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Antifungal effects of sisal leaf juice on *Lasiodiplodia theobromae*, the causal agent of mulberry root rot

Hong Hui Xie^{1*}, Fu Yu Huang², Xia Lan³, Xiao Ling Zhang⁴, Hua Ning Wang⁵, Hui Lv⁶ and Xuan Zhang⁷

¹Guangxi Subtropical Crops Research Institute, Nanning, China.

²Dongfang Farm, Guangxi State Farms, Qinzhou, China.

³College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China.

⁴Shanxu Farm, Guangxi State Farms, Nanning, China.

⁵Xinguang Farm, Guangxi State Farms, Qinzhou, China.

⁶Hongshan Farm, Guangxi State Farms, Yulin, China.

⁷Dongfeng Farm, Guangxi State Farms, Nanning, China.

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This study was carried out to evaluate the antifungal activities of leaf juices (fresh juice, fermented juice, boiled juice and sterile juice) of nine sisal varieties on *Lasiodiplodia theobromae*, the causal agent of mulberry root rot. Results show that all the leaf juices could inhibit the mycelial growth in different degrees (the inhibitory rates ranged from 63.3 to 100%), due to different varieties and treatments. Among the nine varieties, the inhibition effects of hybrid 76416 and *Agave americana* were the best with absolute inhibition of all the leaf juice treatments against the mycelial growth, followed by *Agave Amaniensis*, *Agave viridis*, *Agave angustifolia* and Hybrid 11648. The inhibitory effect of some fresh juices would be cut down after being fermented, boiled and sterilized. The treated mycelia of *L. theobromae* were malformed, enlarged, broken and plasma leaked when observed under the microscope. Most of the leaf juices could inhibit the conidial germination absolutely, except *A. amaniensis*, H.11648 and *A. angustifolia*. The average germination rate of *A. amaniensis*, H.11648 and *A. angustifolia* was 72.4, 16.6 and 13%, respectively. The control efficiency of the fresh juice of H. 11648 against mulberry root rot in the field reached 73.1%.

Key words: Sisal, leaf juice, anti-fungi, anti-fungal activities, mulberry root rot, *Lasiodiplodia theobromae*.

INTRODUCTION

Sisal (*Agave sisalana* Perrine) belonging to the Agavaceae family, is an important hard fiber crop popularly grown in tropical and subtropical regions. Sisal

is grown in more than 20 countries and the area is about 330 thousand hectares in the world (Huang, 2008). The hybrid 11648 was introduced in China in 1963 and quickly

*Corresponding author. E-mail: xhh317@126.com. Tel: +86-771-2539021.

became the major variety in south China (Cai, 2000). The sisal culture is one of the main economic activities and has an important social function due to the unfavorable weather and soil conditions in the hilly grounds of Guangxi province, China. Sisal fiber is the main product from this crop, but it only represent approximately 5% of the leaf fresh weight, the remaining 95% including solid and liquid residues (juice of the sisal leaf, containing 81%), are normally discarded by sisal farmers (Oashi, 1999; Suinaga et al., 2006). A number of waste liquid flowed freely around those scutching spots and strong tart flavor and foul smell arose due to the ferment of the waste which seriously polluted the surroundings. Sisal waste principally contains plant tissue (lignin and cellulose), primary and secondary metabolites such as alkaloids, phenolic compounds, glycosidic saponins, flavonoids, and tannins and water, amongst others (Santos et al., 2009; Chen et al., 2011). In an attempt to utilize this waste, many researches were conducted and researchers have published that the sisal waste or residue contains compounds which can inhibit the growth of *Fusarium mangiferae*, the pathogen of mango malformation disease (Zhang et al., 2010) and the tumor cells of liver and gastric cancer which caused human death (Hu et al., 2010), poison the larvae of mosquitoes (Pizarro et al., 1999), the gastrointestinal nematodes of goats and sheep (Botura et al., 2011; Roberta et al., 2012), the banana burrowing nematode (Jesus et al., 2015) and the *Pomacea canaliculata*, a kind of destructive agricultural pest (Li et al., 2012). And also, the sisal waste could be used as fertilizer to improve crop production (Lacerda et al., 2006).

