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Full Length Research Paper

Physiological mechanism of resistance to anthracnose of different *Camellia* varieties

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Tea oil camellia (*Camellia oleifera*) is an important oil-producing plant which is widely distributed in the Dabie Mountain of Anhui province in China. Anthracnose (*Colletotrichum gloeosporioides*) is a common and serious disease which often cause flower and fruit drop in tea oil, leading to 50% or more loss in yields. Different *Camellia* varieties vary in their resistance to anthracnose. There are few studies on the physiological mechanisms of resistance to anthracnose. In this paper, eight different varieties of *Camellia* in China were selected for measurement of the content of four polyphenols, flavonoids, phenylalanine ammonia lyase and polyphenol oxidase. Among the polyphenols, catechol and salicylic acid content were related to anthracnose resistance, with the content of the resistant varieties been nearly five to ten times higher than that of the more susceptible varieties. Flavonoid content was also significantly higher in resistant varieties than in susceptible varieties. Activities of the defense-related enzymes phenylalanine ammonia lyase and polyphenol oxidase did not differ between different cultivars. However, enzyme activity of resistant cultivars improved markedly after pathogen inoculation, while those of susceptible cultivars did not change. This study broadens the understanding of the mechanisms of disease resistance in *Camellia*.

Keywords: Anthracnose, *Camellia oleifera*, phenylalanine ammonia lyase, polyphenol oxidase.

INTRODUCTION

Tea oil camellia (*Camellia oleifera*), which originated from China, is notable as an important source of the edible oil (known as tea oil or camellia oil) which is obtained from its seeds (Lee et al., 2007; Lee and Yen, 2006). Tea oil has a high content of unsaturated fatty acids which are valued for their health benefits (Soderberg et al., 1996). However, tea oil production is often affected by infection with *Colletotrichum gloeosporioides*, or anthracnose, which is widespread in growing areas and causes severe fruit drop and bud drop, thereby reducing production (Perrett et al., 2003; Weseler et al., 2002). At present, two measures are often selected to control the disease. One is chemical control and the other is pollution-free control, including the use of biological pesticides and breeding resistant varieties. Breeding resistant varieties is the safest and most economical and effective approach to anthracnose control (Campa et al., 2009; Moral et al., 2009).

There are significant differences between different tea oil species in their resistance to anthracnose. For instance, *Camellia yuhsiensis Hu* is highly resistant, while the species used in extensive cultivation of tea oil is highly susceptible. According to the literature, there are a number of reasons why different strains of *Camellia* vary in their resistance to anthracnose (Chen et al., 2007; Crawford et al., 2004). Factors related to the physical structure and chemical composition of plant may confer resistance on the disease. These include the nature of the cell wall and its waxes, lignin, water holes and stomata. Certain cell wall components, such as phyto-alexin, can inhibit the growth of pathogens. Infection with the *Venturia inaequalis* fungus enhanced the metabolism of phenolic compounds at the apple scab spot (Slatnar et al., 2010a , b). Meanwhile, synthesis of pathogenesis-

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Abbreviations: PAL, Phenylalanine ammonia lyase; **PPO,** polyphenol oxidase; **PR-proteins,** pathogenesis-related proteins.

Table 1. The varieties of *C. oleifera.*

Number	Sampling location	Origin	Name	Natural resistance
I	Shucheng	Shucheng	*C. oleifera* Abel var. Shucheng-dahongensis	Medium resistance
II	Shucheng	Shucheng	*C. oleifera* Abel var. Shucheng-daqingensis	Susceptible
III	Huangshan	Hunan	*C. yuhsiensis* Hu	High resistance
IV	Huangshan	Huizhou	*C. oleifera* Abel var. Huizhou-daqingensis	Susceptible
V	Huangshan	Huizhou	*C. oleifera* Abel var. Huizhou-luohanguoensis	Susceptible
VI	Yansi	Huizhou	*C. oleifera* Abel var. Huizhou-xiaoqingensis	Susceptible
VII	Yansi	Huizhou	*C. oleifera* Abel var. Huizhou-dahongensis	Medium resistance
VIII	Yansi	Huizhou	*C. oleifera* Abel var. Huizhou-xiaohongensis	Medium resistance

