

## Development of Tea bush replant disease by red root rot fungus

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### ABSTRACT

The fungal pathogen *Ganoderma philippii* (*Gp*) infects the tea bushes and causes necrotic red root rot. It is more prevalent in mature tea plantations than in younger tea plantations. HPLC analyses of the tea rhizosphere soils for phenolic acids showed a significant increase in the amount of gallic acid and catechin in the rhizosphere soils of older plants. Quantitative RT-PCR results showed that the *Gp* fungus number in the rhizosphere soil increases with plant age. Both gallic acid (GA) and catechin (CA) stimulate the growth of this fungus in *in-vitro* and their addition to the soil along with *Gp*, decreases the tea plant growth.

**Keywords:** *Camellia sinensis*, catechin, chemicals compounds, consecutive monoculture system, disease, gallic acid, *Ganoderma philippii*, phenolic acid, qRT-PCR, red root rot, replant problem, rhizosphere soil, tea bush.

### INTRODUCTION

Oolong tea produced in Wuyi Mountains, Fu-Jian Province, Southeast China, is an important Chinese tea (17,24). However, due to the warm humid climate, elderly tea bushes in monoculture suffers from serious diseases, which reduces yields and cause financial losses (10,14,20, 21). Based on our field observations, the majority of tea diseases are of fungal origin. Of these, the red root rot caused by the fungus *Ganoderma philippii* (*Gp*) is most common affecting the older plants and destroying the mature tea bushes (1,5,7,15).

Several studies have shown that the chemicals released by tea bushes through root exudates, influences the rhizosphere microbiology and affects the tea plants growth (2,3,10). The root exudates contain terpenoids, phenolics, steroids, alkaloids, and cyanogenic glycosides and are involved in interactions in the soil (22,31). Zhou (33) found that *p*-coumaric acid detected in cucumber and soybean root exudates significantly changed the rhizosphere microbial communities and promoted the growth of soil-borne pathogens. Qu and Wang (21) applied phenol 2,4-di-tert-butylphenol (PEDT) and vanillic acid (VA) to the soil and found that VA in particular, influenced the microbial communities and played a major role in soybean monoculture problems.

This study aimed to verify, if the phenolics released in the tea root exudates stimulate the *Gp* fungus and whether they are responsible for the increased disease incidence in older tea plants.

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## MATERIALS AND METHODS

### Soil collection

The rhizosphere soil samples of tea plants were collected in April 2016 from both young (YB, 2 years old) and old bushes (OB, 7-years old) from tea gardens of Oolong Tea in Wuyi Mountains (17°24'12"E, 27°32'36"N), Fujian Province, Southeast China. Both young and elderly bushes were taller than 100 cms and the roots were about 100 cm deep. Ten bushes of each type were selected. After the plants were dug out carefully, the rhizosphere soil samples were collected by brushing the fine soil attached to the root surfaces.

For control (CK), soil from an adjacent uncultivated field was collected from 100 cm depth. After the soil samples were collected, they were air-dried in shade till fully dry.

### I. *Ganoderma philippii* culture

A strain of *G. philippii* (Code No. GP115) was used as the test fungus in these experiments. This strain was isolated from an elderly tea bush showing symptoms typical of red root rot by using fresh potato dextrose agar medium and authenticated by microcopy and Internal transcribed spacer (ITS) sequencing (28) and stored as conidial suspension in 30% glycerine at -80°C.

Prior to *in-vitro* tests, the fungus was revived by sub-culturing spores on fresh potato dextrose agar medium (PDA) at 26°C in dark.

### II. HPLC identification and quantification of chemicals in rhizosphere soil

To quantify the chemicals in soils, 500 g soil samples, passed through 2 mm sieve, was suspended in 1 L distilled water at 90 °C for 30 min on a shaker. The extracts were filtered and the extractions were repeated thrice. The extracts were concentrated by rotary evaporation at 50°C until the final volume was 5 ml and then, the samples were weighed and stored at -80 °C (4,30).

The concentrated extracts were filtered through a 0.22 µm filter prior to analysis and analysed using an HPLC (Waters, USA) with Waters Symmetry fitted with C<sub>18</sub> column (Supelco, USA 4.6 mm × 250 mm, 5 µm) (8,18). The injected sample volume was 10 mL. The columns were eluted with a liner gradient consisting of ortho-phosphoric acid-water (0.05: 99.95, V:V) and acetonitrile at flow rate 0.8 mL/min. Fractions were monitored using a UV detector. The column temperature was maintained at 35°C (18,27). The various phenolics were identified and quantified by their retention time compared with the standards purchased from Sigma.