The fungus, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Pat.) is pleomorphic, plurivorous and ubiquitous soil-borne pathogen in the tropics and subtropics and is associated with up to 500 plant hosts (Úrbez-Torres et al., 2008). Mulberry root rot caused by *L. theobromae* in Heng County of Guangxi Province, China, was first reported in 2014 as a new disease and had seriously influenced the healthy and sustainable development of the local sericulture industry (Xie et al., 2014).

Therefore, with a view to controlling the mulberry root rot and making good use of the waste liquid of sisal industry, the antimicrobial activity of sisal leaf juice of nine varieties on *L. theobromae*, the causal agent of the mulberry root rot was evaluated and the control efficiency of the fresh leaf juice of H. 11648 against mulberry root rot was tested in field in this work.

MATERIALS AND METHODS

Preparation of sisal leaf juice

The fresh juice of nine sisal varieties (*Agave amaniensis*, *Agave fourcroydes*, hybrid 76416, Hybrid 11648, *Agave angustifolia*, *Agave viridis*, *Agave sisalana*, *Agave americana* and hybrid NY1) were extracted from leaves collected from the Germplasm Resources Nursery of Sisal in Nanning, China, by using a juicer. The fermented

juice, boiled juice and sterile juice were made by laying fresh juice at 55°C for 20 days, boiling fresh juice for 90 min and sterilizing for 20 min at 121°C, respectively.

Effects of leaf juice on the mycelial growth of *L. theobromae*

The fresh juice and fermented juice were filtered by bacterial filter ($\Phi=0.22\ \mu\text{m}$). Two milliliters of the four treated juices was mixed into 18 ml sterilized and cool PDA medium and poured into Petri dish (90 mm) to make plate, respectively. The 6-mm-diameter mycelia disc from the edge of the actively growing colonies was put on the center of the dishes, then cultured at $28\pm1^\circ\text{C}$ for 3 days. Each of the treatment was replicated for three times while the control plates (without sisal juice) used equal volume of sterilized water to replace the juice. The mycelial growth of all the treated plates was recorded by measuring the cross diameter of the colonies at three days after inoculation when the upper surface in the control plate was fully covered with the mycelia of *L. theobromae*. The inhibition percentage was calculated using the formula of Opara and Wokocha (2008):

$$\text{Inhibition percentage} = [(dC - dT) / dC] \times 100$$

Where, dC = average mycelial growth of control, dT = average mycelial growth of treated plates.

Effects of leaf juice on the conidial germination of *L. theobromae*

For the spore germination test, spores suspension was prepared from pycnidia collected from 20 days old culture of *L. theobromae* grown on PDA medium with sisal juice of the nine varieties. Sterile distilled water was used to replace sisal juice to make spores suspension for the control. The concentration of all spores suspension treatments were approximately $1.3\times10^8/\text{ml}$ and $10\ \mu\text{l}$ suspension was placed on the slide. All the slides were then placed in an inverted position in moist chambers. Each of the treatment was replicated in three times. Spore germination was recorded under microscope in three microscope fields after 15 h of culture and five hundred spores were observed in each field. Percent spore germination was calculated by using the following formula (Sandipan et al., 2014):

$$\text{Percent spore germination} = \frac{\text{Germination spores}}{\text{Total number of spores}} \times 100$$

Field experiment of leaf juice against mulberry root rot

According to the above inhibition results, fresh leaf juice (leaf liquid residue) of a H. 11648 was used to test the field control efficiency against mulberry root rot. The field experiment was conducted from April 2014 to August 2015 in Heng county, Guangxi province, China. The diseased plants and dead plants in the experimental mulberry yard were surveyed and confirmed to be the root rot trees by surveying the symptoms and identifying the pathogens before sprinkling 25 kg leaf juice on the superficial soil near mulberry plants of each plot at the first time. The diseased and dead rate of mulberry root rot in the experimental yard was about 10%. Each row (about 10 m long, containing 80 plants) of the yard was set to be an experimental plot and five plots of every treatment were prepared that is, five replicates. The juice was used once a month and the control plots were sprinkled with water in equal volume. The plants were calculated again at 15 days after the last time of sprinkling leaf juice and the diseased or dead trees were clarified to be caused by the pathogen of the mulberry root rot. The control

Table 1. Inhibition rates of four treated juice of nine sisal varieties against the mycelia growth of *L. theobromae*.

