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Effect of crude toxins of *Ustilaginoidea virens* on rice seed germination

Rongtao Fu, Jian Wang, Cheng Chen, Xueshu Gong and Daihua Lu*

Institute of Plant Protection, Sichuan Academy of Agricultural Science, No. 20 Jingjusi Road, Chengdu, Sichuan Province, China.

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The plant pathogen *Ustilaginoidea virens* is known to generate secondary metabolite ustiloxins, which are poisonous to humans and domestic animals and to affect plant cells during the growth process. In this study, rice false smut was collected from different locations in Sichuan province and the strains isolated. The crude toxins of *U. virens* were extracted from the liquid culture medium of isolated strains to determine the effects on rice seed germination. Our results, as expected, show that the crude toxins had inhibitory effects on the growth of rice germ and radicle. Moreover, these inhibitory effects were maintained even when the toxin was diluted up to 100 times. Crude toxins caused higher inhibition in the radicle than in the germ. Inhibitory effects of crude toxins on rice germination varied significantly depending on the location of where the isolated strains were extracted. This study also showed that inhibition of rice seed germination by crude toxins was different depending on the type of rice used, which corresponded with the field disease resistance evaluation.

Key words: *Ustilaginoidea virens*, crude toxins, inhibitory effect, significant difference.

INTRODUCTION

Ustilaginoidea virens (Cooke) (Takahashi, 1896) (teleomorph: *Villosiclava virens*) is a causative agent of the false smut of rice (Tanaka et al., 2008). The pathogen initiates infection through rice floral organs in the rice booting stage and forms white spikelet balls, which are called smut balls. The smut balls change their color to yellow, then orange, and finally to greenish black (Fu et al., 2015a; Hu et al., 2014; Tang et al., 2013). False smut not only results in severe yield losses but also contaminates rice grains and straw, which poses a risk of human or animal poisoning (Li et al., 1995; Shan et al., 2012). Previous studies report that *U. virens* produces two types of mycotoxins, named ustiloxins and ustilaginoidins. Ustiloxins belong to the cyclopeptides containing a 13-membered core structure. Six ustiloxins have been identified and named as ustiloxins A, B, C, D, E and F (Zhou et al., 2012; Lu et al., 2014). Ustiloxins is an inhibitor of cell growth, especially by inhibiting microtubule assembly and cell skeleton formation (Koiso et al., 1994). Ustilaginoidins are naphthalene pyrone derivatives, which have shown weak antitumor cytotoxicity in human epidermoid carcinoma (Koyama et al., 1998).

*Corresponding author. E-mail: 453831354@qq.com

Table 1. Isolates of conidiospore used in this study.

Isolate	Locality	Longitude	Latitude	Sample number	Isolates number
UvLZ	Luzhou	105.39	28.91	13	11
UvYA	Yaan	102.97	29.97	8	7
UvPJ	Pujiang	103.29	30.2	10	5
UvXD	Xindu	104.13	30.82	13	6
UvNC	Nanchong	103.47	30.42	5	4
UvYB	Yibing	104.56	29.77	5	5
UvMY	Mianyang	104.73	31.48	8	8
UvZZ	Zizhong	104.85	29.81	10	9
UvQL	Qionglai	103.47	30.42	9	7
UvMZ	Mianzhu	104.19	31.32	12	10
UvJT	Jintang	104.32	30.88	9	8
UvPX	Pixian	103.86	30.8	8	8
UvMY	Miyi	101.41	26.42	5	4
UvWJ	Wenjiang	103.81	30.97	13	10

When livestock was fed with the rice grains and forage contaminated by the rice false smut pathogen, they showed a great variety of symptoms such as poor growth, diarrhea, abortion, and hemorrhage (Nakamura et al., 1994; Luduena et al., 1994). Additionally, ustiloxins, acting as phytotoxins, have a wide range of biological activities in plants, such as growth inhibition of the plumule and the radicle during seed germination of maize and wheat and abnormal swelling of seeding roots (Kois et al., 1994; Tian and Tao, 2000).