### III. DNA extraction and quantitative qRT-PCR of Gp in the rhizosphere soil samples

The total fungal DNA in the soil samples was extracted using the Soil Isolation Kit (Omega Bio-Tek, Inc., GA, USA), as per the manufacturer's instructions and purified with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

Yuskianti' method (28) was used to quantify *G. philippii* DNA by Species-specific PCR (Gphil3f - GTG AAG CGG GCT CTT TGCT and Gphil4r - CAA GTC CAA GCC TAC AAA ACCA) (27). Touchdown PCR reactions were performed in 20 µL reaction mixtures that contain 1 µL of each primer, 1µL total fungal DNA, 1.6 µL dNTP, 2 µL 10 × PCR buffer and 13.4 µL ddH<sub>2</sub>O.

The 'touchdown' PCR assay was done using the following conditions: Eight cycles consisting of denaturation at 95°C for 30 s, annealing at 62-55°C (temperature was decreased by 1°C between consecutive steps) for 45 s and the extension at 72°C for 45 s and then 36 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 45 s and extension at

72°C for 45 s. 10 µL of PCR product were analyzed by agarose gel electrophoresis on 1.2% agarose gel, stained with ethidium bromide, seen under UV trans illuminator and photographed.

The cloned fragment was gel-purified, cloned into the Peasy™-T4 Zero Cloning Kit (Beijing TransGen Biotech Co., Ltd.) and sequenced. The sequenced DNA was re-amplified using Gphil3f/Gphil4r from the plasmid and purified using a TIAN pure Mini Plasmid Kit (Tiangen Biotech Co. Ltd., Beijing). Then the concentration of the target DNA was determined and was then diluted to 5, 4, 3, 2, 1, 0.1, 0.01 and 0.001 ng/mL (31).

The standard curve was developed by plotting the amount of target DNA against the Threshold cycle (Ct) value exported from the Master cycler realplex. The primer sets Gphil3f/Gphil4r were evaluated from the aforementioned established standard curve and melting curve performed by qRT-PCR amplification in four replicates with serial dilutions of the above-mentioned target DNA serving as the template (28, 31).

The standard curve was developed by qPCR analysis and using the equation

$$y = -3.4789x + 13.864 \text{ and } R^2 = 0.997.$$

The qRT-PCR was carried out in 20 µL reaction mixtures (2×SYBR Green PCR Master Mix 12.5 µL, Gphil3f 0.5 µL, Gphil4r 0.5 µL and 4 ng DNA, with ddH<sub>2</sub>O to make up the reaction mixture volume to 20 µL). The PCR programme included 35 cycles at 94 °C for 3 min, 55 °C for 30 s and 72 °C for 3 min (5,28,31).

#### IV. Effects of *Gp* on tea bushes pre-treated with GA and CA (Catechin)

Based on the HPLC analysis of soil extracts, the effect of two phenolics found in the highest concentration in the rhizosphere soils, namely Gallic acid (GA) and Catechin (CA) along with *Gp* was examined on the tea plants. For this, the tea seedlings were pre-treated with the phenolics and then infected with the fungus. *Gp* conidia were cultured in potato sucrose (PS) medium 150 mL, at 28 °C for 6 d and the conidial number per ml was adjusted to 10<sup>4</sup> conidia/mL using the PS medium. The tea seedlings (30 cm tall) used in the experiment were planted in plastic pots (upper dia 16.5 cm, bottom dia 9.5 cm, height 19 cm) with each pot having 2 plants. The pots were cleaned first by washing with tap water and then immersed in 1% potassium permanganate solution for 30 min. The garden soil was disinfected thrice at 121 °C for 20 min, cooled to room temperature and then the pots were filled with 3 Kg of sterilized garden soil. The tea plants were washed in running water and then immersed in 75% alcohol for 3 min and then rinsed with the sterile water several times to remove alcohol. They were then planted in the pots pre-filled with sterilized garden soil and the pots were then placed in a green house at 22°C and at 75% humidity.

GA and CA purchased from Sigma Company were dissolved in 100 % ethanol and then diluted with water to get 0.1 mM solution. Each pot received 10 ml of this solution before inoculation with the fungus.

After 2 d, half the number of plants from each treatment were inoculated with *Gp* by adding the conidial suspension (10<sup>4</sup> conidia/mL) to the nutrient solution (13). The 6 treatments were: Control, 0.1 mM CA, 0.1 mM GA, *GP*, 0.1 mM CA+*GP* and 0.1mM GA+*GP*. Each treatment had 12 tea plants and were replicated thrice.

At 30 days, the first newly sprouted fresh shoots with two leaves and one bud were sampled. Leaf area index was measured using an LAI-2000 Canopy Analysis System (LI-COR Inc., Lincoln, NE, USA) (25).

