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An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae)

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Abstract

Germination of orchid seeds fully depends on a symbiotic association with soil-borne fungi, usually *Rhizoctonia* spp. In contrast to the peaceful symbiotic associations between many other terrestrial plants and mycorrhizal fungi, this association is a life-and-death struggle. The fungi always try to invade the cytoplasm of orchid cells to obtain nutritional compounds. On the other hand, the orchid cells restrict the growth of the infecting hyphae and obtain nutrition by digesting them. It is likely that antifungal compounds are involved in the restriction of fungal growth. Two antifungal compounds, lusianthrin and chrysin, were isolated from the seedlings of *Cypripedium macranthos* var. *rebunense* that had developed shoots. The former had a slightly stronger antifungal activity than the latter, and the antifungal spectra of these compounds were relatively specific to the nonpathogenic *Rhizoctonia* spp. The level of lusianthrin, which was very low in aseptic protocorm-like bodies, dramatically increased following infection with the symbiotic fungus. In contrast, chrysin was not detected in infected protocorm-like bodies. These results suggest that orchid plants equip multiple antifungal compounds and use them at specific developmental stages; lusianthrin maintains the perilous symbiotic association for germination and chrysin helps to protect adult plants.

Keywords: *Cypripedium macranthos* var. *rebunense*; Orchidaceae; Antifungal compounds; Symbiotic germination; Chrysin; Lusianthrin

1. Introduction

Symbiosis with soil-borne fungi is indispensable for germination of orchid seeds, which contain insufficient stores of substances required for germination. In contrast to the peaceful and sustainable symbiotic associations between many other terrestrial plants and mycorrhizal fungi, where the fungi accept photosynthetic products from host plants in exchange for nutritional salts and water, the association between orchids and fungi is strictly a life-and-death struggle. The hyphae of fungi always try to invade the cytoplasm of orchid cells to obtain nutritional compounds, as in a saprophytic infection. On the other hand, orchid cells inhibit the growth of the infecting hyphae to keep them in the apoplast, enclosed by plasma membranes (Peterson et al., 1996), and make the mycelium form dense coils called pelotons. In the developing protocorm, the pelotons are limited to cortical cells and are not found in the apical meristem (Peterson et al., 1998; Shimura and Koda, 2005). Orchid cells appear to obtain nutritional compounds by digesting pelotons through secretion of enzymes (Blakeman et al., 1976). When orchids cells fail to control fungal growth, the fungi prevail and prey on the developing protocorms. In contrast, when the orchids exhibit an excessive resistant response, a hypersensitive reaction occurs and the fungus fails to grow inside the protocorms (Hadley, 1970). In such cases, the developing protocorms starve. Thus, germination of orchid seeds requires quite a delicate balance.

Orchid plants appear to use both chemical and physical devices to control invasion by symbiotic fungi. Thickening

Abbreviations: PLBs, protocorm like bodies.

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of the cell wall and deposition of phenolic compounds seem to act as physical barriers (Beyrle et al., 1995). Blakeman et al. (1976) hypothesized that an increase in oxidative enzyme activity upon fungal infection, which was similar to the oxidative activity of plants against pathogens, may play a role in the defense response of the orchid. As more clear-cut chemical devices, some antifungal compounds are thought to be involved in the control of fungal growth (Hadley, 1982). Although some antifungal compounds (phytoalexin) have been found in adult orchid plants (Fisch et al., 1973; Ward et al., 1975), their role in regulation of fungal growth during symbiotic seed germination remains to be elucidated. Beyrle et al. (1995) reported that a small amount of an antifungal compound was present in aseptic protocorms of *Orchis morio*, but the amount did not change with the inoculation of a symbiotic fungus, suggesting that this compound is not involved in regulating this symbiotic association during germination.

Cypripedium macranthos var. *rebunense*, a kind of lady-slipper orchid, is one of the most famous wild orchids in Japan, because it has become a symbol of threatened-plant conservation. In 1994, the "Law for the Conservation of Endangered Species of Wild Fauna and Flora, Japan" designated this variety as a domestic threatened species, and collection from natural habitats was strictly prohibited. Recently, we have developed two protocols for the micropropagation of this variety: by aseptic culture of protocorm-like bodies (PLBs) (Shimura and Koda, 2004), and by symbiotic germination with a symbiotic fungus (Shimura and Koda, 2005). In the latter study, we found that both nutritional deficiency and a lengthy culture period at a high temperature contribute to loss of the symbiotic association, and as a consequence, the protocorms turn brown and rot. These results suggest that thermal stress and nutrient deficiency reduce the production of antifungal compounds and as a result the protocorms fail to control the invasiveness of the fungus. Some of the characteristics of the symbiotic fungus (designated WO-97) were similar to those of *Rhizoctonia repens* (perfect stage; *Tulasnella* spp), which is known to be a symbiotic fungus for many orchid species. However, the sequence homology of 5.8S rDNA was less than 60% (unpublished data), suggesting that this fungus is different from *R. repens*.