Ustiloxin is the main toxin component that has been isolated and identified from rice false smut balls and mycelia of the *U. virens* (Shan et al., 2013; Fu et al., 2015b). In previous studies, Chen et al. (2004) suggested that the rough ustiloxins extracted from false smut balls strongly inhibited the growth of plant roots. In the present study, the rice seed was used with rough toxins extracted from the liquid culture medium of the pathogen. The aims of the study were to determine the toxins obtained from different isolated strains of *U. virens* at various concentrations, on seed germination of different rice varieties.

MATERIALS AND METHODS

Fungal collection, isolation and culture conditions

Naturally infected rice spikelets showing typical false smut symptoms were collected in a field plot at Luzhou, Yaan, Pujiang, Xindu, Nanchong, Yibin, Mianyang, Zizhong, Qionglai, Mianzhu, Jintang, Pixian, Miyi, and Wenjiang, Sichuan province, China, in 2015 (Table 1). The samples were stored in dry envelopes at room temperature.

The yellowish false smut balls, which were covered with a mass of chlamydospores, were surface-sterilized with 0.1% mercuric chloride solution for 30 s and rinsed four times with sterile water. The treated samples were resuspended into a conidiospores suspension and diluted to 10^3 ml^{-1}. The spore suspension was poured onto potato sucrose agar (PSA, which was made from a boiled extract of 300 g of peeled potatoes, 20 g of sucrose, and 10 g of agar per liter of distilled water) solid medium and subsequently incubated at 28°C in the dark. When visible colonies appeared, the colonies were transferred individually onto fresh PSA plates and incubated at 28°C in the dark (Fu et al., 2014). Each isolate was individually maintained *in vitro* containing 10 ml of a PSA solid medium at 4°C.

Preparation of crude ustiloxins

The fungus was transferred into the PS fluid medium (PS, which was made from a boiled extract of 300 g of peeled potatoes, and 20 g of sucrose) at 28°C under 150 rpm by shaking for 10 days. The nutrient solution contained toxins that had been produced. The mycelium was filtered with sterile gauze then the supernatant was harvested by centrifuging at room temperature for 8 min at 8000 rpm. The supernatant (100 ml) was concentrated under vacuum at 60°C by a rotary evaporator to a concentrated dry matter. The dry matter was dissolved in 100 ml of methanol and shaken for 2 h to fully extract, and then the supernatant was collected by centrifuging. The supernatant was concentrated under vacuum at 55°C by a rotary evaporator to a concentrated dry extract. The dry extract, which was in the form of crude ustiloxins, was dissolved in 100 ml of distilled water. The water extract was stored at 4°C until required.

Rice materials and treatment

Seven rice varieties were tested in this experiment: '9311', 'Guichao 2', 'F you 399', '2 you 838', 'Y liangyou 1', 'Jingyou 127', and 'Chuanyou 6203', respectively. The full rice seeds were chosen and surface-sterilized with 2% sodium hypochlorite solution for 90 s and rinsed three times with sterile water. Rice seeds were germinated in sterile water at 27°C for 48 h.

Bioassays of ustiloxins

For the experiment, 20 sprouted seeds of rice were placed into each sterile Petri dish containing 15 sample solutions and covered with two layers of sterilizing filters. These treatments were placed in a biochemical incubator at 27°C for 4 days in the dark. Then, the length of the germ and radicle of each seed was measured. The crude ustiloxins was diluted into different ratios of the concentration solution: 10, 50 and 100. The length of germ and radicle of treated seeds were compared with those grown in distilled water (control group). In addition, the number of radicle of each processing was counted in this test. The experiments were repeated two times.

Statistical analysis

All of the experiments were carried out two times and the data from the two runs of each test were pooled. All of the statistical analyses of variance were performed by using SPSS statistical analysis software. The statistical comparisons performed among strains, among rice varieties and among toxin concentrations. The treatment means were separated using Fisher's protected least significant difference test (LSD) (α=0.05).