Sisal varieties	Juice concentration (ml/10 ml)	Juice treatments and inhibitory effects							
		Fresh juice		Fermented juice		Boiled juice		Sterile juice	
		D	R	D	R	D	R	D	R
<i>A. amaniensis</i>	1.00	0.80±0.01f	97.60±0.03b	0.80±0.01f	97.60±0.03b	0.80±0.01e	97.60±0.03b	0.80±0.01g	97.60±0.03b
<i>A. fourcroydes</i>	1.00	3.00±0.03b	73.20±0.04f	3.20±0.06b	68.30±0.05h	3.40±0.02b	65.90±0.03g	3.40±0.03c	65.90±0.03g
Hybrid No.76416	1.00	0.00±0.00g	100.00±0.00a	0.00±0.00g	100.00±0.00a	0.00±0.00f	100.00±0.00a	0.00±0.00h	100.00±0.00a
H.11648	1.00	2.00±0.01d	85.40±0.01d	2.30±0.04d	79.30±0.04e	2.30±0.03c	79.30±0.01e	2.50±0.02d	76.80±0.02e
<i>A. angustifolia</i>	1.00	1.30±0.01e	91.50±0.02c	1.80±0.01e	85.40±0.04d	2.00±0.01d	82.90±0.02d	2.20±0.02e	80.50±0.09d
<i>A. virdis</i>	1.00	0.08±0.01f	97.60±0.02b	1.10±0.01f	96.30±0.07c	0.90±0.01e	96.30±0.02c	2.00±0.01f	82.90±0.03c
<i>A. sisalana</i>	1.00	2.60±0.06c	75.60±0.12e	2.80±0.03c	73.20±0.04f	3.30±0.04b	67.10±0.04f	3.30±0.04c	67.10±0.04f
<i>A. americana</i>	1.00	0.00±0.00g	100.00±0.00a	0.00±0.00g	100.00±0.00a	0.00±0.00f	100.00±0.00a	0.00±0.00h	100.00±0.00a
Hybrid NY1	1.00	2.60±0.00c	75.60±0.01e	3.00±0.02c	70.70±0.02g	3.40±0.04b	65.90±0.03g	3.60±0.03b	63.40±0.04h
CK		8.80±0.03a		8.80±0.03a		8.80±0.03a		8.80±0.03a	

Data showed in the table are the average of three replicates. D, Average mycelia growth diameter of treated plates (cm); R, relative inhibitory rate (%). Different lowercases letters in the same column represent significant difference at 5%.

effects were evaluated by using the following formulas (Lai, 1998) and made some appropriate improvements:

$$\text{Diseased and dead percentage (DDP)} = \frac{\text{Diseased plants} + \text{dead plants}}{\text{Total plants}} \times 100$$

$$\text{Control effects percentage} = \frac{\text{CDDP of control plots} - \text{CDDP of treated plots}}{\text{CDDP of control plots}} \times 100$$

$$\text{Correcting DDP (CDDP)} = \text{DDP after sprinkling juice} - \text{DDP before sprinkling}$$

All the collected data in this work were statistically analyzed by using DPS (Version 7.05) software and means were separated by Duncan multiple range test at 5% probability level.

RESULTS

Inhibition of sisal leaf juice on the mycelial growth of *L. theobromae*

All the sisal juice of nine varieties could inhibit the

mycelial growth of *L. theobromae* significantly with the inhibitory rates ranging from 63.4 to 100%, especially the juice of hybrid No. 76416 and *A. americana* which inhibited the mycelial growth absolutely (Table 1). The inhibition effects of H. 11648 and *A. angustifolia* were at a middle level and the inhibitory rates of four juice treatments were 91.5, 85.4%, 82.9, 80.5 and 85.4, 79.3, 79.3, 76.8%, respectively. The inhibitory rates of *A. sisalana*, *A. fourcroydes* and hybrid NY1 ranged from 63.4 to 75.6%; had lower inhibition effects than others. In fresh juice treatments, the inhibition rates of hybrid No. 76416 and *A. americana* were the highest (100%) followed by *A. amaniensis* (97.6%) and *A. virdis* (97.6%) and the inhibition rate of H. 11648 was 85.4%. Though the juice of *A. fourcroydes* was the worst among the nine varieties, its inhibition rate (73%) was still significantly higher than the control. Inhibition effects of fresh juice of some varieties (*A. virdis*, *A. fourcroydes*, *A. angustifolia*, H.11648, *A. sisalana* and hybrid NY1) would be cut down a little while

being fermented, boiled and sterilized, but the inhibitory rates of the four treatments of hybrid 76416, *A. americana* and *A. amaniensis* would not be changed and kept at 100, 100 and 97.6%, respectively. The colonies of *L. theobromae* were blackened with dense substrate mycelia and little aerial mycelia in most treatments (Figure 1). The mycelium was enlarged and malformed (*A. angustifolia*, *A. sisalana*, et al.), broken (H. 11648, *A. fourcroydes*, *A. virdis*, etc), broken with protoplasm leaked (H. 11648, *A. americana*, *A. amaniensis*, etc) (Figure 2).

