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Antifungal Activities of Major Tea Leaf Volatile Constituents toward *Colletorichum camelliae Massea*

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A crude glycosidic fraction was prepared from fresh tea leaves and treated with the crude tea enzyme, fractions of *cis*-3-hexenol, linalool oxide I (*cis*-furanoid), linalool oxide II (*trans*-furanoid), linalool, methyl salicylate, geraniol, benzyl alcohol, and 2-phenylethanol were monitored to be the major aglycone moieties by analyzing the released volatiles. The amount of the released aglycone moieties is 5.8 times higher than those in free form. For investigation of the functions of the glycosidically bound form aroma constituents in tea leaves, their antifungal activities were determined by antifungal assay. Geraniol, linalool, methyl salicylate, benzyl alcohol, and 2-phenylethanol exhibited significant antifungal activities toward *Colletorichum camelliae Massea*, although *cis*-3-hexenol and linalool oxides showed weaker activities by comparison. Among them, geraniol was shown to be the most potential antifungal substance with a MIC value of 440 μg/mL. The crude glycosidic fraction prepared from tea leaves also exhibited significant antifungal activities in a wide range of concentrations from 2 to 25 mg/mL in a PDA medium. It was deduced that the glycosidically bound volatiles are formed and stored in the intact tissue of tea leaf and hydrolyzed by the actions of both the endogenous and the exogenous glycosidases to release volatiles as antifungal substances when exposed to *Colletorichum camelliae Massea*. The results suggested that the higher content of the bound form geraniol in tea leaves of var. *sinensis* might be responsible for their stronger antipathogen properties toward tea leaf blight, as opposed to those of var. *assamica*.

KEYWORDS: Volatile; glycoside; antifungal; *Colletorichum camelliae Massea*; *Camellia sinensis* var. *sinensis* cv. Zhuye

INTRODUCTION

The aroma components of tea are mainly produced as results of biochemical and chemical reactions during tea processing. Fresh tea leaves in living plants are virtually odorless. However, volatile aroma components are released when endogenous enzymes and aroma precursors (generally referring to mono- and diglycosides) separated within the cellular structure are allowed to encounter each other during the oolong tea and black tea manufacturing processing.

So far, diglycosides, such as β-primeverosides (6-O-β-D-xylopyranosyl-β-D-glucopyranosides) of geraniol (*1*), linalool, 2-phenylethanol, and benzyl alcohol (*2*), and both *trans*- and *cis*-linalool 3,6-oxides (*3*), β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) of geraniol (*4*), 6-*O*-β-D-apiofuranosyl-β-D glucopyranosides of methyl salicylate and *cis*- and *trans*-linalool 3,7-oxides (*5*), and (3*R*,9*R*)-3-hydroxy-7,8-dihydro-β-ionol (*6*), have been isolated and identified from fresh tea leaves as tea aroma precursors. β-D-Glucopyranosides of benzyl alcohol (*7*), *cis*-3-hexenol (*8*), and mandelonitrile (*9*) have been isolated and identified as monoglycoside aroma precursors from fresh tea leaves. The mandelonitrile β-D-glucopyranoside, isolated from tea leaves, is readily hydrolyzed by a crude enzyme prepared from the fresh tea leaves to liberate benzaldehyde (*9*).

Early assumptions that glycosidic aroma precursors act as hydrophilic alcohols do not seem to be significantly increased by glycosidation (*10*). An alternative proposition has been put forward by Stahl-Biskup et al. (*11*), which suggests glycosidation is a protective mechanism to prevent lipophilic volatile phenols and alcohols from damaging cell membranes. All the diverse results concerning the roles of these glycosides in tea plants should not lead us to ascribing to them an exclusive role, such as being involved in volatiles metabolism. The range of possible roles should not be underestimated.

Naturally occurring antifungal compounds, such as essential oil components, have received much attention in recent times. The antifugal activity of geraniol toward *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium digitatum*, *Mucor* sp., and *Rhizopus stolonifer* has been investigated. At 100 μg/mL in an agar medium, the inhibition of growth ranges from 39.0 to 81.4% after 48 h of incubation at 28 °C (*12*). To date, there are a few relative reports concerning the functions played by the glycosidically bound form aroma compounds. Plants produce

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stress metabolites when they are exposed to pathogens, abiotic agents such as heavy metal ions, UV light, or mechanical damage. Glycosidic fractions of benzyl β-D-glucoside and 2-phenylethyl β-D-glucoside and of mandelonitrile β-D-glucoside, sambunigrin, and prunasin have been isolated as precursors of antifungal substances from cherry leaves (*Prunus yedoensis Matsumura*) (*13*).

