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Nutritional Characteristics of Normal, *Apolygus lucorum*-Damaged, and Mechanically Damaged Tender Shoots of Tea (*Camellia sinensis*)

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Abstract

Apolygus lucorum is currently a major pest of tea plantations, affecting the normal growth of tea shoots and reducing yield and quality. This study measured the effect of damage by *A. lucorum* alongside mechanical damage using UV spectrophotometry and high performance liquid chromatography. We found that the nutritional characteristics of tea varied with the level of bug damage, which influenced levels of caffeine, theanine, and tea polyphenols. As damage increased, caffeine content gradually increased. In contrast, theanine levels in highly damaged leaves were reduced compared to other levels of damage. The polyphenol level, like caffeine, increased with increasing damage. The levels of five other substances–Epigallocatechin, Catechin, Epigallocatechin gallate, Epicatechin, and Epicatechin gallate were all higher in tea with higher damage. Damage from *A. lucorum* changed the chemical profile of the tea, caused tea water turbidity, taste bitterness and decreased freshness, leading to the tea quality decline.

Keywords: *Camellia sinensis*; Tea quality; *Apolygus lucorum*; Damage; Nutritive substance characteristics

Introduction

Apolygus lucorum Meyer-Dür (Hemiptera: Miridae), named green leaf bug in China, is an omnivorous insect with a broad host range, damaging various plants and weeds by sucking plant sap from plantcotyledons and stems, young leaves, flowers, fruits, and stems [1,2]. In recent years, *A. lucorum* has become the primary pest of tea in China, it damaged tea shoots by sucking sap, with early signs of damage being brown spots followed by perforation, broken stems and malformation of plant parts, ultimately reducing yield [3,4]. Tea is one of the world's three major beverage crops [5], and research on improving the quality of tea is increasing [6,7]. In this study, we examined the changes in the nutrient content of tea that had been mechanically damaged (with needle pricks) or naturally damaged by *A. lucorum*, using UV spectrophotometry and high performance liquid chromatography to determine the effect of such damage onteaconstituents [8].

Caffeine, polyphenols, and theanine levels affect tea quality. Caffeine and tea polyphenols are mainly contained in tea, known as effective ingredients of tea, with high dietary property and medical value [9]. Caffeine (1,3,7-trimethyl-purine) is a natural alkaloid that is an important part of the taste of tea and promotes digestion and diuresis. Tea polyphenols are compounds, mainly forms of catechin, including Gallate Acid (GA), Epigallocatechin (EGC), Catechin(C), Epigallocatechin Gallate (EGCG), Epicatechin (EC), and Epicatechin Gallate (ECG) [10] and tea polyphenolsare the main component determining the taste and color of tea, and the key factor determining tea quality [7]. Theanine is a characteristic amino acid found in tea. Some studies have found theanine solutions to be slightly acidic, with caramel aromas and a taste similar to glutamate, easing the bitterness and enhancing the flavor of tea. Theanine content is directly related to the taste of tea [11,12]. Therefore, clarifying the effects of *A. lucorum* damage on these compounds helps us understand the likely quality of the tea with various levels of bug damage.

Materials and Methods

Study insects and plants

The variety of tea plants was Longjing 13, 60 cm high, lived in a tea plantation in Laoshan district, Qingdao, Shandong Province, China.

The experimental group: Taking 200 tea trees within 20 m^2, netted with a fabric of sufficiently small mesh to contain mirid bugs. Two hundred *Apolygus lucorum* of mixed in stars were introduced per tea plant, and after two days, leaves were collected and graded according to their degree of damage. Leaves with fewer than 10 feeding scars (pits) were designated as having first-level damage; those with 10-20 pits were called second-level damage; those with more than 20 pits were called third-level damage. The first control group was undamaged controls, selected visually from the same tea plantation. The second control group was mechanically damaged leaves, produced by piercing leaves 10 times with a fine (No. 0) insect pin. Such leaves were collected for analysis two days after injury was inflicted.

Sample extraction method

Caffeine, tea polyphenols and catechins were extracted from weighed samples (1 mg) of freshly collected tea leaves (wet weights) by grinding the leaves in liquid nitrogen and placing the material in

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70% methanol. This tube was then placed in a water bath preheated to 70°C where it was held for 20 min (per 5 min shaking 30 s, four times). The solution was then cooled to room temperature, transferred to a 10 ml volumetric flask and centrifuged for 10 min at 3,500 r/min and the residue at the bottom of the tube was extracted once with 70% methanol. These extractions were then held for later analysis via liquid chromatography.

