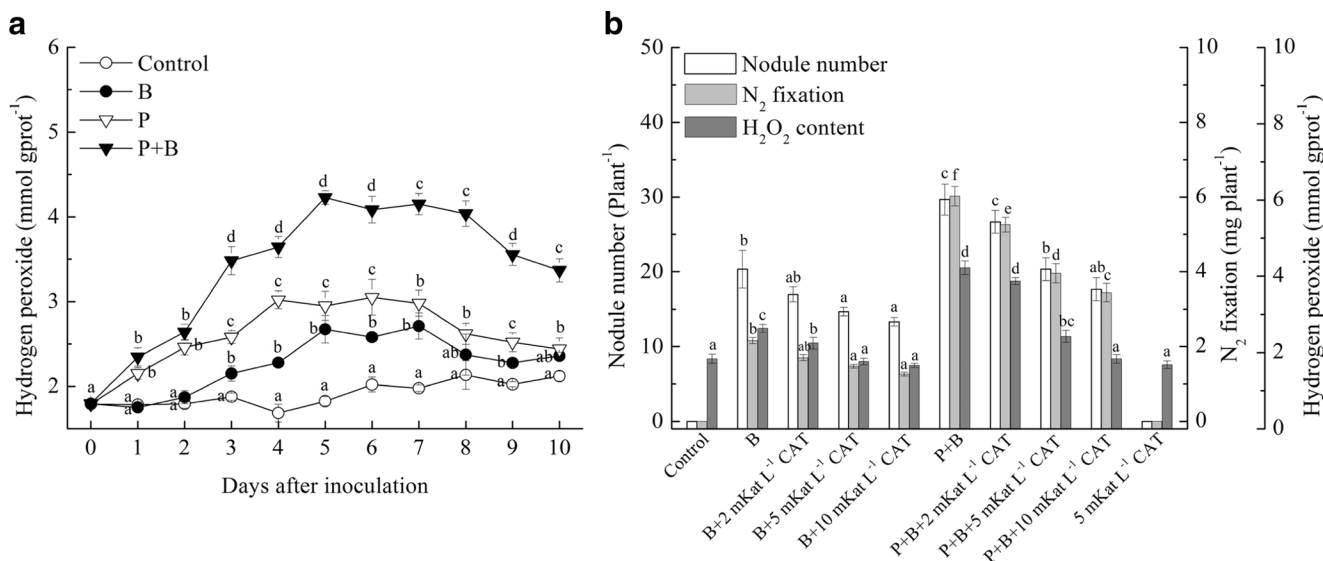
Figure 2a shows that compared with the control, the endogenous H<sub>2</sub>O<sub>2</sub> content in peanut roots increased significantly over time after *Bradyrhizobium* inoculation (B). Further analysis revealed that *P. liquidambari*-infected peanuts (P) consistently had a higher H<sub>2</sub>O<sub>2</sub> content than peanuts that received the B treatment. After 3 days of inoculation, a significant difference in H<sub>2</sub>O<sub>2</sub> content was observed between P + B and the other three treatments, and the highest H<sub>2</sub>O<sub>2</sub> levels occurred from the 5- to 7-day sampling time. This result demonstrated that *P. liquidambari* and *Bradyrhizobium* co-inoculation could further induce the generation of H<sub>2</sub>O<sub>2</sub> in peanut roots. However, after 7 days from inoculation, the levels of H<sub>2</sub>O<sub>2</sub> in the B, P and P + B treatments all gradually declined with longer sampling time.

As shown in Fig. 2b, the addition of H<sub>2</sub>O<sub>2</sub> scavenger (CAT) suppressed not only *Bradyrhizobium*-induced H<sub>2</sub>O<sub>2</sub> production but also *Bradyrhizobium*-elicited nodulation

**Table 1** Peanut nodulation and N<sub>2</sub> fixation parameter analysis

Treatment	Nodule primordium number (plant <sup>-1</sup> )	Nodule number (plant <sup>-1</sup> )	Individual nodule weight (mg nodule <sup>-1</sup> )	Individual nodule size (mm)	Total nodule weight (mg plant <sup>-1</sup> )	Shoot N content (mg plant <sup>-1</sup> )	N <sub>2</sub> fixation (mg plant <sup>-1</sup> )	Shoot dry weight (g plant <sup>-1</sup> )	Root dry weight (g plant <sup>-1</sup> )
Control	—	—	—	—	—	6.2 ± 0.14a	—	0.58 ± 0.05a	0.23 ± 0.03a
B	11 ± 1a	21.33 ± 3.06a	1.7 ± 0.02a	2.08 ± 0.04a	36.21 ± 5.35a	8.35 ± 0.07b	2.16 ± 0.07a	0.69 ± 0.06ab	0.27 ± 0.02ab
P	—	—	—	—	—	6.14 ± 0.28a	—	0.66 ± 0.04ab	0.3 ± 0.03bc
P + B	16.67 ± 1.53b	29.67 ± 1.53b	1.84 ± 0.02b	2.23 ± 0.07b	54.67 ± 2.51b	12.13 ± 0.67c	5.93 ± 0.81b	0.76 ± 0.02b	0.36 ± 0.02c

Values represent mean ± standard deviation. Different lowercase letters within a column indicate significant differences ( $p < 0.05$ )