The inhibitory rate of the germ (radicle) growth (%) = [(Average length of control group germ (radicle) – Average length of treated germ (radicle) / Average legth of control group germ) × 100].

RESULTS

Isolates of *U. virens*

In this study, 128 samples of false smut balls which came from Sichuan province, China, in 2015 were collected and 102 isolates were obtained by using the suspension liquid of chlamydospores (Table 1).

Effect of different concentration of crude ustiloxins on germination of rice seeds

The crude toxins were extracted from 14 *U. virens* strains that were isolated from 14 Sichuan regions (Table 2). As shown in Table 2, the crude toxins strongly inhibited the germ and radicle, and the number of radicle of '9311' and the inhibitory rate of radicle was higher than the germ. The results show that the different concentrations had different effects on rice seed germination and that the inhibition rate, which was diluted 10 times, was significantly higher than the inhibition rate shown for rice germination that was diluted 50 times. The experiment found that the inhibitory effects of the toxin on germ and radicle growth were maintained even after being diluted 100 times but were significantly lower than 10- and 50-times dilutions.

Effect of different crude ustiloxins

In order to investigate the effect of different crude ustiloxins on the growth of rice seed, 14 toxins that were extracted from different *U. virens* strains isolated from 14 Sichuan regions were studied (Table 3). The results show that toxins extracted from UvJT6, UvNC1-1, and UvQL3-2 showed significantly higher inhibitory rates of germ growth compared to the toxins extracted from the other 11 strains. The inhibitory rates of germ growth in these three strains were 76.13, 81.55 and 76.13%. Moreover, for UvJT6, UvNC1-1 and UvQL3-2 strains, the inhibitory rates of the radicle growth were 96.83, 97.53, and 88.47%, respectively, and the inhibitory rates of radicle number were 91.38, 91.88 and 87.96%, respectively. Both of these effects were also significantly higher than most other strains.

As shown in Table 3 and Figures 1 and 2, the crude toxins showed greater inhibition of the radicle growth and numbers, while the inhibition of the germ growth was relatively small. This outcome could be explained because the radicle came in direct contact with the toxin, and the absorption eventually led to the death of seedling.

Effect of crude toxin on the germination of different rice varieties

The results show that the toxins had a certain inhibitory effect on the germ and radicle of each rice variety (Table 4). The inhibitory rate of the radicle growth was higher than that of the germ growth, and the range of inhibitory ratio was 62.78 to 82.06 and 26.71 to 78.8%. The inhibitory action of germination of 'Guichao 2' was significantly higher than the other six varieties, and the inhibitory ratio of the radicle and germ was 82.06 and 78.8%, respectively. The inhibitory effect of '2 you 838' and 'Jingyou127' was relatively poor. In addition, the results indicate that the inhibitory effect was different based on the test varieties and that these results were similar to the disease resistance of rice varieties in the field. Hence, such rice seed varieties can be further considered to identify the mechanisms of resistance to false smut and screen the resistant materials.

DISCUSSION

The ustiloxins may exhibit a broad range of biological activities in animals and plants and can be used for medicinal and agrochemical purposes (Zhou et al., 2012; Li et al., 1995; Shan et al., 2013). It was previously reported that the toxins were mainly obtained from rice false smut (Chen et al., 2004; Fu et al., 2015b; Shan et al., 2013). But in this study, the crude toxins were extracted from the liquid diet of *U. virens* mycelium. The findings show that the crude toxins had inhibitory effects on the germ and radicle growth and that the inhibitory effects on radicle were significantly higher than for the germ. This could be because the radicle directly contracted the toxin through absorption. The results were similar to those of previous findings of Chen et al. (2004). In addition, the experiment found that the toxins maintained their inhibitory effects on the growth of the

Table 2. The different concentration of crude ustiloxins on germination of rice seeds.