Inhibition of sisal leaf juice on the conidial germination of *L. theobromae*

Except the juice of *A. amaniensis*, H.11648 and *A. angustifolia*, the four treated juice of the other six varieties could inhibit the conidial germination absolutely. Although, the conidia could germinate in the juice of H.11648 and *A. angustifolia*, the

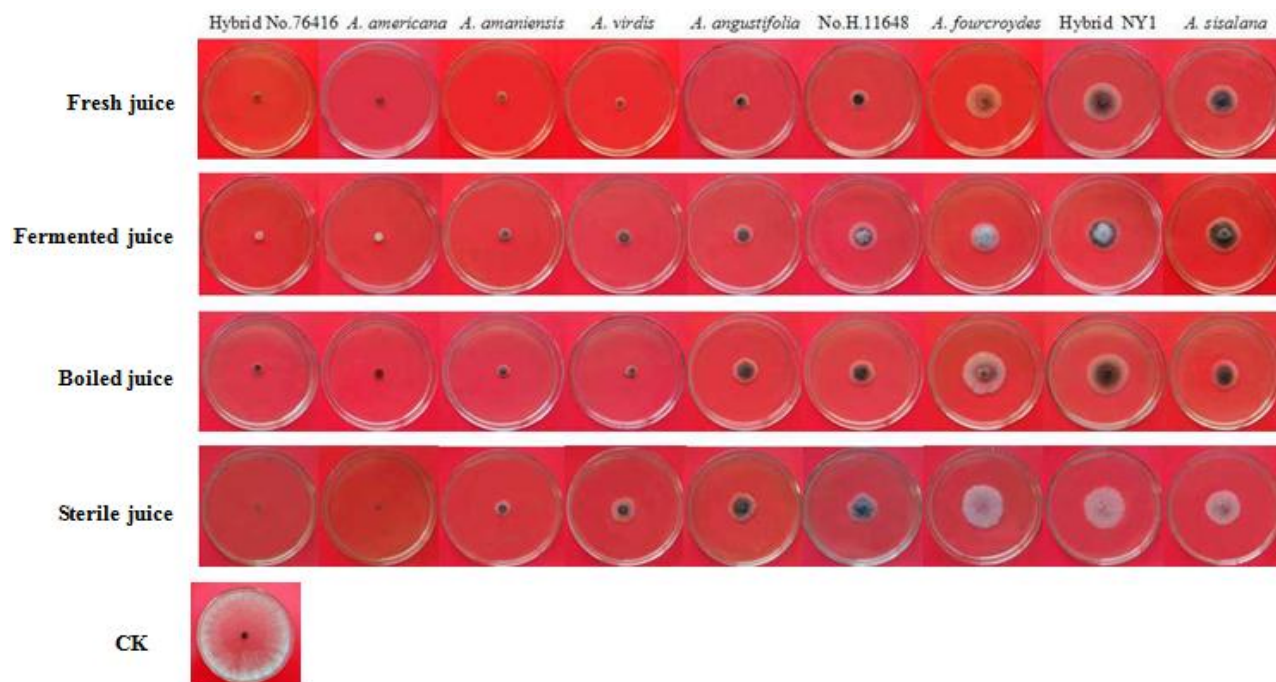


Figure 1. The colonies of *L. theobromae* on the PDA media treated with different sisal juice.