Tea leaf blight disease is one of the most economic diseases of tea plants in all tea-growing countries, especially in var. *assamica* (tea plant variety has large leaves). This disease is caused by *Colletorichum camelliae Massea* which has been described under the name *Guignardia camelliae* (Cooke) E. J. Butler (*14*). So far, the reasons why tea leaf blight disease frequently occurred in var. *assamica* as opposed to var. *sinensis* (tea plant varieties have large leaves) have never been discussed.

The purpose of this experiment is to investigate the major functions of glycosidically bound volatile constituents in tea leaves as antifungal substances against *Colletorichum camelliae Massea*.

MATERIALS AND METHODS

Materials and Chemicals. Fresh tea leaves (one bud and two leaves) of cv. Zhuye were plucked at the campus tea garden of Anhui Agricultural University. *Colletorichum camelliae Massea* was isolated from the tea plants of cv. Zhuye. Emulsifier 0203-B (main ingredient: special nonionic and anionic surfactants, produced by Xingtai Lanxing Auxiliary Factory of China) and reference compounds (purity >95%) of *cis*-3-hexenol, linalool, linalool oxide I and II, methyl salicylate, geraniol, benzyl alcohol, and 2-phenylethanol were purchased from Hexin Chemicals Co. (Shanghai, China).

Tea Leaf Crude Enzyme. The crude enzyme was prepared from the corresponding fresh tea leaves. The fresh juvenile tea leaves were crushed in cooled acetone (−20 °C). After the mixture homogenized for 3 min in a dispersing mixer, the acetone layer was removed by filtration. The debris was washed with cold acetone until it became colorless, then dried at room temperature to give acetone powder, and stored in a freezer (−70 °C) before use.

Preparation of Volatiles in Fresh Tea Leaf. Fresh tea leaves (100 g of wet material) were inactivated by a microwave stove for 2 min and homogenized in water (0.5 L). Free form volatile constituents were extracted from the homogenate. The homogenate was refluxed and vapor-phase-extracted with 50 mL of ether for 30 min in a Likens−Nickerson Simultaneous Steam Extraction (SDE) apparatus as described (*15*). After the homogenate was dried with anhydrous sodium sulfate, the ether was evaporated at 40 °C to give an aroma concentrate for gas chromatography analysis.

Preparation of Glycosidic Fractions. Fresh tea leaves (200 g of wet material) were frozen with liquid nitrogen and homogenized in methanol (1 L). The extract was filtered, and the filtrate was concentrated in vacuo at 50 °C to remove the methanol. The concentrate was extracted with an equal volume of chloroform to remove caffeine and part of the pigments. The aqueous solution was adjusted to pH 7.0 with $NaHCO_3$ and added Pb(OH)Ac to remove polyphenols. The residual supernatant fraction was further concentrated and applied to an Amberlite XAD-2 column (40 mm i.d. × 300 mm) to absorb the glycosidic fraction. After the column was sequentially washed with water and pentane/ether (1/1), the glycoside-rich fraction was eluted with methanol. This methanol-eluted fraction was evaporated to dryness to yield 5 g of the glycoside-rich fraction.

Enzymatic Hydrolysis of the Glycoside-Rich Fraction. One hundred milligrams of the glycosidic-rich fraction was dissolved in 30 mL of a 20 mmol/L sodium citrate buffer (pH 5.0) and mixed with 1 g of tea crude enzyme in a sealed vessel. Twenty milliliters of ether was added onto the top surface of the reaction mixture to extract the released volatiles. The reaction mixture was incubated at 37 °C for 4 h. The released volatiles were extracted three times with 100 mL of ether. The ether extract of the volatiles was dried with anhydrous Na_2SO_4 and concentrated at 40 °C. A control was treated similarly with inactivated tea crude enzyme. Qualitative and quantitative analyses of the compounds were carried out in a gas chromatograph.