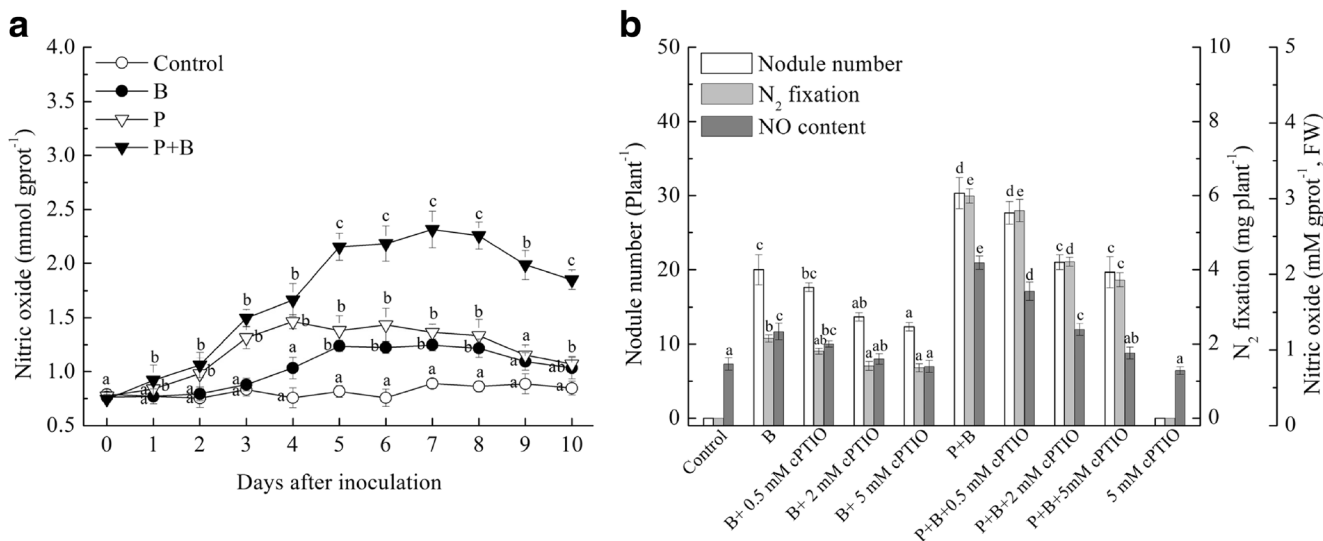


**Fig. 2** Involvement of H<sub>2</sub>O<sub>2</sub> in *Bradyrhizobium*-induced and *P. liquidambari*-enhanced nodulation and N<sub>2</sub> fixation in peanut. **a** *Bradyrhizobium*- and *P. liquidambari*-induced H<sub>2</sub>O<sub>2</sub> production in peanut. **b** Effects of CAT (H<sub>2</sub>O<sub>2</sub> scavenger) on *Bradyrhizobium*-induced and *P. liquidambari*-enhanced H<sub>2</sub>O<sub>2</sub>, nodulation and N<sub>2</sub>

fixation. Values are the means of three independent experiments ± SD. The points on the line graph with different letters indicate significant differences between different treatments at the same sampling time. The data in columns marked by different letters are significantly different between the different treatments (*p* < 0.05)

and N<sub>2</sub> fixation. Nodulation and N<sub>2</sub> fixation in peanuts treated with 2, 5 and 10 mKat L<sup>-1</sup> CAT were 16.39, 27.87 and 34.43% (nodulation) and 20.99, 31.79 and 41.51% (N<sub>2</sub> fixation) lower, respectively, than those in the groups treated with *Bradyrhizobium* alone. A similar phenomenon was also observed in the experimental groups derived from P + B treatment, and the application of 2, 5 and 10 mKat L<sup>-1</sup> CAT also significantly reduced

peanut nodulation and N<sub>2</sub> fixation by 10.11, 31.46 and 40.45% (nodulation) and 12.62, 34.27 and 42.8% (N<sub>2</sub> fixation) compared with the P + B treatment. Therefore, in this study, 5 mKat L<sup>-1</sup> CAT was chosen for the follow-up experiments. The results indicated that the generation of H<sub>2</sub>O<sub>2</sub> indeed played a non-negligible role in *Bradyrhizobium*-elicited and *P. liquidambari*-enhanced peanut nodulation and N<sub>2</sub> fixation.



**Fig. 3** Involvement of NO in *Bradyrhizobium*-induced and *P. liquidambari*-enhanced nodulation and N<sub>2</sub> fixation in peanut. **a** *Bradyrhizobium*- and *P. liquidambari*-induced NO production in peanut. **b** Effects of cPTIO (NO scavenger) on *Bradyrhizobium*-induced and *P. liquidambari*-enhanced NO, nodulation and N<sub>2</sub> fixation.

Values are the means of three independent experiments ± SD. The points on the line graph with different letters indicate significant differences between different treatments at the same sampling time. Data in columns marked by different letters are significantly different between the different treatments (*p* < 0.05)

### Involvement of the Increase in NO in *P. liquidambari*-Induced Nodulation and N<sub>2</sub> Fixation Enhancement

As shown in Fig. 3a, inoculation with *Bradyrhizobium* alone (B) significantly induced the biosynthesis of NO in peanut roots, and elevated NO was observed from 5 to 7 days. Similarly, *P. liquidambari* infection (P) also triggered the production of endogenous NO, while the content of NO in response to P was always relatively higher than that in peanuts that received only B treatment. The highest NO content was observed in co-inoculated peanuts (P + B), which showed significant differences from the other three treatments after 5 days, suggesting that the co-inoculation could further improve the generation of NO. Additionally, after 7 days of incubation, the NO contents all gradually decreased in the B, P and P + B treatments.