Theanine was extracted from similar samples of fresh tea leaves that were ground in liquid nitrogen and placed in a 1 ml centrifuge tubes, to which was added 1.5 ml of filtered, deionized water (MilliQ water, room temperature). The solution was allowed to stand at 80℃ for 1 h, at which point it had cooled to room temperature, and was then spun at 1,300 r/min for 10 min, and 1.5 ml of the supernatant removed to a fresh centrifuge tube for storage and further analysis. Extracted samples were stored at 4°C, after which 1 ml of solution was passed through a 0.45 µm membrane filter to obtain a 2 ml sample for chromatographic analysis. These extractions were then held for later analysis via liquid chromatography.

Caffeine, theanine, tea polyphenols and catechins determination

Levels of caffeine, theanine, and catechins (polyphenols) in extractions from tea leaf samples were determined by high performance liquid chromatography (Tables 1-3) and samples were compared with the respective standard series to calculate the levels of caffeine, theanine and catechins [13,14].

Tea polyphenols are phenolic compounds that reacted with ferrous ions in the tea water extract to form a purple-blue complex [15]. The absorbance (A) of each solution was measured and the content of tea polyphenols extracted from tea leaves was measured at 765 nm wavelength.

Peak areas of different concentration gradients of caffeine and theanine were determined and used to produce standard curves. Determination of the absorbance (A) of different concentrations of gallic acid solution at 765 nm wavelength was used to produce standard curves based on the absorbance at different concentrations.

Peak areas of different concentrations of Gallic Acid (GA), table Epigallocatechin (EGC), catechin (C), (-) table Epigallocatechin Gallate (EGCG), Epicatechin (EC) and Epicatechin Gallate (ECG) were determined under the chromatographic conditions of catechins, and the respective standard curves were made according to their peak areas at different concentrations.

Data processing was used by SPSS20.0 data processing software of Deng Kenxin Duncan detecting.

Parameter	Condition
Instruments	Vacuum degasser, P4000pump, AS1000 auto-sampler UV6000LP photo Diede Array detector
Column	Phenomenex column: LUNR 5 µ C18 (2) 30 × 4.6 mm, (Guard column) Nova-Pak column: C18, 3.9 × 300 mm; 4 µ
Mobile phase	H_2O: MeOH (v/v7:3)
Other condition	Scan wavelength: 192-798 nm; wavelength: 280 nm; Column Temp: 40℃; Flow rate: 1.2 ml/min; Injection volume: 20 µl; Run time: 60 min

Table 1: HPLC conditions for detecting caffeine.

Parameter	Condition		
Instruments	Agilent 1100 series		
Column	Column: Symmetry C18 3.5 µm 2.1 × 150 mm		
Mobile phase	Time (min)	0.05 TFAA%	ACN%
	0.00	100%	0%
	3.00	100%	0%
	3.01	0%	100%
	6.00	0%	100%
	6.01	100%	0%
	10.00	100%	0%
Other conditions	Scan wavelength: 192-798; Wavelength: 203 nm; Column temperature: 20℃; Flow rate: 1.0 ml/min Injection volume: 20 µl; Run time: 10 min		

Table 2: HPLC conditions for detecting theanine.

Parameter	Condition		
Instruments	Agilent 1100 series		
Column	Column: Symmetry C18 5 µm 4.6 × 250 mm		
Mobile phase	Time (min)	Mobile phase A%	Mobile phase B%
	0.00	99%	1%
	20.00	97%	3%
	30.00	12%	88%
	30.01	0%	100%
	38.00	0%	100%
	38.01	99%	0%
	45.00	99%	0%
Other conditions	Scan wavelength: 192-798; Wavelength: 278 nm; Column temperature: 30℃; Flow rate: 1.0 ml/min; Injection volume: 10 µl; Run time: 45 min		

Table 3: HPLC conditions for detecting catechins.

Results

Effect of damage on tea constituents

Caffeine, theanine, and polyphenol levels in tea varied with levels of bug or mechanical damage (Table 4). As the level of damage by *Apolygus lucorum* on tea increased, caffeine level increased as well. Caffeine content under the highest level of bug damage (third level) (13.23 ng/kg) was significantly higher than those of mechanical damage (df=14; F=23.495; P=0.037), first-level bug damage (df=14; F=23.495; P=0.000) and the control leaf (df=14; F=23.495; P=0.000). Compared with control leaves, caffeine levels were greater in leaves with third-level damage (increasing by 43%) > second-level damage (by 33%) > mechanical damage (by 30%) > first-level damage (by 9%) > control.

