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Production of volatile substances by rhizomorphs of *Marasmius crinisequi* and its significance in nature

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Abstract Twigs entangled by rhizomorphs of *Marasmius crinisequi* inside tea bushes are mostly devoid of leaves. This was found to be due to the emission of a defoliation-inducing volatile by the rhizomorphs, because when tea twigs were enclosed with rhizomorph sections of *M. crinisequi*, leaf fall occurred within 5 days. Leaves on control twigs remained healthy and attached. The volatile substances emitted from rhizomorphs were passed through acetone and the volatile compounds in acetone were analyzed by GC/MS. The emitted volatiles were identified as 3-oxo-β-ionol, 2,4,6-tri-ter-butyl-4-methyl-cyclohexadien-2,5-one and 2-phenyl-3,4,5,6-tetramethylpyridine. These compounds have not been reported from fungi or plants prior to this report. The significance of the production of a defoliation-inducing volatile by rhizomorphs of *M. crinisequi* in its acquisition of nutrients from tea leaves in the competitive environment is discussed.

Keywords Defoliation · Horse-hair blight · Rhizomorphs · Tea bush · Volatile substances

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Introduction

Marasmius is a worldwide distributed genus and its members are an important litter-decomposing agaric component of tropical ecosystems (Desjardin and Ovrebo 2006; Wannathes et al. 2009a,b). Some species of *Marasmius* produce abundant black, hair-like rhizomorphs as a means of exploration for colonization of twigs and leaves (Seaver 1944; Singer 1976; Redhead 1989). Rhizomorphs of several *Marasmius* species have been used as nesting material by various bird species (Foster 1976; Hedger 1990; McFarland and Rimmer 1996; Young and Zuchowski 2003; Freymann 2008; Chen et al. 2010) and even flying squirrels (Prange and Nelson 2006).

Horse-hair blight of tea (*Camellia sinensis* (L.) Kuntze) caused by *Marasmius crinisequi* Mull. ex Kalch. (=*Marasmius equicrinis* Mull.) is known to occur in India, Sri Lanka, Java, Australia, China, Japan and Taiwan (Petch 1923; Hara 1931; Sarmah 1960; Hu 1984; Chen and Chen 1990. Ezuka and Ando 1994; Dassanayake et al. 2009). *M. crinisequi* has been considered epiphytic on the tea bush, obtaining its food from the dead outer back of the older stems, and from the dead leaves and twigs in the tangle (Petch 1923; Sarmah 1960; Dassanayake et al. 2009).

Decline of tea bushes associated with *M. crinisequi* (Fig. 1) has become serious in recent years in a number of tea farms in central Taiwan. The affected tea bushes became unthrifty and the production of harvestable tea leaves by these plants is greatly reduced (Hu 1984). During a survey conducted in the tea farms with decline problem, it was observed that twigs of lower branches entangled with rhizomorphs of *M. crinisequi* were devoid of leaves. It was, therefore, hypothesized that *M. crinisequi* rhizomorphs may have caused defoliation of affected twigs. Our study

Fig. 1 A tea twig entangled by rhizomorphs of *Marasmius crinisequi*

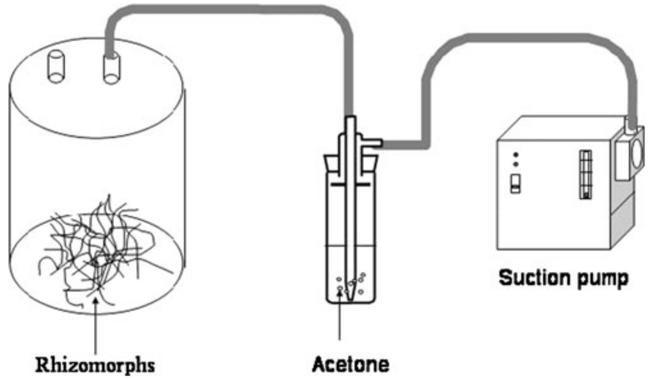

Fig. 2 Sketch of equipment used for collection in acetone (*centre*) of volatile substances released from rhizomorphs of *Marasmius crinisequi* (*left*) using a suction pump (*right*)

revealed the involvement of volatile substances produced by rhizomorphs of *M. crinisequi* in the defoliation of affected twigs. The chemistry of the released volatile substances were subsequently determined. Details of the study are reported herein.