Figure 3b shows that NO scavenger (cPTIO) suppressed NO accumulation in peanut roots, and the nodulation and N<sub>2</sub> fixation in the B + 2 mM cPTIO and B + 5 mM cPTIO treatments were both significantly decreased by 31.67 and 38.33% (nodulation) and 34.57 and 36.9% (N<sub>2</sub> fixation), respectively, compared with the B treatment. A similar trend was observed in the P + B treatments supplemented with cPTIO; the application of 2 and 5 mM cPTIO significantly reduced nodulation by 30.77 and 35.16% and N<sub>2</sub> fixation by 29.66 and 37.67% compared to the P + B treatment. Therefore, 2 mM cPTIO was used for the follow-up experiments. These data implied that the enhancement of peanut nodulation and N<sub>2</sub> fixation induced by *P. liquidambari* infection likely depends on the improvement of endogenous H<sub>2</sub>O<sub>2</sub> and NO biosynthesis in peanut roots.

### Interaction Between H<sub>2</sub>O<sub>2</sub> and NO Biosynthesis in *P. liquidambari*-Induced Nodulation and N<sub>2</sub> Fixation Enhancement

The results obtained above indicated that H<sub>2</sub>O<sub>2</sub> and NO biosynthesis are involved in *Bradyrhizobium*-induced or *P. liquidambari*-stimulated peanut nodulation and N<sub>2</sub> fixation; however, their relationship and involvement in this process remained unclear. As shown in Fig. 4a, b, in the B and P + B treatments, *Bradyrhizobium*-induced or *P. liquidambari*-stimulated NO generation was strongly inhibited by the H<sub>2</sub>O<sub>2</sub> scavenger CAT, whereas the NO scavenger (cPTIO) did not significantly affect *Bradyrhizobium*-induced or *P. liquidambari*-stimulated H<sub>2</sub>O<sub>2</sub> production, indicating that the H<sub>2</sub>O<sub>2</sub> and NO signalling pathways were closely linked and that H<sub>2</sub>O<sub>2</sub> might act as an upstream signalling molecule during *Bradyrhizobium*-induced or *P. liquidambari*-stimulated NO biosynthesis. In addition, the application of exogenous H<sub>2</sub>O<sub>2</sub> and SNP could reverse the inhibition of *Bradyrhizobium*-induced or *P. liquidambari*-stimulated

peanut nodulation and N<sub>2</sub> fixation by CAT and cPTIO, which further confirmed the results described above.

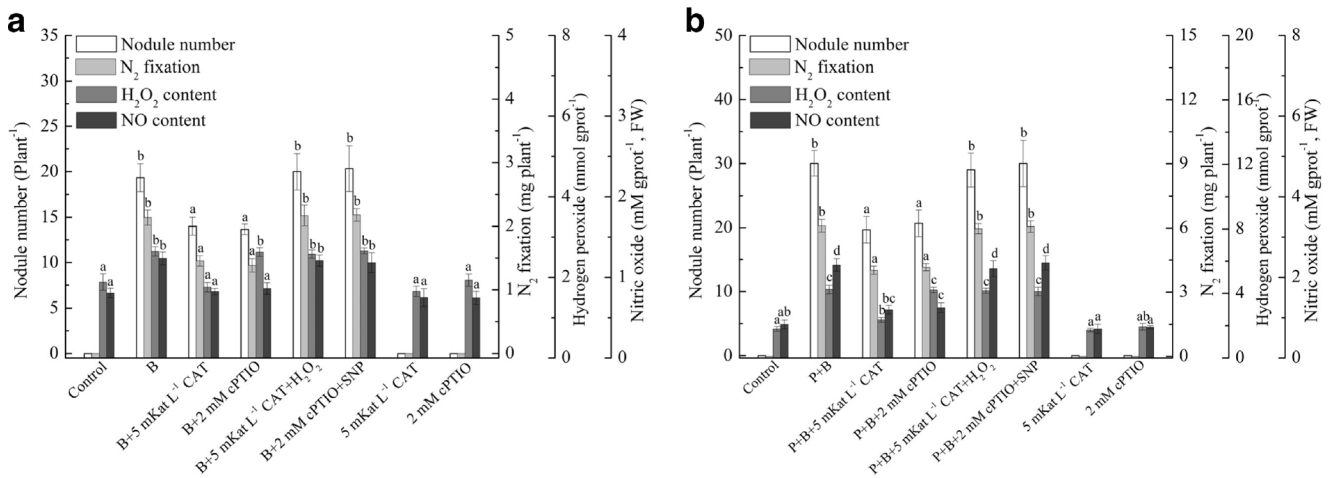
### *P. liquidambari*-Induced NO Dependence on H<sub>2</sub>O<sub>2</sub> Production