### V. Effects of GA and CA on *Gp* growth

To investigate the effects of GA and CA on the growth of *Gp*, after the medium was cooled and filter sterilized (through 0.22  $\mu\text{m}$  membrane filter), the tests were done using PDA medium in Petri dishes (9 cm dia). The CA and GA, dissolved in 100% ethanol and then diluted with  $\text{H}_2\text{O}$  to 0.1 M concentration, respectively, were added to the medium, mixed and poured into Petri dishes. The final concentration of CA and GA was 100 and 250  $\mu\text{mol}\cdot\text{L}^{-1}$ .

To test the effects of CA and GA on the growth of the fungus, 0.7 cm wells were made in the centre of PDA plates and an agar plug of 0.7 cm dia from a 7-day-old growth of *Gp* on PDA was placed in the well. After 72 h of incubation at 26 °C in dark, the diameter of the mycelia growth was determined by calculating the distance (cm) from one edge to another. All treatments were replicated thrice.

### Statistical Analysis

Statistical analysis of the samples data was based on the complete sample profiles. The data were tested for normality with SPSS 19.0 (3).

## RESULTS AND DISCUSSION

Chemicals in the rhizosphere have an important ecological role in plant-microbe-soil interactions (4). These chemicals not only exert detrimental impacts on plants but also predispose the roots to infection by pathogenic fungi (7,23). Their influence increases with plant age in continuous monocrop cultivation (22). To assess this in tea plantations, we first analysed the rhizosphere soils of young and old tea plants for their phenolic acid contents by HPLC. Analysis of the soil extracts showed the presence of several phenolics of which the gallic acid, theophylline, theacrine, caffeine and catechin were the most abundant (Table 1). The content of these phenolics in the rhizosphere soils increased with the age of plants. The rhizosphere soil samples from the 2-years old plants had less GA and CA compared to the 7-years old tea plants and this was much higher than in control soil, confirming that these two phenolics accumulate in the rhizosphere of tea plants and their content increases with age.

Table1. Chemicals found in rhizospheres soils of young and old tea bushes determined by HPLC

Chemicals compounds	Retention time (min)	Contents of chemicals compounds in soils ( $\text{mg}\cdot\text{kg}^{-1}$ )		
		Control Soil (CK)	YB Soil	OB Soil
Gallic acid	3.54	0.191+0.01	0.513+0.02	1.235+0.01
Theophylline	8.46	ND	ND	0.003+0.01
Theacrine	10.32	0.013+0.01	0.016+0.01	0.018+0.01
Caffeine	13.16	0.072+0.02	3.423+0.02	0.069+0.01
Catechin	25.59	0.161+0.02	0.731+0.02	2.762+0.02

CK: Soil from the adjacent uncultivated field. YB : Rhizosphere soil samples from young tea plants (2 years old); OB: Rhizosphere soil samples from elderly bushes (7-years old); ND: Not determined

### qRT-PCR of *GP* collected from tea bush rhizosphere soils

To determine if the increase in the concentration of these chemicals has a relationship with the level of the pathogenic fungus, using qRT-PCR, the amount of fungal pathogen DNA in the rhizosphere of the tea bushes was determined.

The melting curve showed only one peak and had to be adjusted for further qRT-PCR analysis. The quantity of *Gp* DNA significantly increased with years of monoculture (Fig. 1).

These results showed that with increasing years of tea bush monoculture, the amount of pathogenic *Gp* significantly increased. The amount of *Gp* DNA was  $1.2 \times 10^5 \cdot \text{g}^{-1}$  in fresh soil, while it was  $2.1 \times 10^5 \cdot \text{g}^{-1}$  in young bush soil after 2-years and  $3.2 \times 10^5 \cdot \text{g}^{-1}$  in older bush soil after 7-years growth. This is consistent with the observed phenomenon that the *Gp* is more prevalent in mature tea plantations than in young tea plantations. Our results are also consistent with reports that accumulation of plant root exudates in the rhizosphere, encourages the proliferation of the pathogens and this plays a crucial role in soil quality, crop health and yield (2,5,8,10,22,28).

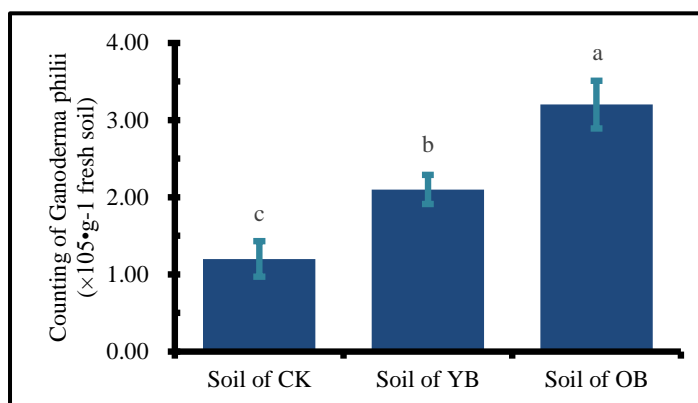


Figure 1. Quantity of *G. philippii* in young and old tea bush rhizosphere soils by using qRT-PCR

#### Effects of GA, CA and GP on tea bush biomass production

To verify, if there is a relationship between the concentration of the two major phenolics in the rhizosphere and the presence of fungus, on plant development was determined (Table 2).