Measurement of β-Glucosidase Activities in Tea Leaf and in a PDB Culture Medium of *Colletorichum camelliae Massea*. The blight-diseased tea leaves were plucked and the diseased parts of the leaves were removed. The uninjured leaf parts (1.00 g) were treated with liquid nitrogen, crushed with PVPP (poly(vinylpyrrolidone) powder), and extracted with 4 mL of 20 mmol/L citrate buffer (pH 5.0). The extracted materials were centrifuged at 8000*g* for 20 min, and the supernatant was then applied for enzyme assay. The same part of the fresh juvenile tea leaf was removed and treated in the same manner as the control. The activated *Colletorichum camelliae Massea* was mixed with 50 mL of a PDB culture medium and cultured at 25 °C at 105 rpm for 7 days. After filtration, the culture medium was applied for enzyme assay. The enzyme assay was carried out using the method described in (*16*). The β-glucosidase samples were measured for the hydrolysis activities toward *p*NP β-D-glucopyranoside. The incubation mixture (1 mL) was composed of 0.9 mL of 20 mmol/L citrate buffer (pH 5.0) containing 0.1% BSA, 50 μL of an enzyme sample solution, and 50 μL of 10 mmol/L *p*NP β-D-glucopyranoside [in 20 mmol/L citrate buffer (pH 5.0)]. Reaction was started by adding an enzyme sample at 37 °C and stopped by adding 0.5 mL of 1 mol/L Na_2CO_3. The liberated *p*-nitrophenol was determined spectrophotometrically at 405 nm. One unit was defined as the amount of enzyme capable of liberating 1 μmol of *p*-nitrophenol per minute under the assay conditions.

Instrumental Analysis. The resulting ether extract was concentrated to 0.5 mL under a stream of nitrogen, and 1 μL of the sample was injected into a GC. The gas chromatography was the Shimazu GC 9A model, equipped with a PEG-20M fused silica capillary column (50 m × 0.25 mm i.d.), carrier gas, and N_2 (1 mL/min) at a split ratio of 18:1. The GC oven was maintained at 50 °C for 5 min, then programmed to increase at 3 °C/min until it reached 190 °C, and maintained at 190 °C for 8 min. The injection temperature was 200 °C. The components were identified by comparing their GC retention times and those of the authentic commercial samples. Quantitative analysis data were acquired by establishment of the standard curves with individual reference commercial volatiles.

Antifungal Assay of Volatile Constituents in an Agar Medium. Seven volatile constituents were evaluated for antifungal activity based on their capacity to inhibit the growth of *Colletorichum camelliae Massea*. The method of mycelial growth tests was applied to this assay (*17*). The individual volatile constituents of *cis*-3-hexenol, linalool oxide I and II, linalool, methyl salicylate, geraniol, benzyl alcohol, and 2-phenylethanol were emulsified by emulsifier 0203-B. The solution was mixed with potato dextrose agar (PDA) culture media respectively to give a series of 100, 500, 1000, 1500, and 2000 μg/mL concentrations of culture media containing the compounds described above. The media were dispensed into sterilized Petri plates (9 cm). A mycelial disk of 4 mm diameter of the test pathogens taken from 4 days old culture, with the help of a sterilized cork borer, was placed at the center of the medium. The control and emulsifier control without any test volatile constituents were performed in the same way. Radial growth of colonies was measured at two points along the diameter of the plate and the mean of these two readings was taken as the diameter of the fungal colony. The growth of the colonies in control sets was compared with that of various treatments and the difference was converted into percent inhibition $[(C - T) \times 100/C]$ where C and T are the radial diameters of the colony in control and treatment, respectively. The minimal concentration that completely inhibited the growth of fungal was defined as the minimal inhibitory concentration (MIC). Three replicates were kept for each treatment; the MIC values were obtained after incubation for 5 days at 25 °C.

Antifungal Assay of Crude Tea Leaf Glycosides in an Agar Medium. The glycoside-rich fraction extracted from tea leaves as described above was employed for antifungal assay in an agar medium to evaluate the antifungal activity against *Colletorichum camelliae Massea*. The test crude glycosides were dissolved in water and incorporated in the molten agar media at concentrations of 0, 2, 5, 10, 15, 20, and 25 mg/mL. The media were then dispensed in 2 mL quantities in sterile Petri plates (3.5 cm). A mycelial disk of 4 mm