In this paper, we describe the isolation and structural elucidation of antifungal compounds from plantlets of this orchid, and discuss the involvement of these compounds in symbiotic germination.

2. Results and discussion

Ethanol extract of plantlets was sub-fractionated into *n*-hexane, EtOAc, butanol, and aqueous soluble fractions. Each solvent fraction obtained from plantlets (equivalent to 3 g fresh weight) was added to medium and their antifungal activities against WO-97 were examined. The EtOAc fraction showed the strongest antifungal activity,

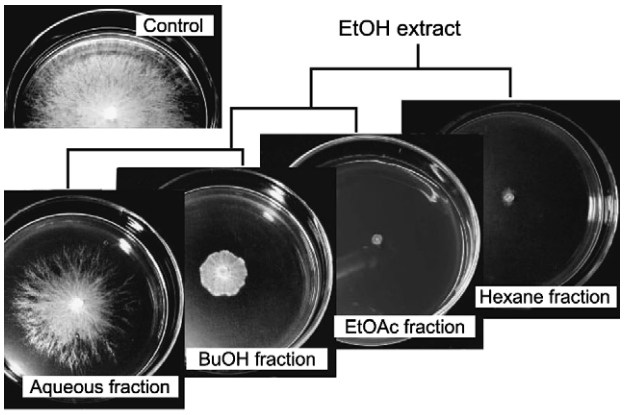
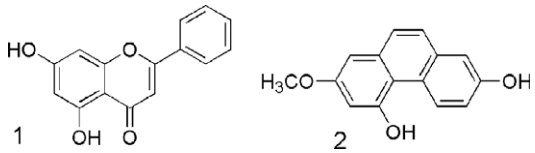


Fig. 1. Antifungal activities of solvent fractions obtained from plantlets of *Cypripedium macranthos* var. *rebunense* against its symbiotic fungus, WO-97. Fractions (each equivalent to 3 g fr wt) were added to 10 ml of assay medium. WO-97 was inoculated onto the medium and cultured for 4 weeks.

and the fungus failed to grow completely (Fig. 1). Considerable activity was also found in the *n*-hexane fraction.

The EtOAc fraction was subjected to further bioassay-guided purification based on the presence of antifungal activity. The activity was separated into two fractions after chromatography on a Sephadex LH-20 column, and the fractions were designated A and B in the order of elution. Fraction A was subjected to TLC and silica gel column chromatography because it showed low solubility in MeOH. One mg of active compound was obtained from fraction A. The ^{1}H NMR spectroscopic data for this compound were identical to those of chrysin (**1**) (Chen et al., 2003), a commercially available flavonoid (Acros Organics, NJ, USA). This compound had been reported to be an anticonvulsant found in *Passiflora* (Medina et al., 1990), a chemical component of an orchid, *Bulbophyllum odoratissimum* (Majumder and Sen, 1991), and a phytoalexin found in the sapwood of Rosaceae (Kokubun and Harborne, 1995).



Final purification of the active compound in fraction B was performed by successive HPLC procedures, and 1.2 mg of pure compound was obtained. The ^{1}H and ^{13}C NMR spectroscopic data for compound B agreed with those of lusianthrin (**2**) (Majumder and Lahiri, 1990). Although this compound has previously been isolated as a chemical component of a dried sample of an orchid plant, *Lusia indivisa* (Majumder and Lahiri, 1990), it has not been shown to have antifungal activity. The position of the methoxyl group was supported by NOE's between the

methoxyl group and H-1 (8.4%) and H-3(2.2%) in a differential NOE experiment. The chemical structure of lusianthrin (**2**) resembles those of orchinol and hircinol, which have been isolated as phytoalexins from adult orchid plants (Fisch et al., 1973; Ward et al., 1975). Lusianthrin (**2**) showed slightly stronger antifungal activity than chrysin (**1**); the ED_{50} values of both compounds against WO-97 were ca. 10 μM.