related (PR) proteins is induced by pathogen infection and various enzymes such as chitinase and glucanase (Lorang et al., 2007; Nagy and Bennetzen, 2008; Zhuang and Liu, 2004). In addition, the plant generates defense responses to resist disease when infected by the pathogen, which include the release of various reactive oxygen species, expression of defense genes and hypersensitive responses (Leister, 2004; Takakura et al., 2008). Some substances that are contained in the plant host itself, such as saponins, glucosinolates and cyanogenic glycosides also have disease resistance properties (Asgary et al., 2008; Kim et al., 2009). Therefore this study, carried out in the Dabie Mountain area of local tea oil cultivars, investigated the relationship between disease resistance and various enzymes and their free radical scavenging capacity, and provides a scientific basis for resistance mechanism of the disease.

MATERIALS AND METHODS

Materials including tea leaves and fruits of the Shexian cultivar of the Special Economic Forest Park, southern Anhui, and widely grown cultivars from Shucheng, western Anhui, were used in this study. The cultivars and their relative susceptibilities to infection are listed in Table 1.

Determination of fruit coat polyphenols in different strains of *C. oleifera*

Chlorogenic acid (40 mg), ferulic acid (39.5 mg), salicylic acid (39.4 mg) and catechol (40.8 mg), were placed in 10 ml volumetric flasks, dissolved in 70% ethanol, diluted to the mark and shaken. The standard solutions were made up by different dilutions in 70% ethanol. Healthy fruit coat was taken from disease-free tea, rinsed with distilled water, dried at 50°C, crushed and screened. Samples of 2.0 g were weighed, placed in a Soxhlet extractor with 50 ml of 75% ethanol at 85°C in a water bath and extraction was carried out for 2 to 3 h, then the filtered volume was made up to 50 ml. Analysis of the extract was carried out on Column µ-Bondapak C18 (4.6 x 150 mm 10 µm, Agilent), with mobile phase component A acetic acid : water (1:99) solution, mobile phase component B methanol, mobile phase elution flow rate 1 ml/min; washing program as follows: 0 to 25 min, the A-phase ratio was decreased from 100 to 10%, while the relative proportion of B was increased from 0 to 90%; from 25 to 30 min, these proportions were kept steady; from 30 to 40 min, the A phase ratio was increased from 10 to 100%, while the B phase gradually decreased from 90 down to 0%, by the end of the elution. The detection wavelength was set at 280 nm. A standard curve was produced under these conditions from 10, 20, 30 and 40 µg/ml of chlorogenic acid, ferulic acid, p-hydroxybenzoic acid and catechol standard solutions.

Analysis of flavonoids

A standard dried weight of rutin reference (20 mg) was placed in a 100 ml volumetric flask and dissolved in 50% ethanol to the mark. The standard solutions were prepared from this by dilution. Spectrophotometry at 510 nm wavelength absorbance, gave the regression equation y = 0.0223x + 0.1293 (R2 = 0.9905), where x is the flavonoid concentration and y is the absorbance.

Healthy fruit coat was obtained from disease-free tea oil, rinsed with distilled water, dried at 50°C then crushed and screened. 50% ethanol (50 ml) was added to 0.5 g of the tissue and the mixture was subjected to reflux extraction in a water bath for 1 h, and then was filtered. The filtered volume was made up to the mark in a 50 ml flask with 50% ethanol, shaken, and then set aside. A standard curve was then determined at 510 nm wavelength absorbance. The flavonoids content (M µg/g) was calculated as: M = (93.05D + 0.463 1) × V / m, where D is the absorbance of the extract at 510 nm, V is the extract volume (ml) and m represent the quality of fruit coat (g) of healthy oil tea. The determination of activity of flavonoids was repeated three times.