Table 1. Free and Bound Form Volatile Constituents in Fresh Tea Leaves of Cv. Zhuye

volatile compounds	retention time (min)	free form[a]	bound form[b]	ratio
cis-3-hexenol	24.682	12 ± 0.5	24 ± 5	1.9
linalool oxide I	27.498	11 ± 0.2	32 ± 5	3.0
linalool oxide II	29.035	22 ± 0.7	68 ± 8	3.1
linalool	32.900	70 ± 0	116 ± 9	1.7
methyl salicylate	42.737	17 ± 1.4	66 ± 24	3.8
geraniol	45.863	125 ± 0.8	455 ± 64	3.6
benzyl alcohol	46.630	14 ± 0.2	227 ± 21	16.6
2-phenylethanol	48.023	13 ± 0.3	652 ± 49	48.9

[a] μg/100 g of fresh tea leaves (data are mean ± SE of two experiments). [b] μg/100 g fresh tea leaves (data are mean ± SE of six experiments).

Table 2. Antifungal Activities of Seven Major Tea Aroma Constituents against *Colletorichum camelliae Massea*[a]

	Fungal Growth Inhibition (%)					
	concentrations of volatile constituents in PDA (μg/mL)					MIC
volatile constituents	100	500	1000	1500	2000	(μg/mL)
cis-3-hexenol	42.6	44.4	61.1	74.1	79.6	>2125
linalool oxide I and II	37.0	40.7	44.4	48.1	57.4	>2375
linalool	37.0	46.3	50.0	100	100	1376
methyl salicylate	42.6	50.0	55.6	100	100	1770
geraniol	58.1	100	100	100	100	440
benzyl alcohol	44.4	46.3	61.1	68.5	83.3	2600
2-phenylethanol	51.9	46.3	70.4	85.2	92.6	2040

[a] Data are the mean of three replicates. The inhibition of growth is measured after 5 days of cultivation at 25 °C. Standard deviation in all cases never exceeds ±5%.

diameter of the test pathogens taken from 4 days old culture, with the help of a sterilized cork borer, was placed at the center of the medium. The mycelial disks on PDA without any test chemical served as control. Three replicates were kept for each treatment and incubated for 1−3 days at 25 °C. Percent inhibition was measured as described above.

RESULTS

Aglycone Moieties Released by Enzymatic Hydrolysis. The glycosidically bound form aroma compounds occurred in tea leaves. A glycoside-rich tea extract prepared by chromatography of methanol extracts from fresh tea leaves on an Amberlite XAD-2 column was incubated with the crude enzymes (tea leaf acetone powder). Gas chromatographic analyses of the released aglycones was summarized in **Table 1**. Significant amounts of *cis*-3-hexenol, linalool oxide I (*cis*-furanoid), linalool oxide II (*trans*-furanoid), linalool, methyl salicylate, geraniol, benzyl alcohol, and 2-phenylethanol were yielded. These eight kinds of glycosides were revealed to be the major bound form aroma components in fresh tea leaves. These aroma constituents exist in fresh tea leaves in both free and bound form. The amount of glycosidically bound form aroma compounds released by the crude enzymes was approximately 16 μg/g fresh tea leaves total, which is about 5.8 times higher than those in free form.

Antifungal Activities of Volatile Constituents. The antifungal activities of seven volatile constituents toward the mycelial growth of *Colletorichum camelliae Massea* are shown in **Table 2**, in which the inhibition was enhanced with the increase of volatiles concentrations. Each MIC value of the volatile constituents was obtained. All of the compounds tested exhibited antifungal activities against *Colletorichum camelliae Massea*. Among these seven active compounds, geraniol was the most potential, with a MIC of 440 μg/mL (**Figure 1**). MICs of methyl salicylate, linalool, 2-phenylethanol, and benzyl alcohol were from 1170 to 2600 μg/mL, respectively, while linalool oxides and *cis*-3-hexenol were the least effective, having MICs over 2125 and 2375 μg/mL, respectively.

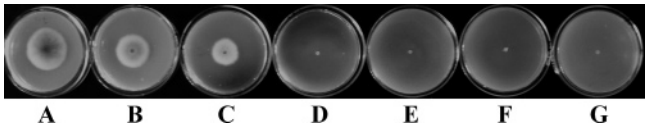

Figure 1. Mycelium growth of *Colletorichum camelliae Massea* on culture media containing geraniol. (A) The control plate medium without emulsifier 0203-B and geraniol; (B) the control plate medium with an equal amount of 0.01% emulsifier 0203-B; (C) culture medium containing 100 μg/mL of geraniol and 0.01% emulsifier 0203-B; (D) culture medium containing 500 μg/mL of geraniol and 0.01% emulsifier 0203-B; (E) culture medium containing 1000 μg/mL of geraniol and 0.01% emulsifier 0203-B; (F) culture medium containing 1500 μg/mL of geraniol and 0.01% emulsifier 0203-B; (G) culture medium containing 2000 μg/ mL of geraniol and 0.01% emulsifier 0203-B. The photographs were taken 5 days after inoculation.