The antifungal spectra of lusianthrin (**2**) and chrysin (**1**) were studied using five kinds of fungi, including three representative pathogens. Lusianthrin (**2**) showed strong antifungal activity against WO-97 and its closely related fungi, *Rhizoctonia repens*, but weak or no activity against the three pathogens (Fig. 2). Chrysin (**1**) also showed its strongest activity against WO-97 (Fig. 3).

To confirm whether or not lusianthrin (**2**) and chrysin (**1**) help to defend against infection by a symbiotic fungus,

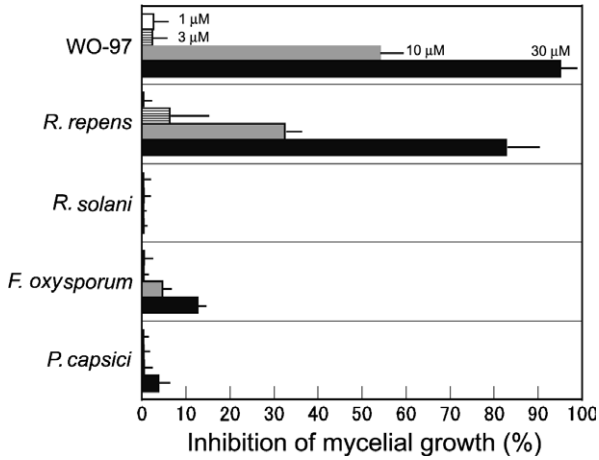


Fig. 2. Antifungal spectrum of lusianthrin (**2**). The fungi used were; WO-97, a symbiotic fungus; *Rhizoctonia repens* R.r.1, a nonpathogenic fungus; *Rhizoctonia solani* AG2-2-IV, beet root-rot disease; *Fusarium oxysporum* f.sp. *cepae*, onion bulb dry-rot disease; *Phytophthora capsici*, gray blight disease. Numbers at the tops of the bars show the molar concentrations of the compounds. Bars indicate ±SD ($n = 5$).

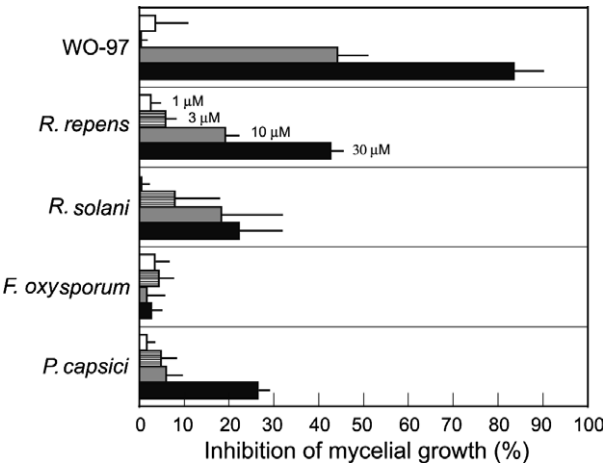


Fig. 3. Antifungal spectrum of chrysin (**1**). The fungi used were the same as in Fig. 2. Bars indicate ±SD ($n = 5$).

changes in the levels of these two compounds after the inoculation of WO-97 were examined. Due to the shortage of seeds, it is practically impossible to produce a sufficient amount of aseptic protocorms. Therefore, this experiment was carried out using aseptic protocorm-like bodies (PLBs) derived from germinated seeds (Shimura and Koda, 2004). To facilitate fungal infection, PLBs that had begun to differentiate roots were selected and inoculated with WO-97, and then sampled monthly. Microscopic observation revealed that invasion by hyphae into the cortical cells of the roots occurred at 1–2 months. After 3 months, pelotons were observed in the cells. Four months after inoculation, some of the PLBs turned brown and then rotted, and some remained white and healthy. Pelotons and digested pelotons were found in the cells of healthy PLBs (Fig. 4a). In contrast, no pelotons were found in the cells of brown PLBs, and monilioid cells, which are nutritional storage hyphae, were observed (Fig. 4b), suggesting that the fungus preyed PLBs. Monilioid cells were not found in the cells of healthy PLBs.

Healthy PLBs were examined for the presence of antifungal compounds. The level of lusianthrin (**2**) in aseptic PLBs remained low (Fig. 5). On the other hand, the level in inoculated PLBs began to increase markedly after 3 months. The simultaneous occurrence of a large increase in the level of lusianthrin (**2**) and the formation of pelotons seems to indicate that the increased level of lusianthrin (**2**) inhibits mycelial growth and helps to enclose them within the plasma membrane to form pelotons. The level of lusianthrin (**2**) in PLBs 4 months after inoculation was twice the ED_{100} (Fig. 5), yet hyphae and pelotons were found in the cells (Fig. 4a). This result suggests that the compound is localized in the cytoplasm of orchid cells and the concentration in the apoplast remains low to allow fungal growth. Since PLBs began to turn brown and die at this point in culture, it is possible that browning is due to autointoxication by the overproduction of lusianthrin (**2**).