The results shown in Fig. 5a indicated that in the group inoculated with *Bradyrhizobium* alone, both exogenous H<sub>2</sub>O<sub>2</sub> and SNP significantly increased nodulation and N<sub>2</sub> fixation. The increased nodulation and N<sub>2</sub> fixation produced by H<sub>2</sub>O<sub>2</sub> were completely inhibited by cPTIO, whereas the enhancement induced by SNP remained almost unaffected by CAT. Exogenous H<sub>2</sub>O<sub>2</sub> could promote NO generation, but the application of SNP did not influence H<sub>2</sub>O<sub>2</sub>. However, compared with the P + B treatment (Fig. 5b), exogenous H<sub>2</sub>O<sub>2</sub> addition enhanced the levels of endogenous H<sub>2</sub>O<sub>2</sub> and NO but significantly decreased nodulation and N<sub>2</sub> fixation, while SNP addition had no significant differential effect on nodulation and N<sub>2</sub> fixation between the P + B and P + B + SNP groups. In addition, the application of cPTIO significantly inhibited nodulation and N<sub>2</sub> fixation in the P + B + H<sub>2</sub>O<sub>2</sub> group, whereas in the P + B + SNP group, CAT did not appear to have any effect. Further analysis also indicated that H<sub>2</sub>O<sub>2</sub> stimulated NO generation, but no impact on H<sub>2</sub>O<sub>2</sub> was observed in response to exogenous SNP for the P + B treatment. These findings further demonstrated that H<sub>2</sub>O<sub>2</sub> may act upstream of NO production in *P. liquidambari*-induced nodulation and N<sub>2</sub> fixation; however, excessive H<sub>2</sub>O<sub>2</sub> accumulation could have unfavourable effects on peanut nodulation and N<sub>2</sub> fixation.

### Transcriptional Responses of the Symbiosis-Related Genes *SymRK* and *CCaMK* to Different Experimental Treatments

As shown in Fig. 6a, following *Bradyrhizobium*-only infection, exogenous H<sub>2</sub>O<sub>2</sub> and SNP significantly up-regulated the transcription of *SymRK* and *CCaMK*, which were decreased following the application of CAT and cPTIO. H<sub>2</sub>O<sub>2</sub> supplementation could alleviate this inhibition by CAT but did not affect cPTIO inhibition. However, SNP supplementation reversed the transcriptional suppression of *SymRK* and *CCaMK* produced by both CAT and cPTIO. For the P + B treatment (Fig. 6b), exogenous H<sub>2</sub>O<sub>2</sub> down-regulated the transcription of *SymRK* and *CCaMK*, but no significant influence was observed for SNP addition. Both CAT and cPTIO decreased the expression levels of *SymRK* and *CCaMK*. Similarly, exogenous H<sub>2</sub>O<sub>2</sub> reversed the suppression produced by CAT, but no obvious effect was observed following cPTIO addition. Additionally, exogenous SNP supplementation could significantly up-regulate the expression levels of *SymRK* and *CCaMK* in response to CAT and cPTIO inhibition. These findings were consistent with the observations described





**Fig. 4** Interactions between H<sub>2</sub>O<sub>2</sub> and NO signalling pathways for *Bradyrhizobium*-induced (a) and *P. liquidambari*-enhanced (b) nodulation and N<sub>2</sub> fixation in peanut. Values are the means of three

independent experiments ± SD. Data in columns marked by different letters are significantly different between the different treatments (*p* < 0.05)

above. Additionally, *P. liquidambari*-only infection did not affect the transcription of *SymRK* and *CCaMK*.

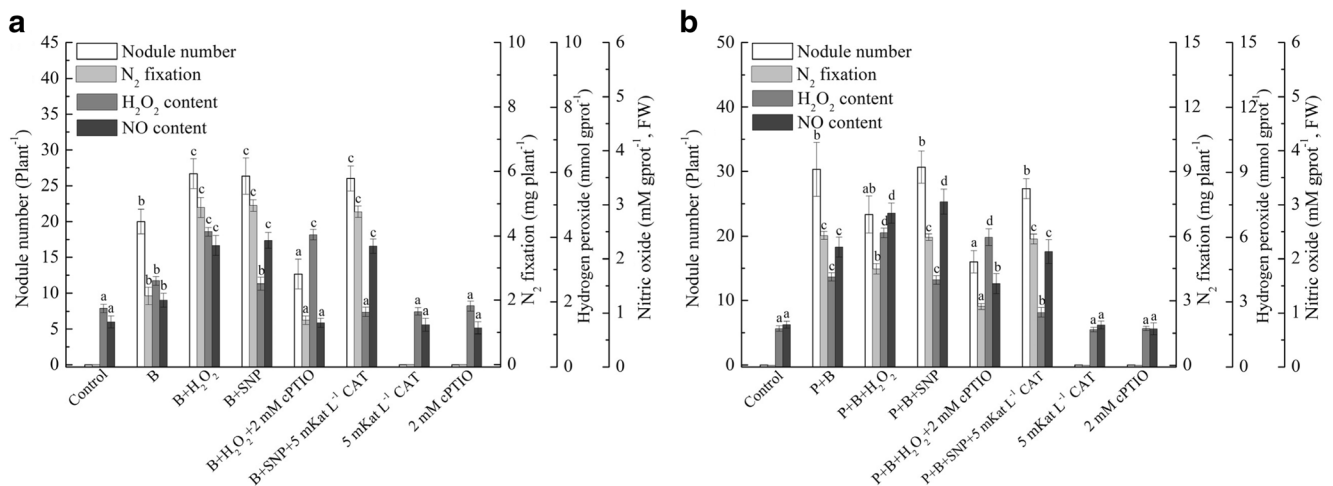
***P. liquidambari*-Induced Nodulation and N<sub>2</sub> Fixation Increase via Enhancement of the H<sub>2</sub>O<sub>2</sub> and NO Signalling Pathway Under Pot Conditions**

In the pot experiment, *P. liquidambari* inoculation significantly increased peanut nodule primordium formation, nodulation and N<sub>2</sub> fixation, and exogenous H<sub>2</sub>O<sub>2</sub> and SNP could effectively reverse the inhibition of nodulation and N<sub>2</sub> fixation produced by CAT and cPTIO, respectively (Fig. 7a). Figure 7b shows that the levels of H<sub>2</sub>O<sub>2</sub>, NO and *SymRK* and *CCaMK* transcription were all significantly increased after *P. liquidambari* infection. Exogenous CAT slightly inhibited *P. liquidambari*-stimulated H<sub>2</sub>O<sub>2</sub> and NO, while cPTIO reduced NO generation but did not affect H<sub>2</sub>O<sub>2</sub> generation. Moreover, both CAT and cPTIO effectively down-