Determination of PAL and PPO activity

High resistance, medium resistance and high susceptibility strains of tea oil plants were subjected to an anthracnose inoculation experiment, with PAL and PPO disease response activity measured every 12 h. Weighed leaves (5 g), were ground into a homogenate with 0.5 g polyvinylpyrrolidone, quartz sand and ice. Then 15 ml pH 8.8 borate buffer was added and the mixture was centrifuged at 8000 *g* and 4°C for 10 min, to obtain the supernatant, which contained the crude enzyme extracts. Then, 4 ml 0.02 mol/L phenylalanine solution and 5 ml pH 8.8 borate buffer solution was added to 1 ml of the supernatant and the mixture was placed at 40°C in a water bath for 30 min, and the reaction was terminated by adding 1 ml 6 mol/L HC1. Absorbance changes at 290 nm were measured to obtain enzyme activity of 0.001 to 1 unit of enzyme activity (U); enzyme activity was expressed as U/ (min. g fresh weight).

5 g leaves were ground into a homogenate with quartz sand in low temperature. Then, 15 ml phosphate buffer (pH 7.2) was added and the mixture was centrifuged at 8000 *g* and 4°C for 10 min to obtain the crude enzyme extract in the supernatant. Then, 1.5 ml phosphate buffer (pH 7.2), 0.10 mol/L catechol solution, and 2 ml of

Table 2. The regressive equations and linear range of different polyphenols.

Standard solution	Regression equation	R^2	Linear range (mg/mL)
Ferulic acid	Y=3227.2X-170.57	0.9995	0.0988-0.4938
Catechol	Y=3399.3X-138.15	0.9997	0.1020-0.5100
Salicylic acid	Y=2520.1X-125.41	0.9995	0.0985-0.4925
Chlorogenic acid	Y=5696.5X-315.05	0.9996	0.1000-0.5000

Table 3. Main phenolic content of different varieties of *C. oleifera.*

Variety	Chlorogenic acid (mg/100g)	Ferulic acid (mg/100g)	Salicylic acid (mg/100g)	Catechol (mg/100g)
C. oleifera Abel var. Shucheng-dahongensis	0. 62±0.03	5.60±0.02	0.09±0.02	0.56±0.01
C. oleifera Abel var. Shucheng-daqingensis	0.51±0.03	5.09±0.02	0.04±0.01	0.13±0.01
C. yuhsiensis Hu	0.72±0.01	0.81±0.01	0.17±0.01	0.92±0.03
C. oleifera Abel var. Huizhou-daqingensis	0.63±0.02	2.82±0.02	0.04±0.01	0.17±0.02
C. oleifera Abel var. Luohanguoensis	0.25±0.02	0.35±0.02	0.07±0.01	0.44±0.01
C. oleifera Abel var. Huizhou-xiaoqingensis	0.85±0.01	0.60±0.03	0.04±0.01	0.19±0.02
C. oleifera Abel var. Huizhou-dahongensis	0.01±0.00	0.54±0.01	0.13±0.02	0.64±0.02
C. oleifera Abel var. Huizhou-xiaohongensis	0.01±0.00	0.27±0.02	0.11±0.02	0.73±0.02

crude enzyme extract were mixed and heated at 40°C for 30 min, afterward, 50 µl HCl was added to terminate the reaction. Absorbance changes at 450 nm were measured within 1 min. We regarded 0.001 absorbance changes as one enzyme activity unit (U). The determination of activity of PAL and PPO was repeated three times.

RESULTS

Polyphenol content of different *C. oleifera* varieties

The peak times of the standard polyphenols by HPLC analysis were: 9.8 ± 0.3 min for salicylic acid; 13.3 ± 0.5 min for catechol; 17.8 ± 0.5 for chlorogenic acid min; and 32.2 ± 0.3 min for ferulic acid. Standard curves were prepared for these major phenolic compounds and the results are shown in Table 2.