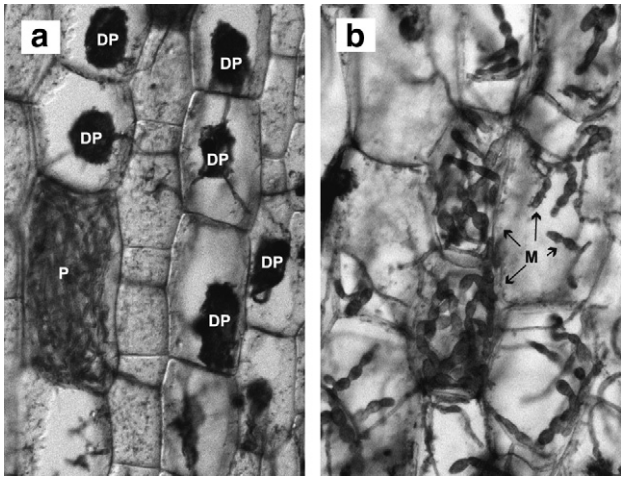


Fig. 4. Microscopic observations of healthy (a) and brown (b) roots of protocorm-like bodies 4 months after inoculation. DP, digested peloton; M, monilioid cells; P, peloton.

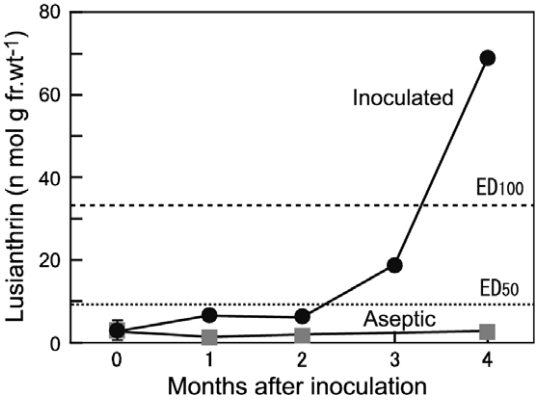


Fig. 5. Time-course changes in the level of lusianthrin (**2**) in protocorm-like bodies after inoculation of a symbiotic fungus. Broken lines represent ED_{50} and ED_{100}. SD ($n = 5$) of the aseptic control was 1.35 nmol g fr wt^{-1}.

Therefore, the toxicity of lusianthrin (**2**) against the host *C. macranthos* var. *rebunense* was examined using PLBs. PLBs were transferred to Hyponex–Peptone medium (Shimura and Koda, 2004) that contained 0, 10, 30 or 100 μM lusianthrin (**2**). Even at the highest concentration, browning of the PLBs was not observed until 4 months of culture (data not shown). This result suggests that the browning of PLBs cannot be attributed to autointoxication by the overproduction of lusianthrin (**2**).

Chrysin (**1**) that was present in the initial sample was not detected in the infected PLBs. The initial sample mostly consisted of plantlets that had already developed shoots. It is likely that chrysin (**1**) is an antifungal product of adult plants and is not formed in protocorms. We are now raising more than 500 micropropagated plants of this orchid in our Botanic Garden (Nagatani et al., 2006). Studies to verify this speculation are now in progress using some of these plants.

3. Concluding remarks

Two antifungal compounds, lusianthrin (**2**) and chrysin (**1**), were isolated from the seedlings of *C. macranthos* var. *rebunense* that had developed shoots. The large increase in the level of lusianthrin (**2**) in PLBs after infection with the symbiotic fungus suggested that the compound is involved in regulating the symbiotic association during germination. In contrast, chrysin (**1**) was not detected in infected PLBs. Since orchid cells inevitably allow fungal infection during symbiotic germination, they have to be constitutively susceptible to fungal infection. It is likely that orchid plants equip multiple antifungal compounds and deploy them in specific developmental stages: lusianthrin (**2**) maintains the perilous symbiotic association for germination and chrysin (**1**) helps to protect adult plants as a phytoalexin. This is the first report on antifungal compounds that appear to be involved in symbiotic germination of orchid seeds.