Materials and methods

Detection of volatile substances released from rhizomorphs

Two-year-old cv. Chin Hsin Oolong tea plants each with three twigs were used. Rhizomorphs of *M. crinisequi* collected from declining tea bushes were cut to 3-cm sections with a pair of scissors, and 50 rhizomorph sections were fastened to each twig with a Parafilm band (5 mm wide) about 10 mm away from the main stem. Twigs similarly treated with Parafilm bands were used as controls. Each treated plant was sprayed with distilled water and enclosed in a plastic bag (27 cm diam., 37 cm high). Test plants were kept outdoors under shade with the temperature ranging from 28°C to 34°C. Plastic bags were removed after 5 days. Five plants were used for each treatment and the experiment was performed three times.

Chemical identification of volatile substances

For collection of volatile substances, approximately 80 g of rhizomorphs of *M. crinisequi* gathered from affected tea bushes was placed in a 5-l glass jar connected through Teflon tubing to a glass tube with a pointed tip immersed in 25 ml acetone in a 70-ml glass vial which was connected to a suction pump (Fig. 2). After equilibration of volatile substances, released from rhizomorphs in the jar at 24–26°C, was attained, the headspace gas was sucked through acetone at the flow rate of 8 ml min^{-1} for 3 h (Ioffe and Vitenberg 1984).

Analysis of the volatile substances in acetone was carried out using a Varian 4000 GC/MS system (Varian, Inc., Palo Ato, CA, USA Anonymous 1996a). The injection temperature was 250°C for 5 min. A non-polar VF-5-MS column (30 m×0.25 mm i.d., 0.25 mm film thickness, Varian, Inc., Palo Ato, Ca, USA) was operated under the following conditions: 70°C for 3 min, 20°C min^{-1} to 180°C for 3 min, and 10°C min^{-1} to 230°C for 20 min. The MS was operated at 70 eV. Full scan of masses was from m/z 20 to 400 with a scan rate of 470 μ/s.

Results

When plastic bags were removed from the tea plants 5 days after exposure to rhizomorphs of *M. crinisequi*, some leaves fell from the twigs. Fallen leaves lost vigor and turned brown gradually. Leaf fall continued and none of the leaves remained on the treated twigs after 4 weeks (Table 1). Control plants remained healthy during the experiment. None of the rhizomorphs used formed adhesive mycelial discs to stick to the twigs, indicative of ability of the rhizomorphs to emit defoliation inducing volatiles.

Based on the GC/MS analysis, the volatile compounds released from *M. crinisequi* rhizomorphs were identified as 3-oxo-β-ionol, 2,4,6-tri-ter-butyl-4-methyl-cyclohexadien-

Table 1 Incidence of tea twig defoliation following exposure to volatile substances released from rhizomorphs of *Marasmius crinisequi*

Treatment	No. of twigs defoliated [a]/ No. of twigs exposed		
	Exp. 1	Exp. 2	Exp.3
Exposed to volatile substances	15/15	15/15	15/15
Control	0/15	0/15	0/15

[a] Data were recorded 4 weeks after the treatment

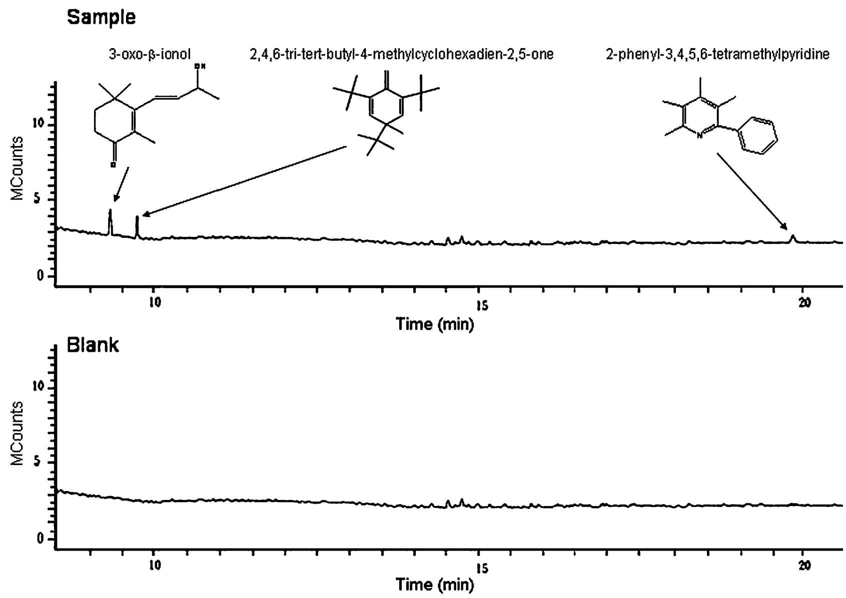

Fig. 3 Structures and chromatograph of volatile compounds released from rhizomorphs of *Marasmius crinisequi* based on the analysis using the Varian 4000 GC/MS system

2,5-one and 2-phenyl-3,4,5,6-tetramethylpyridine (Fig. 3). These constituents have more than 80% similarity with the known compounds in NIST standard reference database (Anonymous 1996b) (Table 2).