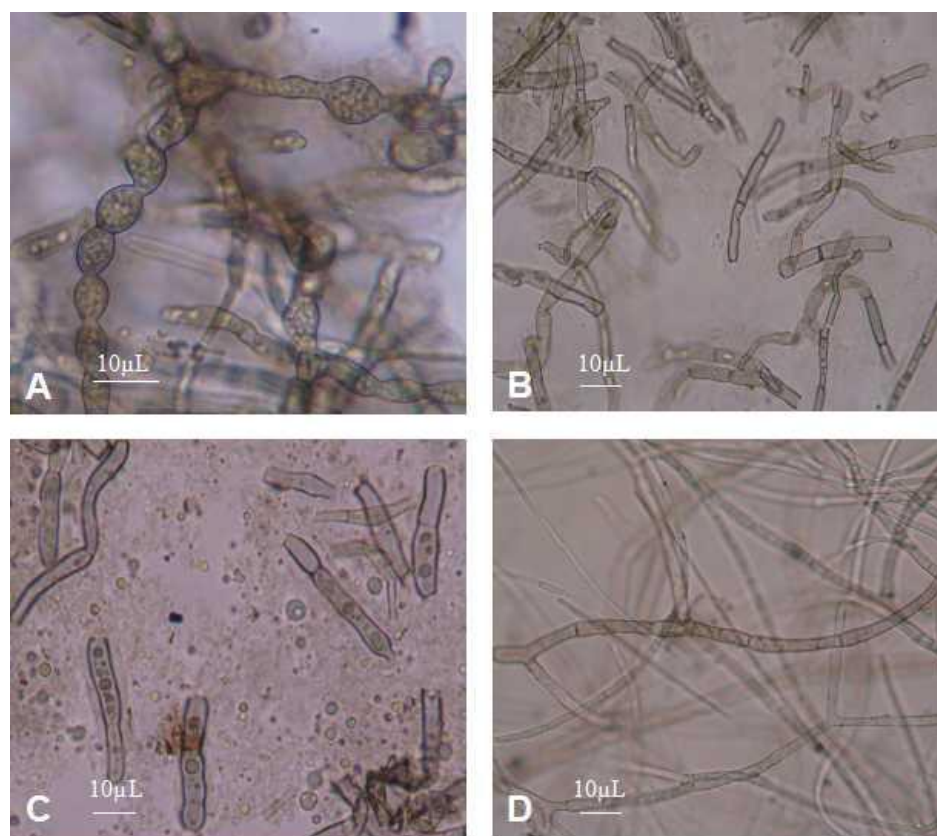


Figure 2. Mycelia characteristics of *L. theobromae* treated with leaf juice of sisal. **A.** Enlarged and malformed mycelia. **B.** broken mycelia. **C.** broken mycelia with protoplasm leakage. **D.** normal mycelia.

Table 2. Conidia germination rates of *L. theobromae* in different treatments of sisal leaf juices.

Varieties	Conidial germination rate				Average germination rate
	Fresh juice	Fermented juice	Boiled juice	Sterile juice	
<i>A. amaniensis</i>	70.40±0.16b	72.60±0.21b	72.00±0.14b	74.40±0.15b	72.40±0.13b
<i>A. fourcroydes</i>	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
Hybrid No.76416	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
H.11648	14.00±0.06c	15.40±0.17c	17.60±0.04c	19.20±0.12c	16.60±0.15c
<i>A. angustifolia</i>	10.80±0.09d	12.60±0.11d	12.00±0.10d	16.60±0.21d	13.00±0.08d
<i>A. virdis</i>	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
<i>A. sisalana</i>	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
<i>A. americana</i>	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
Hybrid NY1	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e	
CK	98.40±0.07a	98.40±0.07a	98.40±0.07a	98.40±0.07a	98.40±0.07a

Data shown in the table are the average of three replicates. Different lowercase letters in the same column represent significant difference at 5%.

Table 3. Control efficiency of fresh juice of H. 11648 against mulberry root rot in field.

Treatments	DDP before sprinkling juice	DDP after sprinkling juice	CDDP	Control efficiency
Leaf juice	14.54±0.05%b	18.78±0.05%b	4.28±0.03%b	73.10%
CK	15.56±0.06%a	31.47±0.02%a	15.91±0.03%a	

Different lowercases letters in the same column represent significant different at 5%. DDP, diseased and dead percentage, CDDP, correcting DDP.

germination rates were very low. The average conidial germination rate in the fresh juice of H. 11648 and *A. angustifolia* was 16.6 and 13% respectively. But the conidia in the juice of *A. amaniensis* had relatively high germination rates (above 70%) and the average germination rate of four treated juice was 72.4% (Table 2).