4. Experimental

4.1. General

Silica gel (Wakogel C-300), Sephadex LH-20, and Sep-Pak C_{18} cartridge (Waters) were used for column chromatography. TLC was conducted on Silica gel 70 F_{254} Plate (Wako). A preparative column (Nova-Pak HR C_{18}, 100 × 25 mm, Waters) and an analytical column (Nova-Pak C_{18}, Radial-Pak cartridge 100 × 8 mm, Waters) were used for HPLC. The elution profile was monitored by *A* at 210 nm. The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were obtained using a Bruker AMX500 spectrometer. CD_3OD or $CDCl_3/CD_3OD$ (1/1) was used as a solvent for NMR experiments. The proton signal of CHD_2OD (δ 3.30 ppm) was used as an internal standard in ^{1}H NMR experiments, whereas the carbon resonance of CD_3OD (δ 49.0 ppm) was used as an internal standard in the ^{13}C NMR experiments. The MS spectrum was recorded on a JEOL JMS-AX500 spectrometer.

4.2. Plant material

Since the collection of the orchid plants from the natural habitat is prohibited by law and their commercial value is extremely high, it is difficult to obtain sufficient plant material. For the past 7 years, we have been studying the micropropagation of this orchid species through PLBs (Shimura and Koda, 2004) and symbiotic germination with symbiotic fungi (Shimura and Koda, 2005). For the subculture of micropropagated plantlets, normal and healthy plantlets were always selected, and plantlets that seemed to be inadequate for transplanting were dipped in EtOH and stored at −20 °C. The plantlets obtained by both methods were mixed and subjected to the extraction of antifungal compounds.

4.3. Isolation of chrysin and lusianthrin

The plantlets (200 g fr wt) were homogenized with a sufficient amount of EtOH to give a final EtOH–H_2O (4:1, v/v) extract and then passed through filter paper (Advantec, No.2). The filtrate was concentrated to 30 ml and the resultant aqueous residue was partitioned 3 times against equal volumes of *n*-hexane, EtOAc and *n*-BuOH, respectively. The EtOAc fraction (920 mg) was fractionated by a silica gel column (100 × 25 mm, EtOAc/toluene, stepwise). Strong antifungal activity was found in the EtOAc/toluene (1:4, v/v) fraction. This fraction (167 mg) was applied to a Sephadex LH-20 column (450 × 20 mm). Elution was carried out with MeOH (300 ml) and then with MeOH–AcOH (999:1, 200 ml) at a flow rate of 1.5 ml min^{-1}. Antifungal activity was found in fractions eluted at 135–150 ml and 375–435 ml, and these fractions were designated A and B, respectively. Fraction A (13 mg) was subjected to TLC (hexane–EtOAc 1:1, R_f 0.45), and a final purification was carried out by silica gel column (85 × 3 mm, $CHCl_3$–EtOAc

15:1). Twenty-five 0.3-ml fractions were collected and fractions 7–11 contained 1.0 mg of the active compound, chrysin **1**, yellow powder. ^{1}H NMR (500 MHz, CD_3OD:$CDCl_3$ = 1:1): δ 7.94 (2H, *dd*, J = 1.6, 7.6 Hz, H-2 and H-6), 7.57 (1H, *tt*, J = 1.6, 7.6 Hz, H-4), 7.55 (2H, *br t*, J = 7.6 Hz, H-3 and H-5), 6.68 (1H, *s*, H-3), 6.49 (1H, *d*, J = 2.2 Hz, H-8), 6.28 (1H, *d*, J = 2.2 Hz, H-6); EIMS, 70 eV, *m*/*z* (rel. int.): 254 $[M]^+$ (100), 226 $[M-CO]^+$ (23), 152 $[M-C_8H_6]^+$ (17), 124 $[M-C_9H_6O]^+$ (11). Fraction B (43 mg) was successively subjected to a Sep-Pak C_{18} cartridge (Vac 20 cc, eluted with MeOH–H_2O, 4:1, v/v), prep. HPLC (MeOH–H_2O, 4:1, v/v, including 0.1% AcOH, 4 ml min^{-1}, R_t 10.4 min), and analytical HPLC (MeCN–H_2O, 3:7, v/v, including 0.1% AcOH, 1 ml min^{-1}, R_t 30.2 min) to give lusianthrin **2** (1.2 mg, light yellow oil). ^{1}H NMR (500 MHz, CD_3OD): δ 9.49 (1H, *d*, J = 9.2 Hz, H-5), 7.53[a] (1H, *d*, J = 9.0 Hz, H-9), 7.49[a] (1H, *d*, J = 9.0 Hz, H-10), 7.12 (1H, *d*, J = 3.0, H-8), 7.06 (1H, *dd*, J = 3.0, 9.2 Hz, H-6), 6.84 (1H, *d*, J = 2.8 Hz, H-1), 6.67 (1H, *d*, J = 2.8 Hz, H-3), 3.87 (3H, *s*, CH_3O–); ^{13}C NMR (125 MHz, CD_3OD): δ 158.7 (*s*, C-1), 158.0 (*s*, C-7), 155.4 (*s*, C-4), 135.9 (*s*, C-8a), 134.7 (*s*, C-10a), 130.5 (*d*, C-5), 128.4[b] (*d*, C-9), 128.3[b] (*d*, C-10), 125.9 (*s*, C-4b), 117.1 (*d*, C-8), 116.1 (*s*, C-4a), 112.1 (*d*, C-6), 103.3 (*d*, C-1), 102.1 (*d*, C-3), 55.7 (*q*, OCH_3); EIMS, 70 eV, *m*/*z* (rel. int.): 240 $[M]^+$ (100), 197 $[M\text{-}C_2H_3O]^+$ (35). [a,b]Values are interchangeable with each other.