Discussion

This study shows that rhizomorphs of *M crinisequi* are able to induce defoliation of tea twigs by releasing volatile substances. Induction of defoliation by aerial rhizomorphs of *M. crinisequi* may explain the absence of leaves on most twigs entangled by rhizomorphs inside the affected tea bush (Petch 1923; Hara 1931; Hu 1984; Chen and Chen 1990). *M. crinisequi* is considered epiphytic and not plant pathogenic, obtaining its nutrients from dead bark, and from dead leaves and twigs of the tea bush (Petch 1923; Sarmah 1960; Dassanayake et al. 2009). Results from this study suggest that *M. crinisequi* employs a unique strategy in evading competitors during its acquisition of nutrients from dying leaves of the tea bush. The rhizomorphs of this fungus produces adhesive discs to stick on living leaves when crossing them during its exploration (Hedger 1990; Ko, unpublished observations). These leaves will fall from twigs due to defoliation-inducing volatiles emitted by the rhizomorph. The falling leaves will be captured by the fungus with its adhesive discs and suspended in the air. Since detached leaves are less disease resistance (Liu et al. 2007) and macromolecules will be degraded (Quirino et al. 2000), it will be easier for *M. crinisequi* to obtain nutrients from fallen tea leaves. Suspending fallen leaves in the air also can prevent the captured food source being eaten by soil animals (Coleman and Wall 2007) and/or colonized by other soil microorganisms (Alexander 1977). It is not known if emission of volatiles by rhizomorphs is common among fungi. Production of volatile antimicrobial compounds by endophytic *Muscodor* including *Muscodor albus* (Ezra et al. 2004), *Muscodor crispans* (Mitchell et al. 2008, 2010) and *Muscodor yucatanensis* (Macias-Rubalcava et al. 2010) in cultures has been reported in recent years. The volatile compounds produced by *M. yucatanensis* are also inhibitory to plant seed germination and root elongation (Macias-Rubalcava et al. 2010). Whether the volatile substances emitted from rhizomorphs of *Marasmius* are antimicrobial remains to by investigated.

The volatile substances produced by *M. crinisequi* and identified as 3-oxo-β-ionol, 2,4,6-tri-ter-butyl-4-methyl-cyclohexadien-2,5-one and 2-phenyl-3,4,5,6-

Table 2 Volatile chemical constituents, released from rhizomorphs of *Marasmius crinisequi*, identified by the Varian 4000 GC/MS system

No.	Retention time (min)	Compounds	Molecular formula	Similarity (%)[a]
1	9.313	3-oxo-β-ionol	$C_{13}H_{20}O_2$	95
2	9.742	2,4,6-tri-ter-butyl-4-methyl-cyclohexadien-2,5-one	$C_{19}H_{32}O$	82
3	19.855	2-phenyl-3,4,5,6-tetramethylpyridine	$C_{15}H_{17}N$	81

[a] Compared with the known compounds in NIST standard reference database

tetramethylpyridine have not been reported from fungi or plants previously (Kaiser 2006; Pichersky et al. 2006) and thus are different to those produced by *Muscodor* species which are primarily alcohols, organic acids, esters, ketones and lipids (Macias-Rubalcava et al. 2010). Only a closely related 3-oxo-α-ionol has been detected in passion fruit (Winterhalter 1990), grapes (Strauss et al. 1987) and starfruit (Herderich et al. 1992). Since authentic compounds are not available, it is still not known which of the three volatile compounds detected in this study is involved in the induction of defilation. It appears to be a new kind of plant growth regulator. The mechanism of defoliation induction and possible application in agriculture for stimulation of flower production deserves further investigation. The functions of the other two volatile compounds also remains to be studied. It would also be interesting to know if the volatiles emitted by the rhizomorphs are involved in the selection by birds as the nesting material (Foster 1976; Hedger 1990; McFarland and Rimmer 1996; Young and Zuchowski 2003; Freymann 2008; Chen et al. 2010).

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