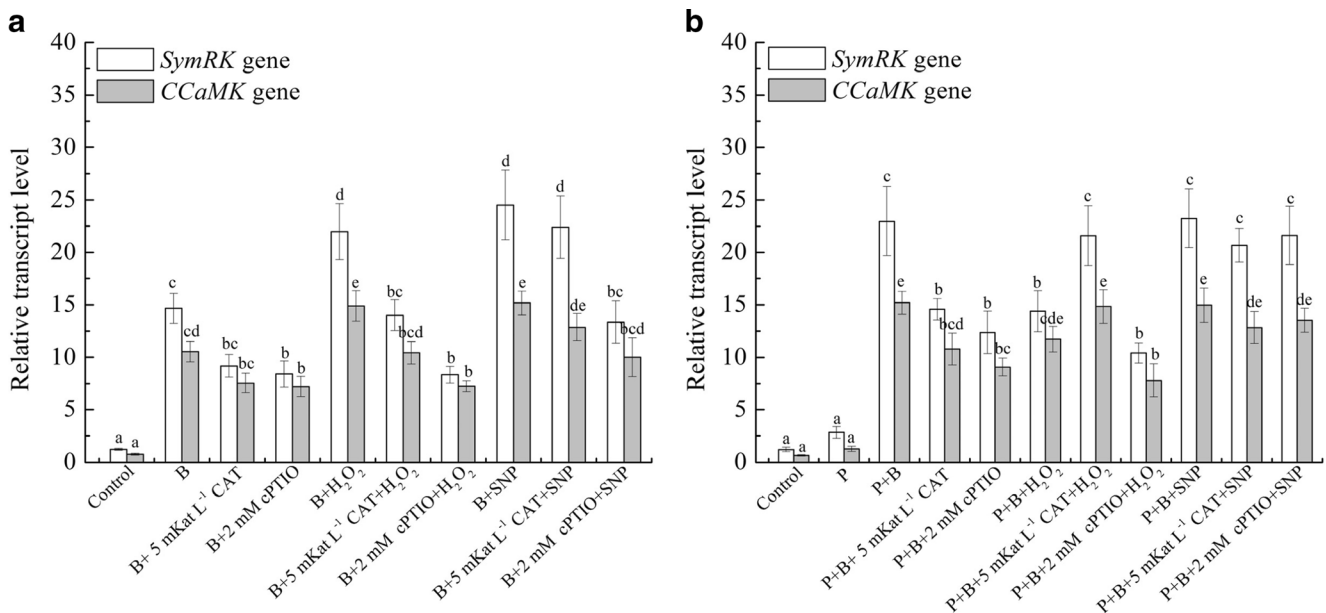
regulated *SymRK* and *CCaMK* gene expression. However, exogenous H<sub>2</sub>O<sub>2</sub> and SNP not only reversed the inhibition of H<sub>2</sub>O<sub>2</sub> and NO production by CAT and cPTIO but also clearly up-regulated the transcription of *SymRK* and *CCaMK*. This further demonstrated that the *P. liquidambari*-induced H<sub>2</sub>O<sub>2</sub> and NO signalling pathway improved the peanut–*Bradyrhizobium* interaction, ultimately resulting in increased peanut nodulation and N<sub>2</sub> fixation.

**Discussion**

The use of beneficial microorganisms to increase leguminous plant nodulation and N<sub>2</sub> fixation is a promising strategy and supplies an irreplaceable advantage over other conventional methods [8, 32–35]. Garg and Pandey [35] showed a positive correlation between arbuscular mycorrhizal fungal (AMF) colonisation and the increased legume–rhizobium symbiosis,



**Fig. 5** *Bradyrhizobium*-induced (a) and *P. liquidambari*-enhanced (b) NO dependence on H<sub>2</sub>O<sub>2</sub> production in peanut. Values are the means ± SD of three independent experiments. Data in columns marked by different letters are significantly different between the different treatments (*p* < 0.05)