In the concentration range used, the concentration and peak area of each standard sample showed a good linear relationship, so the samples can be quantitatively analyzed using the earlier mentioned regression equations.

The content of these four phenolic compounds in the eight varieties of *C. oleifera* fruit is shown in Table 3 and their relative susceptibilities to infection is shown in Table 1. Among the eight varieties of *Camellia*, there was one highly resistant, three medium resistant and four susceptible varieties to anthracnose infection (Table 1). Table 3 shows that the catechol and hydroxybenzoic acid contents of the resistant cultivars were nearly five to ten times higher than the susceptible ones in the field survey. The chlorogenic acid content was not significantly different between the eight tea-oil cultivars. The hydroxybenzoic acid and catechol content in *C. yuhsiensis Hu* were 0.0017 and 0.0092 mg/g which is significantly higher than those in the other cultivars. Therefore, catechol and p-hydroxybenzoic acid can be regarded as the major tea polyphenols. There was a negative relationship between salicylic acid and/or catechol contents and the degree of susceptibility to infection of the cultivars studied in this field investigation.

Flavonoid content of different varieties of *C. oleifera*

The flavonoid content of the fruit coat of the eight varieties of Camellia is shown in Table 4 and their relative susceptibilities to infection are shown in Table 1. There are differences in flavonoid content between disease-resistant varieties and susceptible ones, with that in disease-resistant varieties been significantly higher than that in the susceptible varieties. These results can guide further study of the mechanisms of disease resistance in *C. oleifera*.

PAL and PPO activity in different varieties of *C. oleifera* leaves infected with anthracnose

High resistance, medium resistance and susceptibility strains of leaves were stab inoculated with anthraconose, and PAL activity was determined after inoculation. The results in Figure 1 show that in the different tea oil varieties infected with anthracnose, PAL activity increased initially and then decreased. These peaks were more obvious in the resistant strains, and occurred earlier. With lower levels of disease resistance, the initial

Table 4. Flavonoid content of different varieties of *Camellia oleifera*.

Cultivar	Flavonoid content (mg/g)
C. oleifera Abel var. Shucheng-dahongensis	1.83±0.02[d]
C. oleifera Abel var. Shucheng-daqingensis	1.07±0.01[h]
C. yuhsiensis Hu	2.43±0.03[b]
C. oleifera Abel var. Huizhou-daqingensis	1.61±0.02[f]
C. oleifera Abel var. Luohanguoensis	1.73±0.03[e]
C. oleifera Abel var. Huizhou-xiaohongensis	2.51±0.01[a]
C. oleifera Abel var. Huizhou-dahongensis	2.35±0.00[c]
C. oleifera Abel var. Huizhou-xiaoqingensis	1.41±0.01[g]

The different letters show significant differences among treatments at 95% confidence level using the Duncan multiple comparisons.