4.4. Assay for antifungal activity

All cultures were carried out at 20 °C in the dark. WO-97, a symbiotic fungus (Shimura and Koda, 2005), was used for the assay of antifungal activity in the extracts. The fungus was cultured for 8 weeks on green pea medium, and inocula for assays were taken as 5-mm discs from the advancing edge of the colonies on agar plates. The sample to be tested was dissolved in MeOH and added to a test tube. After MeOH was removed under reduced pressure, green pea medium (10 ml) was poured into the tube and autoclaved for 7 min. Before gelatinization, the medium was sonicated for 3 min to disperse water-insoluble compounds and then poured into a Petri dish. At the center of the dish, the fungal inoculum was placed upside-down so that the hyphae were in contact with the agar surface. Since WO-97 showed very slow growth, the diameter of the colony was measured 4 weeks after inoculation. Antifungal activity was expressed as inhibition of mycelial growth that was calculated by [100-(average radius of colony on test medium)/(average radius of colony on basal medium) × 100].

In addition to WO-97, four kinds of fungi were used to assess the antifungal spectra of lusianthrin **2** and chrysin **1**; (1) *Rhizoctonia repens* R.r.1, a nonpathogenic fungus isolated from roots of *Spiranthes sinensis*; (2) *Rhizoctonia solani* AG2-2-IV, a pathogen of beet root-rot disease isolated from infected beet roots; (3) *Fusarium oxysporum* f.sp. *cepae*, a pathogen of onion bulb dry-rot disease isolated from infected onion bulbs; (4) *Phytophthora capsici* (ATCC 58208), a pathogen of gray blight disease. The Laboratory of Plant Pathology of our Graduate School preserves these fungal stocks on potato–sucrose–agar medium. Since these fungi show quite different growth rates, the diameter of the colony of each fungus was measured 7, 3, 6 and 3 days after inoculation, respectively. All experiments were carried out with five replicates.

4.5. Changes in the levels of antifungal compounds in PLBs after inoculation of the symbiotic fungus

WO-97 was inoculated at the center of a Petri dish that contained 10 ml green pea medium and had been cultured for 4 weeks. To facilitate fungal infection, PLBs that had developed roots were selected, and transferred to the Petri dish. The PLBs (ca. 4 g fr wt) were harvested every month and subjected to extraction with 80% EtOH followed by EtOAc extraction after concentration. The EtOAc fraction was then purified by TLC (hexane–EtOAc 1:1, R_f 0.25). The amounts of lusianthrin **2** in these samples were measured by HPLC on an analytical column (MeCN–H_2O, 3:7, v/v, including 0.1% AcOH, 1 ml min^{-1}, *A*254 nm, R_t 30.2 min). Since practical difficulties involved in obtaining a sufficient amount of uniform PLBs did not permit the replication of each experiment, the levels of aseptic control were determined 5 times with different lots. For light microscopy, the inoculated PLBs were fixed in a mixture of formaldehyde–AcOH–EtOH–H_2O (2:1:10:7), embedded in 5% agar and sectioned by a micro-slicer at 80 μm. Hyphae were stained with 0.05% trypan blue in lactophenol (PhOH–glycerol–lactic acid–H_2O 1:1:1:1).

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