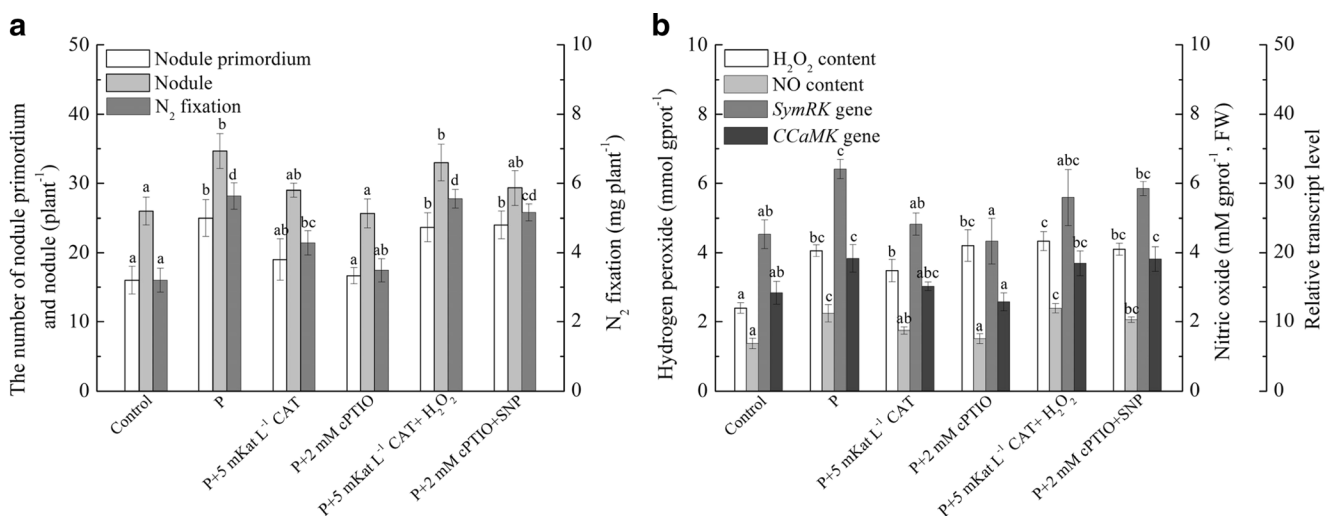


**Fig. 6** The transcriptional activities of the symbiosis-related genes *SymRK* and *CCaMK* during *Bradyrhizobium*-induced (a) and *P. liquidambari*-enhanced (b) nodulation and  $N_2$  fixation in peanuts.

Values are the means  $\pm$  SD of three independent experiments. Data in columns marked by different letters are significantly different between the different treatments ( $p < 0.05$ )

nodulation and  $N_2$  fixation. Therefore, efficient infection or colonization is pivotal for the ability of exogenous microorganisms to exert their potential ecological functions [8, 27, 35, 36]. Compared with our previous study [8], this study further indicated that endophytic fungus *P. liquidambari* could successfully infect and colonise peanut roots in a relative short incubation time. *P. liquidambari* infection effectively increased individual nodule weight and size and total nodule weight, implying that the improvement of nodule characteristics should be responsible for increasing the peanut  $N_2$  fixation capacity. Although many studies have demonstrated that some bacteria, fungi and other rhizobial helper microbes could

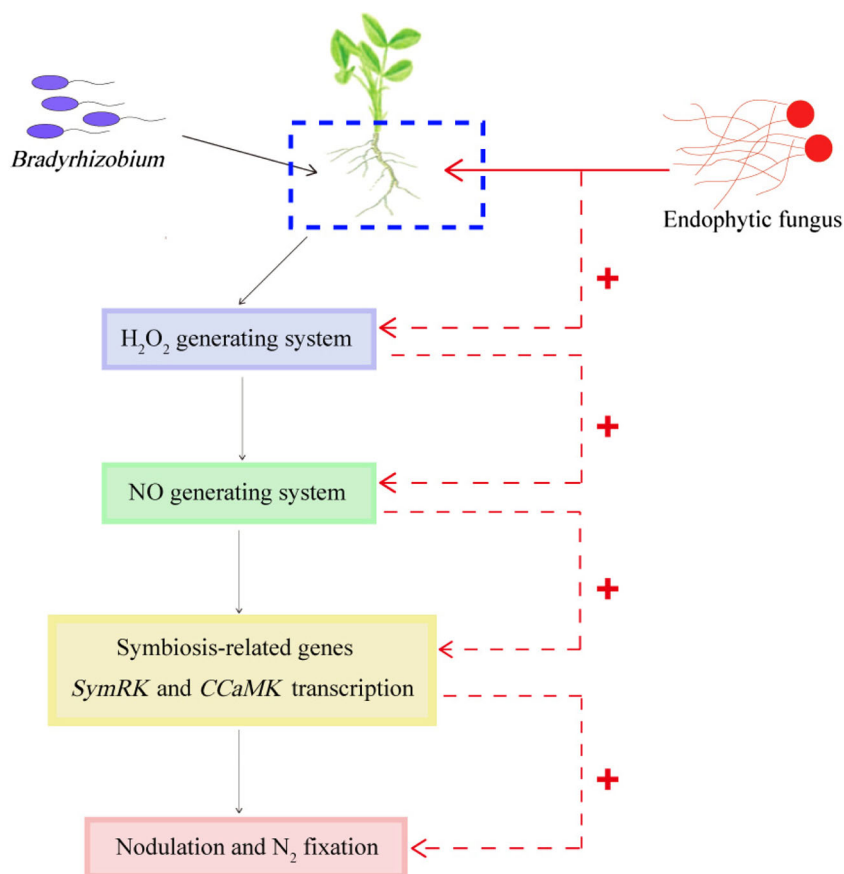
significantly increase plant nodulation and  $N_2$  fixation [32, 33, 35, 37–39], little information was available concerning the impact of endophytes on legume–rhizobium symbiosis interaction. Several studies have suggested the positive effects of endophytic actinomycetes *Streptomyces* sp. based on their ability to influence legume nodules by increasing the root-nodulating frequency [34, 40–42]. Subramanian et al. [43] demonstrated that plant endophytic bacteria *Bacillus megaterium* LNL6 isolated from the root nodules of *Lespedeza* sp. and endophytic bacteria isolated from rice leaves significantly improved nodule function and  $N_2$  fixation in soybean co-inoculated with *Bradyrhizobium japonicum*



**Fig. 7** Effects of CAT, cPTIO and exogenous  $H_2O_2$  and SNP on *P. liquidambari*-induced nodulation and  $N_2$  fixation,  $H_2O_2$  and NO, and *SymRK* and *CCaMK* transcription under pot conditions. Values are the

means  $\pm$  SD of three independent experiments. Data in columns marked by different letters are significantly different between the different treatments ( $p < 0.05$ )

**Fig. 8** A model in which *P. liquidambari* infection enhances H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk in peanut, resulting in an increase in peanut–*Bradyrhizobium* interactions, nodulation and N<sub>2</sub> fixation. The symbol “+” represents a positive effect on peanut–*Bradyrhizobium* interactions in response to *P. liquidambari* infection in peanut roots



MN110. In comparison to these studies, we first reported that an endophytic fungus has the potential to increase peanut–*Bradyrhizobium* interactions, and *P. liquidambari* appear to provide improved nodulation and N<sub>2</sub> fixation activity than those previously reported microbes.