Control efficiency of fresh leaf juice of H. 11648

As mentioned before, H. 11648 is the main cultivated variety and others are just planted and preserved as germplasm resources in China and, the leaf juice of H. 11648 could significantly inhibit the mycelial growth and conidial germination of *L. theobromae*. Therefore, the fresh leaf juice of H. 11648 collected from the Shanxu Farm of Guangxi State Farms was used for the field experiment due to its adequate sources and significant inhibitions. Results show that the fresh leaf juice of H. 11648 had significant control efficiency (73.1%) against the disease after continuously sprinkling of the juice for about 16 months (Table 3).

DISCUSSION

The search for natural products from plants and agro-

industrial waste, which may become useful to society, has been the subject of intense research in recent years (Harvey, 2007; Lee et al., 2007), and botanical pesticides with low toxicity, little or no residue and environment-friendly due to the source of the plant itself had become the highlight of researchers (Corato et al., 2010; Wang et al., 2012). The anti-fungi activities of ethanol extracts of 77 plants containing *Alpinia chinensis*, *Eleutherine plicata*, *Vatica xishuangbannensis*, *Morindu cochinchinensis* and *A. americana* against 12 plant pathogenic fungi were studied; results showed that the ethanol extracts of 15 plants had inhibitory effects on the target pathogens and the extracts of *A. americana* could significantly inhibit the mycelia growth of *Exserohilum turcicum* with the inhibition rate of 76.6% (Zhang et al., 2011). The extracts of *Acalypha hispida*, *Chromolaena odorata*, *Azadirachta indica* and *tetrapleura tetraptera* could effectively inhibit the mycelia growth of *L. theobromae* and the inhibition rates ranged from 30.2 to 88.44% (Adeniyi and Joseph, 2015). Ethanol extracts and anthraquinones isolated from the root extract of *Coccoloba mollis* showed anti-fungi activity against *L. theobromae*, the inhibition rate of the most active compound (emodin) was up to 44% (Barros et al., 2011). As a cosmopolitan soil-borne fungus, *L. theobromae* could cause both field and storage diseases on more than 280 plant species including fruits, crops and

plantation trees (Talukdar, 1974; Singh et al., 1977; Domsch et al., 1980). In most cases, the pathogen and its induced diseases were managed by chemical fungicides, such as Dithane M-45 (Jayanta and Raj, 1989; Bhadra et al., 2014), carbendazim and thiophanate methyl (Banik et al., 1998; Mahmood and Gill, 2002; Shahbaz et al., 2009; Sultana and Ghaffar, 2010) but chemical fungicides may cause phytotoxicity to the mulberry trees and poison the silkworms due to the high sensitive to many chemicals. Therefore, searching for the botanical fungicide and applying biocontrol become very important in controlling the diseases of mulberry. In our work, leaf juice of sisal was used for antifungal tests and showed promising results for their use as fungicides and all leaf juices of the nine sisal varieties had strong antifungal activities against *L. theobromae*. The results also revealed that anti-fungi compounds extensively existed in the plants of *Agave*. The fresh juice of H. 11648 could effectively control the mulberry root rot in field. Leaf juice of sisal made the mycelia of *L. theobromae* to be enlarged, malformed, broken and protoplasm leaked, which is similar to the antagonistic mechanisms of some biocontrol microbes (Brain et al., 1945; Chen et al., 2009; Ikeda et al., 2012; Levy et al., 2015). Different inhibition effects on the target pathogen indicated that the concentrations of the antifungi active substances contained in those leaf juices might be different. Antifungal activities of some leaf juices would be cut down after being fermented, boiled and sterilized, but the others would not be changed, indicating that differences existed in the types and physicochemical properties of the antifungi substances in the leaf juices extracted from the same variety and the different varieties; some antifungal substances have thermal stability and others do not. The result of the present work is similar to that of Zhang et al. (2010). So, it may not be a simple antifungal substance but a kind of antifungal composites or compounds contained in the sisal leaf juice. Promising purification work of the antifungal compounds from sisal waste will be conducted in subsequent studies.

Conclusion

Liquid residue (leaf juice), the by-product of sisal industry showed significant anti-fungal activities on *L. theobromae* and control efficiency against the mulberry root rot disease, which revealed a new way not only for raising the reasonable and comprehensive utilization of sisal waste, but also for controlling the mulberry root rot and other diseases caused by *L. theobromae*.

Conflict of interests

The authors have not declared any conflict of interest.

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