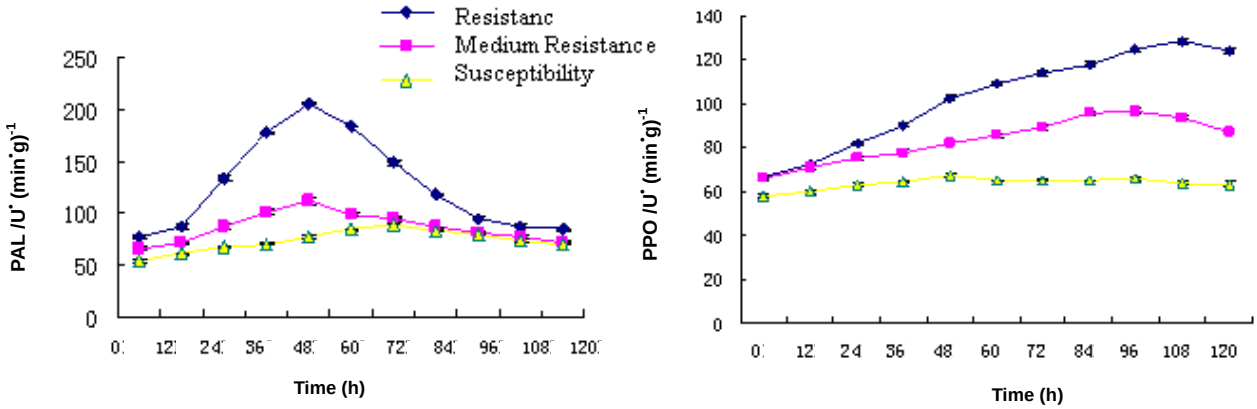

Figure 1. The changes of PAL and PPO enzymes after inoculation with anthracnose.

PAL activity response time of leaves was longer, and the degree of response was significantly reduced.

PAL activity of resistant strains rose slowly in the first few hours, and then gradually increased. Between 36 and 48 h after inoculation, it reached a peak three times that of the initial peak value. After reaching this peak, PAL activity decreased rapidly, reaching a steady value at about 120 h after inoculation.

The peak of PAL activity in medium resistant strains was close to that of resistant varieties, both been about 48 h. However, the peak value was significantly lower than that of the resistant strain, at only about twice that of the initial value. After 48 h, PAL activity decreased slowly to the initial level in all strains. When compared with resistant and medium resistant strains, PAL activity of highly susceptible strains increased slowly after inoculation of anthracnose. Three days after the inoculation, it reached its highest value, which was only about 50% higher than the initial enzyme activity value.

After inoculation, the PPO activity of different resistant varieties gradually increased over time. PPO values of resistant varieties increased more rapidly after inoculation. 72 h after inoculation, the rate of increase began to slow and about 100 h after inoculation, PPO levels reached a peak and then declined rapidly. The response time of medium susceptible varieties after inoculation was significantly longer than that of resistant varieties. PPO activity values increased slowly and reached a peak at 96 h, which was earlier than the resistant varieties. There was no significant change in PPO levels in the highly susceptible cultivars.

DISCUSSION

Polyphenols and flavonoids are the main active components of *C. oleifera*. Research shows that tea oil contains polyphenols and flavonoids which have a variety of physiological functions (Masella et al., 2005; Schmitt and Dirsch, 2009). They are not only antioxidants, but also have anti-cancer, anti-tumor, anti-radiation, blood sugar-lowering and other properties. Our results show an obvious difference in the polyphenol and flavonoid content in the fruit coat of different strains. The hydroxybenzoic acid, catechol and flavonoid content of resistant strains were higher than that of the susceptible ones. Plants accumulated a large number of phenolic compounds that are synthesized by acetic acid or

shikimic acid (Balasundram et al., 2003; Wong et al., 2008). Many of these have antimicrobial properties and can trigger a defense response from the plant. Phenylalanine ammonia lyase is a key enzyme of the shikimate pathway, whose activity and production of phenolic compounds are closely related when PAL and PPO activity and the amount of polyphenol content increase synchronously. Therefore, the activity of these enzymes or phenolic compounds, in turn, reflected the level of plant disease resistance at an early stage (Shang and Zhang, 2008; Wei et al., 2004). In this paper, PAL and PPO activity were measured after inoculation of different strains with anthracnose, and the findings show the change trends of enzyme activity after inoculation as a single peak curve. The occurrence of the peak time of disease-resistant varieties was generally earlier than the susceptible ones, indicating that the resistant varieties had a rapid defense response when attacked by the pathogen. Whether polyphenols and flavonoids increase the inhibition of pathogen infection and reproduction, or whether they reduce the accumulation of reactive oxygen by eliminating H_2O_2 and O_2^- to reduce injury to the cell membrane system requires further study (Zhang et al., 2009). In addition, the study only covers PAL and PPO activity of different strains after inoculation of anthracnose. Whether there is a correlation between the change of the two enzymes' activity and the incidence of the disease, the mechanism requires further study.

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