The signalling crosstalk between H<sub>2</sub>O<sub>2</sub> and NO appears to be considered an essential factor that allows plants to adapt to complicated external conditions and respond to different biotic and abiotic stresses [16–19, 44]. H<sub>2</sub>O<sub>2</sub> may act as a cofactor to promote endogenous NO synthesis [45, 46]. However, NO may also induce H<sub>2</sub>O<sub>2</sub> production [47, 48]. Therefore, the forms of signalling crosstalk between H<sub>2</sub>O<sub>2</sub> and NO are varied and mainly depend on the plants and the external stresses. Although an increasing number of studies have demonstrated that H<sub>2</sub>O<sub>2</sub> and NO may have important functions in legume–rhizobium symbiosis, nodulation and N<sub>2</sub> fixation [15–20], no studies have investigated the relationship between increased nodulation and the regulation of H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk by co-inoculation with beneficial microorganisms.

To date, research investigating the important roles of H<sub>2</sub>O<sub>2</sub> and NO during legume–*Rhizobium* symbiosis and nodulation have mainly focused on the pair-wise interactions between hosts and their corresponding symbiotic microorganisms [16, 19] while overlooking the interplay among multiple endosymbionts and their combined effects on host plants.

Regarding N<sub>2</sub> fixing interactions, several studies have also shown that co-inoculation with symbiotic microorganisms (AMF or endophytes) can effectively increase legume–*Rhizobium* symbiosis, nodulation and N<sub>2</sub> fixation [33, 35, 37, 41–43, 49]. However, the detailed mechanisms underlying symbiosis interaction by multiple endosymbionts, and the involvement of H<sub>2</sub>O<sub>2</sub> and NO crosstalk in this process had not been previously assessed. The present findings demonstrated that enhanced H<sub>2</sub>O<sub>2</sub> and NO signalling are involved in *P. liquidambari*-induced peanut–*Bradyrhizobium* symbiosis, nodulation and N<sub>2</sub> fixation. To our knowledge, this is the first report to demonstrate the enhanced signalling crosstalk between H<sub>2</sub>O<sub>2</sub> and NO in endophytic fungal promotion of peanut–*Bradyrhizobium* interactions. However, we also observed that excessive H<sub>2</sub>O<sub>2</sub> accumulation may induce oxidative damage in roots, resulting in unfavourable conditions for nodulation and N<sub>2</sub> fixation.

In this study, compared with only *Bradyrhizobium* inoculation, *P. liquidambari*-infected peanuts consistently had a higher H<sub>2</sub>O<sub>2</sub> and NO contents in roots, which suggested that *P. liquidambari* infection significantly improved the production of H<sub>2</sub>O<sub>2</sub> and NO. Many studies showed that inoculation with AMF could significantly increase H<sub>2</sub>O<sub>2</sub> and NO accumulations in the early stages of AMF–host interactions [16, 24, 50], which played an important role involved in symbiosis,

growth promotion, defence reaction and secondary metabolite synthesis. Our previous studies indicated that endophytic fungus- and bacterium-triggered H<sub>2</sub>O<sub>2</sub> and NO could directly increase total volatile oil accumulation and oxygenous sesquiterpenoid diversity in *A. lancea* [22, 51]. Therefore, we believe that the improvement of H<sub>2</sub>O<sub>2</sub> and NO production in peanut roots may be due to the induction of systemic acquired resistance responded to endophytic fungus *P. liquidambari* infection; thus, the elevated H<sub>2</sub>O<sub>2</sub> and NO can be used to enhance peanut–*Bradyrhizobium* interactions. In addition to responding *P. liquidambari*-infected stress, the stimulated H<sub>2</sub>O<sub>2</sub> and NO might be considered as important signalling pathway to mediate peanut growth promotion, secondary metabolite production and even the other potential ecological functions produced from *P. liquidambari*. Therefore, the detailed action mechanisms for enhancing H<sub>2</sub>O<sub>2</sub> and NO biosynthesis in peanut roots with *P. liquidambari* infection need further investigation.