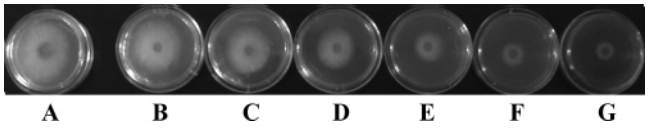

Figure 2. Mycelium growth of *Colletorichum camelliae Massea* on culture media containing crude tea leaf glycoside-rich fractions. (A) The control plate medium without test compounds; (B) culture medium containing 2 mg/mL compounds; (C) culture medium containing 5 mg/mL compounds; (D) culture medium containing 10 mg/mL compounds; (E) culture medium containing 15 mg/ mL compounds; (F) culture medium containing 20 mg/ mL compounds; (G) culture medium containing 25 mg/mL compounds. The photographs were taken 2 days after inoculation.

Table 3. Antifungal Activity of Tea Leaf Glycoside-Rich Extracts against *Colletorichum camelliae Massea*[a]

concentrations of glycosides in PDA (mg/mL)	inhibition of growth (%) after culture for 1 day	inhibition of growth (%) after culture for 2 days	inhibition of growth (%) after culture for 3 days
2.0	14.3 ± 0.07	8.7 ± 0.03	3.2 ± 0.04
5.0	27.3 ± 1.11	26.1 ± 1.17	16.1 ± 0.68
10.0	42.9 ± 1.14	34.8 ± 1.08	29.0 ± 1.60
15.0	61.9 ± 2.24	50.0 ± 1.69	43.5 ± 2.03
20.0	71.4 ± 1.83	60.9 ± 2.93	48.4 ± 1.44
25.0	86.4 ± 2.37	82.6 ± 2.01	72.6 ± 1.72

[a] The inhibition of growth is measured after 1–3 days of cultivation at 25 °C. Each value is the mean of three experiments.

It should be noted that throughout this experiment the emulsifier 0203-B was used since most of the lipophilic flavor compounds tested are not soluble in water. In fact, this compound shows weaker antifungal activities when tested using the method employed.

Antifungal Activities of Crude Tea Leaf Glycoside-Rich Fractions. To evaluate the antifungal activity of glycosidically bound tea aroma constituents against *Colletorichum camelliae Massea*. The glycoside-rich fraction extracted from tea leaves was employed for antifungal assay in an agar medium. As shown in **Figure 2** and **Table 3**, the test crude glycosides also exhibited significant antifungal activities in a wide range of concentrations from 2 to 25 mg/mL in a PDA medium. None of the above concentrations completely inhibited the test fungus. The inhibition ranged from 3.2 to 86.4%. Inhibition percent of mycelium growth was positively related to the concentration of the crude glycosides in a medium. The antifungal activities of the compounds in the PDA plates declined to different extents as time passed.

Table 4. β-Glucosidase Activities in Tea Leaves and in a Culture Medium of *Colletorichum camelliae Massea*[a]

β-glucosidase activities in tea leaves (U/g fresh leaves)		β-glucosidase activities in a 50 mL culture medium (U/mL)
healthy leaves	tea leaf blight-injured leaves	
1.44 ± 0.07	1.65 ± 0.08	0.0156 ± 0.0016

[a] Data are the mean of three experiments.

***β*-Glucosidase Activities in Tea Leaf and in a Culture Medium of *Colletorichum camelliae Massea*.** As described above, both the glycosidically bound form and free form aroma components show significant antifungal activities toward *Colletorichum camelliae Massea*. The glycosidically bound form aroma components were expected to be hydrolyzed by glycodidases to release volatiles as an antifungal substance when exposed to the pathogen. After tea leaves were injured by the blight disease, endogenous β-glucosidase activity was monitored and showed a significant increased from 1.44 to 1.65 U/g (**Table 4**). When the activated *Colletorichum camelliae Massea* was cultured in 50 mL of a PDB culture medium for 7 days, significant β-glucosidase was germinated and secreted in a culture medium with enzymatic activity of 0.0156 U/mL. It was strongly suggested that greater amounts of volatiles would be released from tea leaves by catalysis of both endogenous and exogenous glycosidases when the leaves were pathogen-injured, although since the variations of released volatiles remain to be investigated, a definite conclusion could not be verified.