Effect of damage on theanine in tea

For theanine, as the level of damage by *A. lucorum* increased levels of the compound decreased, such that theanine content in first and second-level damage was not significantly different from that of normal tea, but the theanine content of third-level damage (1.08 ng/kg) was lower than that of normal tea (1.39 ng/kg), or first-level damage (1.64 ng/kg) (stats go here), or second-level damage (1.37 ng/kg). Interestingly, the theanine content (1.80 ng/kg) in the mechanically damaged leaves was significantly higher than that in third-level damage (df=14; F=1.473; P=0.049). The percentage change in theanine content (compared to control leaves) in the mechanical damage treatment was greater (up 30%) than in the first-level damage treatment (up 18%), the second-level bug damage treatment (down 1%) or the third-level damage (down 22%).

Effect of damage on tea polyphenols in tea

Levels of tea polyphenols increased as the level of damage by *A. lucorum* rose, being greatest in the second-level bug damage treatment (517.97 ng/kg), significantly higher than that in control leaves (326.20 ng/kg) (df=14; F=5.519; P=0.001), and also higher than that in first-level damage (427.57 ng/kg) or the mechanical damage treatment (415.13 ng/kg). Compared to the normal, the percentage change in polyphenol content was greatest in the second-level bug damage treatment (up 59%), greater in the third-level bug damage treatment (up 43%), and less great in the first-level bug damage treatment (up 30%) or the mechanical damage treatment (27%).

Effects of damage on specific polyphenols

The specific tea polyphenols examined (GA, EGC, C, EGCG, EC and ECG) were differently affected by bug damage. We found that with the exception of GA, increasing *A. lucorum* damage was associated with changing levels of each of these components. Specifically, ECG content in third-level damage (10.53 ng/kg) was significantly higher than that of the control (5.70 ng/kg) (df=14; F=10.424; P=0.000), while the other compounds did not differ from the control at this damage level. In leaves with third-level damage, the content of ECG increased most (by 85%), in leaves with second-level bug damage, EGC increased most (by 53%), while C increased most (by 64%) in leaves with first-level bug damage. Otherwise, GA content decreased in the leaves damaged by *A. lucorum*, particularly after second-level damage, with GA content decreasing by 25%.

After mechanical damage, changes to these six compounds differed, with GA increased by 100% and C increased by 93%, significantly higher than that of the control (GA: df=14, F=6.108, P=0.007; C: df=14, F=2.813, P=0.023), and ECG content increasing only by 1.20%. Meanwhile, EGCG, EC, and EGC levels all decreased, EGCG by -23.04%, EC by -2.54 % and EGC by -8.12%, respectively, compared to the control (Table 5).

Discussion and Conclusion

As *Apolygus lucorum* damage to tea increased, so too did caffeine content. There may be two reasons for this phenomenon: Many studies have found caffeine content in resistant varieties of plants to be significantly higher than that in conventional varieties. In addition, increased levels of caffeine distributed in the leaves, fruit, flowers and buds of plants might protect these plants against pest damage [16,17]. Caffeine is a bitter tasting alkaloid which might have an inhibiting effect on subsequent feeding behavior by insects due to toxicity. This is a part of the complex chemical defense system of plant to resist pests in which secondary metabolites like caffeine work alone or in combination to help plants resist insect feeding behavior [18-23]. On the other hand, the increased caffeine content might be due to the damage caused by *A. lucorum* arousing the activity of some enzymes in tea. When tea plants are in a poor external environment, they often induce or suppress different secondary plant compounds or enzymes to better adapt to the environment. Many studies have found that protective enzymes such as Phenylalanine Ammonialyase (PAL), Peroxidase (POD), and Polyphenol Oxidase (PPO), are closely related to the insect resistance of plants [24]. Further research is needed to determine the mechanism behind this change in caffeine content.

The reduction in theanine that we found with increasing bug damage might be due to the activity of some enzymes related to the metabolism of theanine stimulated by *A. lucorum* feeding. L-theanine synthetase and L-theanine hydrolase have both been found to affect the metabolism of theanine, and L-theanine synthetase could catalyze the synthesis of tea ammonia by glutamic acid and amine as a substrate catalyst, which is the opposite effect of theanine hydrolase [25-27]. From this we inferred that the damage from *A. lucorum* might inhibit

Damage classification \ Content changes	Caffeine		Theanine		Tea polyphenols	
	Content (ng/kg)	Rate of change (%)	Content (ng/kg)	Rate of change (%)	Content (ng/kg)	Rate of change (%)
Normal	9.27 ± 0.23c	-	1.39 ± 0.08ab	-	326.27 ± 11.29c	-
First-level	10.07 ± 0.57c	8.63	1.64 ± 0.14ab	17.99	427.57 ± 15.57ab	30.43
Second-level	12.37 ± 0.30ab	33.44	1.37 ± 0.08ab	-1.44	517.97 ± 43.54a	58.76
Third-level	13.23 ± 0.26a	42.72	1.08 ± 0.10b	-22.30	467.23 ± 47.11ab	43.20
Acupuncture	12.07 ± 0.23b	30.20	1.80 ± 0.47a	29.50	415.13 ± 8.34b	27.24

Note: The data in the table showed the mean ± standard error by Duncan's multiple range test, the same column after the data lowercase letters indicate significant differences at *P*<0 05

Table 4: Effect of damage of *Apolygus lucorum* on the content of caffeine, theanine and tea polyphenols in tea (Qingdao, China).