Strain	Concentration ratio	Inhibitory rate of germ (%)	Inhibitory rate of radicle (%)	Inhibitory rate of radicle number (%)
	100	46.41^{cB}*	62.39^{cA}	49.58^{c}
UvNC1-1	50	61.95^{bB}	82.98^{bA}	82.07^{b}
	10	76.13^{aB}	97.53^{aA}	91.88^{a}
	100	$29.79^{c\ B}$	49.04^{cA}	57.98^{c}
UvJT6-1	50	42.64^{bB}	71.3^{bA}	72.55^{b}
	10	81.55^{aB}	96.83^{aA}	91.38^{a}
	100	35.28^{cB}	36.17^{cA}	49.58^{c}
UvQL3-2	50	62.31^{bB}	77.24^{bA}	77.59^{b}
	10	79.54^{aB}	93.81^{aA}	87.96^{a}
	100	44.97^{cB}	34.69^{cA}	55.74^{c}
UvYA1-2	50	58.72^{bB}	74.22^{bA}	71.43^{b}
	10	70.57^{aB}	88.47^{aA}	83.75^{a}
	100	34.6^{cA}	28.01^{cB}	56.3^{c}
UvPJ1-1	50	48.56^{bA}	43.94^{bB}	62.75^{b}
	10	74.37^{aB}	92.08^{aA}	82.07^{a}
	100	42.28^{cB}	45.57^{cA}	64.99^{c}
UvPX5-2	50	47.74^{bB}	67.54^{bA}	65.83^{b}
	10	62.31^{aB}	91.34^{aA}	80.11^{a}
	100	25.23^{cB}	45.57^{cA}	34.73^{c}
UvXD5-1	50	35.39^{bB}	62.89^{bA}	49.58^{b}
	10	60.34^{aB}	90.1^{aA}	71.99^{a}
	100	21.03^{cB}	44.09^{cA}	13.17^{c}
UvMY1-2	50	36.11^{bB}	66.35^{bA}	66.39^{b}
	10	50.11^{aB}	90.1^{aA}	76.75^{a}
	100	22.83^{cB}	48.49^{cA}	40.34^{c}
UvWJ2-1	50	32.99^{bB}	62.35^{bA}	57.42^{b}
	10	53.02^{aB}	84.36^{aA}	77.59^{a}
	100	31.41^{cB}	3.51^{cA}	50.14^{c}
UvLZ10-2	50	46.16^{bB}	31.82^{bA}	61.34^{b}
	10	53.34^{aB}	85.16^{aA}	75.49^{a}
	100	13.85^{cB}	68.43^{cA}	0.28^{c}
UvYB5-2	50	44.15^{bB}	71.3^{bA}	53.22^{b}
	10	58.47^{aB}	82.48^{aA}	71.99^{a}
	100	26.42^{cB}	63.48^{cA}	39.22^{c}
UvMY1-1	50	31.8^{bB}	71.75^{bA}	59.94^{b}
	10	41.53^{aB}	63.73^{aA}	76.47^{a}
	100	44.62^{cB}	46.71^{A}	63.87^{c}
UvZZ9-1	50	47.7^{bB}	49.14^{bA}	71.99^{b}
	10	57.43^{aA}	54.38^{aB}	77.79^{a}
	100	24.12^{cB}	49.47^{cB}	52.1^{c}
UvMZ9-2	50	$37.44^{b\ B}$	53.71^{bB}	63.87^{b}
	10	$49.75^{a\ B}$	58.44^{aB}	70.59^{a}
Control group	-	27.86 mm	20.21 mm	3.57

*Different lowercase letters indicate significant difference among toxin concentration for each strain. *Different capital letters indicate significant difference between germs and radicle.

Table 3. Effect of crude ustiloxins on germination of '9311' rice seed.