Many studies have demonstrated that H<sub>2</sub>O<sub>2</sub> and NO generation have important functions in regulating the expression of some symbiosis-related genes during the early stages of the legume–*Rhizobium* interaction, which are important for initiating the subsequent *Rhizobium* infection and formation of the nodule primordium [15, 16, 18, 52]. Regarding the symbiotic interaction between legumes and their cognate *Rhizobium* symbionts, Arthikala et al. [17] suggest that the symbiotic signals are transduced by the so-called *Rhizobium* symbiosis pathway, in which the symbiotic marker genes *SymRK* and *CCaMK* participate in the early signalling pathway that recognizes *Rhizobium* endosymbionts and further induces downstream signalling events to regulate the symbiotic process. The study reported by Sinharoy et al. [30] demonstrated that the early stages of the symbiotic peanut–*Bradyrhizobium* interaction are also regulated by the symbiotic marker genes *SymRK* and *CCaMK*. Therefore, the transcriptional activities of *SymRK* and *CCaMK* can directly reflect the interaction strength between peanut and *Bradyrhizobium* symbiosis. In this study, *P. liquidambari* co-inoculation significantly increased transcription of the *SymRK* and *CCaMK* genes, consistent with the enhanced peanut nodulation and N<sub>2</sub> fixation. However, the enhanced *SymRK* and *CCaMK* transcription was completely abolished by specific H<sub>2</sub>O<sub>2</sub> and NO scavengers, and exogenous supplementation of H<sub>2</sub>O<sub>2</sub> and SNP could effectively reverse their transcriptions. Therefore, these results further confirm that the enhanced H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk in response to *P. liquidambari* infection might be responsible for the increased symbiotic peanut–*Bradyrhizobium* interaction. In addition to *SymRK* and *CCaMK* genes, some other symbiosis-related genes, such as *CASTOR*, *POLLUX*, *NUP85* and *NUP133*, which are also required and may be significantly regulated by H<sub>2</sub>O<sub>2</sub> and NO during the early stages of the legume–*Rhizobium* interaction [15, 17, 18]. However, no information is available about

the functions of these symbiosis-related genes or their homologous genes in the peanut–*Bradyrhizobium* symbiosis interaction. Therefore, more studies are needed to further investigate those genes involved in the early peanut–*Bradyrhizobium* symbiosis interaction and their possible relationships with enhanced H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk.

Although endophytic fungi are broad-spectrum endosymbionts that are widely distributed in various hosts, very few studies have investigated improvements in legume nodulation and N<sub>2</sub> fixation in response to endophytic fungal infection. As an environmentally adaptive endophytic fungus, *P. liquidambari* can efficiently improve soil biochemical and microbiological properties, which could provide a beneficial habitat for plant–microbe interactions [28, 53–56]. As a symbiotic fungus, *P. liquidambari* has been observed to significantly increase nitrogen uptake and accumulation in rice (*Oryza sativa* L.) by establishing a symbiotic system [27, 36, 56]. The discovery of the ability of *P. liquidambari* to increase peanut nodulation and N<sub>2</sub> fixation may provide a new research field to elaborate novel ecological functions of endophytic fungi. The controlled pot experiment further confirmed that the increase in peanut nodulation and N<sub>2</sub> fixation mainly resulted from the enhancement of H<sub>2</sub>O<sub>2</sub> and NO signalling crosstalk induced by *P. liquidambari* (Fig. 8). Additionally, exogenous H<sub>2</sub>O<sub>2</sub> and SNP addition effectively increased nodulation and N<sub>2</sub> fixation. Therefore, we speculate that for practical agricultural production, direct application of H<sub>2</sub>O<sub>2</sub> or SNP can improve symbiotic peanut–*Bradyrhizobium* interactions. However, this effect requires further investigation because of the presence of various factors in the natural agri-ecosystem. In contrast, based on the results of our previous studies and in this study, we believe that *P. liquidambari* can be directly used as an endosymbiotic elicitor to alleviate the long-term continuous cropping obstacles associated with the peanut monoculture field by facilitating the peanut–*Bradyrhizobium* interaction, nodulation and N<sub>2</sub> fixation. However, additional research is needed to further understand the detailed molecular regulation of the peanut–*Bradyrhizobium* interaction in response to *P. liquidambari*-induced H<sub>2</sub>O<sub>2</sub> and NO.

## Conclusions

Consecutive monoculturing of the peanut has seriously affected its yield and quality. Different measures used for improving peanut production should be explored to ensure sustainable development of the peanut industry. In this work, we demonstrated that infection by the endophytic fungus *P. liquidambari* significantly increased H<sub>2</sub>O<sub>2</sub> and NO generation compared with *Bradyrhizobium* inoculation alone. Specific H<sub>2</sub>O<sub>2</sub> and NO scavengers and exogenous H<sub>2</sub>O<sub>2</sub> and SNP supplementation experiments showed that *P. liquidambari*-induced



increases in peanut nodulation and N<sub>2</sub> fixation were attributed to enhanced signalling crosstalk between H<sub>2</sub>O<sub>2</sub> and NO. The transcriptional levels of the symbiosis-related genes *SymRK* and *CCaMK* also increased significantly in response to *P. liquidambari* infection. The pot experiment further confirmed that the *P. liquidambari* infection-enhanced H<sub>2</sub>O<sub>2</sub> and NO signalling pathway was responsible for the increased symbiotic peanut–*Bradyrhizobium* interaction, which was required for improving nodulation and N<sub>2</sub> fixation. This is the first study to report that *P. liquidambari* acts as an endophytic elicitor to increase peanut–*Bradyrhizobium* interactions via enhanced H<sub>2</sub>O<sub>2</sub>/NO-dependent signalling crosstalk, alleviating the long-term continuous cropping obstacles associated with peanut by increasing nodulation and N<sub>2</sub> fixation.

**Acknowledgements** We would like to acknowledge the National Natural Science Foundation of China (NSFC No. 31370507), the Ph.D. Programs Foundation of Ministry of Education of China (No. 20133207110001), the Major Natural Science Research Programs of Jiangsu Higher Education Institutions (No. 13KJA180003), the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions of China, and the Graduate Education Innovation Project of Jiangsu Province (KYZZ15\_0215). We also express our great thanks to the anonymous reviewers and editorial staff for their time and attention.

#### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Research Involving Human Participants and/or Animals** This article does not contain any studies with human participants or animals performed by any of the authors.

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