Table 2. Effects of gallic acid (GA), catechin (CA) and *G philippii* (*Gp*) on tea bush growth

Treatment	Shoot height (cm)	Number of branches	Leaf area ( $\text{cm}^2 \cdot \text{bush}^{-1}$ )
Control	28a	6a	16.8 a
0.1mM CA	22b	5a	15.8a
0.1mM GA	20b	5a	16.0a
<i>GP</i>	25a	4a	13.6b
0.1mM CA+ <i>GP</i>	21b	5a	12.2b
0.1mM GA+ <i>GP</i>	20b	4a	12.4b

a and b indicate significant differences between groups according to a one-way ANOVA followed by a post-hoc LSD test ( $P < 0.05$ ).

Treatment of tea plants with either GA or CA marginally reduced the total leaf area. However, the treatment with *Gp* significantly, the plant growth decreased which was further enhanced by the addition of phenolics. These results support our hypothesis that the phenolics may be enhancing the infection of tea plants by *Gp*. Thus chemicals in the rhizosphere have an ecological role in plant-microbe-soil interactions.

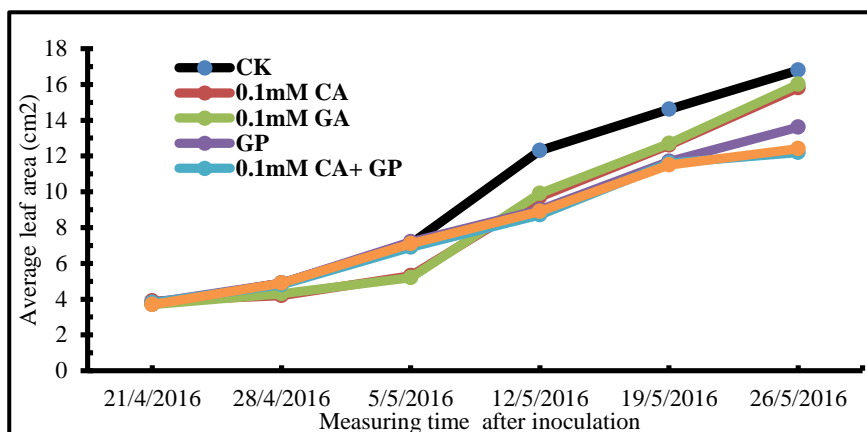


Figure 2. Effects of gallic acid (GA), catechin (CA) and *G philippii* (GP) on leaf area of the tea bush.

#### Effects of GA and CA on fungal growth

To verify if the soil phenolics stimulate the fungal growth, *in-vitro* tests were done. Both GA and CA promoted the growth of fungus and the extent of stimulation increased with increased concentrations of the added GA and CA (Fig. 3). Between the two, GA was more stimulatory. These results showed that the GA and CA found in the soil from the root exudates, had positive effects on the growth of the pathogen.

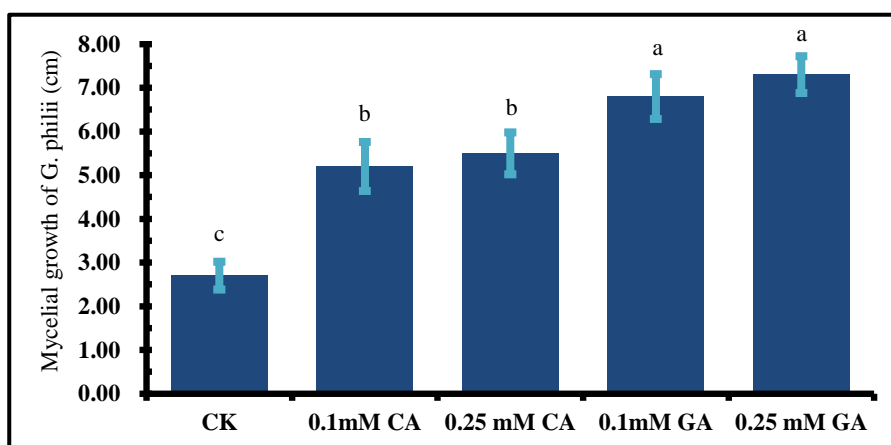


Figure 3. Effects of gallic acid (GA) and catechin (CA) concentrations on the growth of *G. philippii* (GP).

## CONCLUSIONS

We found that the increased incidence of *Gp* disease in monoculture fields of tea bushes is stimulated by the accumulated phenolics released from the tea plant root exudates. The two phenolics that we have examined, namely CA and GA, are of major importance in the host-pathogen interactions.

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