DISCUSSION

Incubation of a glycoside-rich tea extract with the tea leaf crude enzyme has been shown to yield *cis*-3-hexenol, linalool, linalool oxides I and II, methyl salicylate, geraniol, benzyl alcohol, and 2-phenylethanol, which were revealed to be the major aglycone moieties in fresh leaves of a wide plant tea cultivar (*Camellia sinensis* var. *sinensis* cv. Zhuye) in China. These aglycone moieties were observed to be very rich in bound form while few volatile constituents were observed in free form in the fresh tea leaves. This result is in agreement with the findings of Wang and You (*18*).

Qualitative and quantitative analyses of glycosidically bound aroma precursors in tea leaves showed that about 25% of these glycosides are β-D-glucosides with all sorts of aglycone moieties present in forms of diglycosides (*19*).

In tea leaves, endogenous β-primeverosidase is responsible for the hydrolysis of the naturally occurring diglycosides into the corresponding disaccharide and aglycone (*20*, *21*). Additionally, two kinds of endogenous β-glucosidases have been purified from *Camellia sinensis* var. *sinensis* cv. Yabukita and revealed that these enzymes were responsible for the liberations of glucosically bound form aroma precursors (*22*).

These glycosides and corresponding glycosidases have been confirmed as a concern in tea aroma formations during tea manufacture processing.

Our investigation of the major functions of glycosidically bound volatile constituents in tea leaves by antifungal screening has shown that both the glycosidically bound form and free form aroma components showed significant antifungal activities toward *Colletorichum camelliae Massea*. The β-glucosidase activity in the tea leaf blight-injured leaves increased significantly. The β-glucosidase activity was also detected in the culture medium of *Colletorichum camelliae Massea*. It was strongly suggested that the precursors of antifungal substances existing as glycosides in tea leaves would be hydrolyzed by both the endogenous and the exogenous glycosidases when the leaves were pathogen-damaged. These results implied that the glycosidically bound form volatiles in fresh tea leaves served as potential antifungal substances against *Colletorichum camelliae Massea*, which is responsible for causing the disease of tea leaf blight.

It is well-known that tea leaf blight mainly infects mature leaves in the late period of summer and early autumn when tea leaves are aged. Serious disease is seen mostly in var. *assamica* with large leaves (*23*).

The employed seven volatile constituents exhibited significant antifungal activities against *Colletorichum camelliae Massea*, although *cis*-3-hexenol and linalool oxides showed weaker activity than the others. Geraniol, a monoterpene alcohol with the highest content in leaves of var. *sinensis*, as opposed to var. *assamica* which mainly grow in China and Japan (*24*), is revealed to be the most potential substance against this fungi.

Monoterpene alcohols of geraniol and linalool are the major aroma components in tea products. The ratio of these two alcohols is different according to the geographical location of the product. *Camellia sinensis* cultivated for tea production has two varieties, i.e., var. *assamica* and var. *sinensis*. The former with a high content of linalool is cultivated in southeast Asia and has large leaves, while the latter with a high content of geraniol is grown in China and Japan and has small leaves. Consequently, the geographical difference in the linalool and geraniol ratio could be caused by differences between these two cultivars or their hybrids (*25*).

The higher content of bound form geraniol in tea leaves may be one of the reasons why tea leaf blight is not a serious disease in China and Japan, as opposed to that in India, Sri Lanka, and Malaysia.

It has been revealed that higher amounts of geraniol were found in juvenile tea leaves than in mature tea leaves (*18*, *26*) which conforms with the fact that tea leaf blight mainly infects mature leaves in late summer and early autumn.

Thus, we postulate that these glycosides are formed and stored in the intact tissue of tea leaf and hydrolyzed by the actions of both the endogenous and the exogenous glycosidases to release volatiles as antifungal substances when exposed to *Colletorichum camelliae Massea*. These glycosides, such as geranyl glycoside, act as the self-defense fractions against tea leaf blight in tea plants.

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