Strain	Concentration ratio	Length of germ (mm)	Inhibitory rate of germ (%)	Length of radicle (mm)	Inhibitory rate of radicle (%)	Number of radicle	Inhibitory rate of radicle number (%)
UvNC1-1	10	6.65	76.13a^{b*}	0.5	97.53^{a}	0.29	91.88^{a}
UvJT6-1	10	5.14	81.55^{a}	0.64	96.83^{a}	0.31	91.38^{ab}
UvQL3-2	10	5.7	79.54^{a}	1.25	93.81^{ab}	0.43	87.96^{bc}
UvYA1-2	10	8.2	70.57^{c}	2.33	88.47^{cde}	0.58	83.75^{ab}
UvPJ1-1	10	7.14	74.37^{bc}	1.6	92.08^{bc}	0.64	82.07^{cde}
UvPX5-2	10	10.5	62.31^{d}	1.75	91.34^{bc}	0.71	80.11^{def}
UvXD5-1	10	13.09	60.34^{de}	2.01	90.1^{cd}	1	71.99^{gh}
UvMY1-2	10	13.9	50.11^{g}	2	90.1^{cd}	0.83	76.75^{fg}
UvWJ2-1	10	13.09	53.02^{fg}	3.16	84.36^{de}	0.8	77.59^{ef}
UvLZ10-2	10	13	53.34^{g}	3	85.16^{de}	0.875	75.49^{fg}
UvYB5-2	10	11.57	58.47^{e}	3.47	82.48^{e}	1	71.99^{h}
UvMY1-1	10	16.29	41.53^{h}	7.33	63.73^{f}	0.84	76.47^{fg}
UvZZ9-1	10	11.86	57.43^{ef}	12.06	54.38^{g}	0.66	77.59^{fg}
UvMZ9-2	10	13.4	49.75^{g}	8.41	58.44^{f}	1.3	63.59^{i}
Control group	-	27.86	-	20.21	-	3.57	-

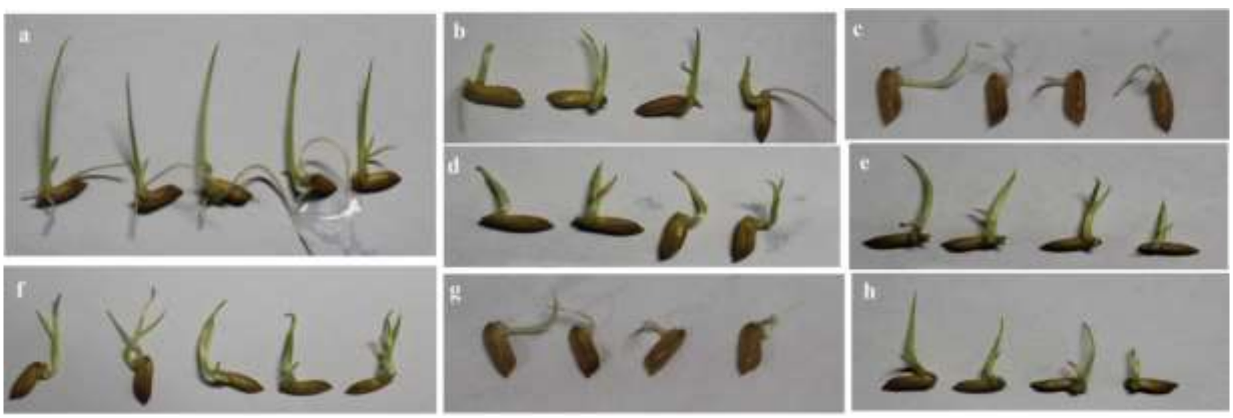

Figure 1. Effect of crude ustiloxins on the germ and radicle of '9311'. a. CK; b. UvZZ9-1; c. UvQL3-2; d. UvPJ1-1; e. UvWJ2-1; f. UvMY1-1; g. UvJT6-1; h. UvYA1-2.