Damage classification \ Content change's	GA		EGC		C		EGCG		EC		ECG	
	The average fresh weight (ng/kg)	Rate of change (%)	The average fresh weight (ng/kg)	Rate of change (%)	The average fresh weight (ng/kg)	Rate of change (%)	The average fresh weight (ng/kg)	Rate of change (%)	The average fresh weight (ng/kg)	Rate of change (%)	The average fresh weight (ng/kg)	Rate of change (%)
Normal	0.40 ± 0.03 b		1.97 ± 0.10 ab		0.14 ± 0.01 b		14.41 ± 0.86 ab		1.18 ± 0.14 a		5.70 ± 0.22 b	
First-level	0.32 ± 0.05 b	-20.00	2.49 ± 0.60 ab	26.40	0.23 ± 0.06 a	64.29	15.24 ± 3.52 ab	5.60	1.52 ± 0.33 a	28.81	8.85 ± 0.83 ab	55.26
Second-level	0.30 ± 0.04 b	-25.00	3.00 ± 0.31 a	52.28	0.14 ± 0.08 b	0	18.37 ± 2.80 ab	27.48	1.35 ± 0.16 a	14.41	6.66 ± 0.82 ab	16.84
Third-level	0.37 ± 0.06 b	-7.50	2.64 ± 0.25 a	34.01	0.24 ± 0.08 ab	71.43	18.74 ± 0.63 a	30.05	1.20 ± 0.08 a	2.54	10.53 ± 0.71 a	84.76
Acupuncture	0.80 ± 0.16 a	100.00	1.81 ± 0.21 b	-8.12	0.27 ± 0.05 a	92.86	1.09 ± 1.48 b	-23.04	1.15 ± 0.10 a	-2.54	5.82 ± 0.47 b	1.20

Note: Gallate Acid (GA), Epigallocatechin (EGC), Catechin (C), Epigallocatechin Gallate (EGCG), Epicatechin (EC), and Epicatechin Gallate (ECG). The data in the table shows the mean ± standard error by Duncan's multiple range test, while different lowercase letters indicate significant differences at P<0 05

Table 5: Effect of damage by *Apolygus lucorum* on the content of catechins in tea (Qingdao, China).

L-theanine synthetase activity or stimulate L-theanine hydrolase activity, leading to a decrease in theanine. Another reason might be the increase in tea polyphenols with increasing damage, which could lead to a decrease in theanine synthesis because an ethylamine needed for production of theanine is also a synthesis precursor of catechin phloroglucinol nucleusin new tea shoots [28]. Theanine has a caramel aroma and a taste similar to glutamate, easing the bitterness and increasing the sweetness of tea. Thus a decrease in theanine due to *A. lucorum* damage would reduce tea quality.

Of the six catechins tested, ECG, C, EGC and EGCG increased the most. The damage of *A. lucorum* may activate some enzymes related to the synthesis of catechins in tea, thereby increasing the content of tea polyphenols at the same time. Because tea polyphenols are one of the main factors determining tea color and taste, their levels directly affect tea quality [29]. Damage by *A. lucorum* increasing the levels of tea polyphenols could lead to an increase in tea color turbidity, decrease in sweetness, increase bitter taste and a general decline in tea quality [30].

What's more, we found that there were some differences between the effects of damage by *A. lucorum* on tea and the mechanical damage on tea. After the mechanical damage, the content of caffeine was higher than that of the first-level damage by *A. lucorum* and lower than that of the third-level damage, the content of the anine was higher than that of *A. lucorum* damage, and the content of tea polyphenols was lower than that of *A. lucorum* damage. The main difference of *A. lucorum* damage and mechanical damage lies in secreting salivary enzyme during *A. lucorum* sucking the leaf tissue. It is supposed that the salivary enzyme secreted into the tea tissue by *A. lucorum* affected the activity of some enzymes in tea, which caused the change of nutritional characters.

In summary, caffeine, theanine, and polyphenols were all affected by bug damage, and each of these changes can influence tea color, taste and nutritional value. The mechanism of `nutrition change in tea leaves caused by *A. lucorum* would be studied based on the salivary enzyme secreted into the tea tissue in molecular level.

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