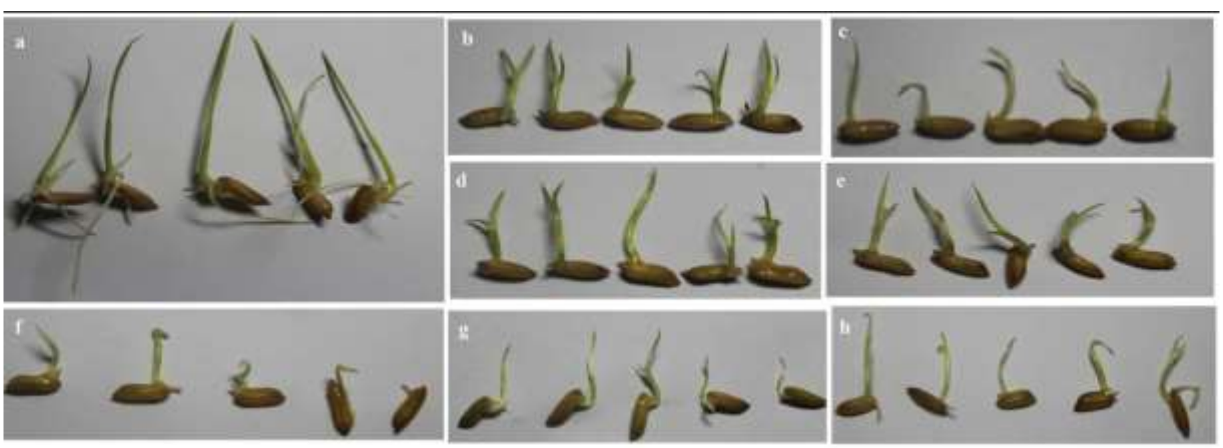

Figure 2. Effect of crude ustiloxins on the germ and radicle of '9311'. a. CK; b. UvLZ10-2; c. Uv MY1-2; d. Uv MZ9-2; e. UvYB5-2; f. UvNC1-1; g. UvPX5-2; h. UvXD5-1.

Table 4. Effect of crude toxin of UvJT6-1 on the germination of different rice varieties.

Rice varieties	Concentration ratio	Germ length (mm)	Inhibitory rate of germ (%)	Length of radicle (mm)	Inhibitory rate of radicle (%)	Number of radicle	Inhibitory rate of radicle number (%)
Guichao 2	10	5.28	78.8^{aB}*	3.23	82.06^{aA}	0.48	77.14^{a}
9311	10	8.33	66.55^{bB}	3.56	80.22^{abA}	0.5	76.19^{a}
F you 399	10	8.11	67.43^{bB}	4.56	74.67^{cdA}	0.75	64.29^{b}
Y liangyou 1	10	7.89	68.31^{bB}	4.8	73.33^{cdA}	0.69	67.14^{b}
Chuanyou 6203	10	11.18	55.1^{cB}	4.23	76.5^{bcA}	0.89	57.62^{c}
2 you 838	10	14.86	40.32^{dB}	5.31	70.5^{dA}	1.24	40.95^{d}
Jingyou127	10	18.25	26.71^{eB}	6.7	62.78^{eA}	1.56	25.71^{e}

germ and radicle even after being diluted 100 times. However, this finding was not consistent with the former reports in which the low concentration of toxin stimulated the germination of rice seed (Wang et al., 1995). Because ustiloxins have inhibitory effects on rice radicle, it is possible that the inoculation by a spikelet with conidial suspension may hurt the young ear, eventually leading to death of a section or the whole spikelet. Therefore, the conidial suspension should undergo dialysis to remove toxins before inoculation.

This study utilized toxin extracts from different isolates with rice seeds. The results show that these toxins, depending on the location of the strain, had significantly different physiological effects on germ and radicle. If there is a certain direct correlation between the toxicity of ustiloxins and the pathogenicity of *U. virens*, it needs further research. To identify false smut resistant rice varieties, inoculums that can generate stronger toxicity of ustiloxins can be chosen to obtain more effective and reliable results.

For now, the resistance identification method mainly adopts artificial inoculation with spore suspensions or natural inducements in the field (Fu et al., 2015a; Yang et al., 2011). This study found that the toxin had different inhibitory actions on the germination of rice varieties and that there was significant difference among the varieties. The findings are similar to resistance identification of rice varieties in the field (Fu et al., 2015a). If this method can reduce manpower, financial resources and identification time by using the toxins as selection pressure of resistant material, it will become our focus in field research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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