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In vitro selection of sheath blight resistance germplams in rice

Liang XiaoXing, Li Ping and Zheng AiPing*

Rice Research Institute of Sichuan Agricultural University, Chengdu, 611130, China.

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An effective rice callus screening method for breeding disease-resistant regenerated plants of sheath blight caused by *Rhizoctonia solani* Kühn is yet to be developed. In this study, callus-resistant rice was screened using pathogenic crude toxins of highly virulent isolates, *R. solani* (Anastomosis Group 1-IA) collected from Sichuan Province, China. Highly susceptible rice cultivars, Lemont and moderate resistant rice cultivar, Kasalath were used for callus screening. The detached leaf and micro-chamber methods were used to evaluate the resistance of the regenerated plants (R_0) and their offspring (R_1). The disease resistance levels of R_0 and R_1 obviously improved when compared with the control (P>0.05). Rice seedlings were inoculated with 0.25 g of *R. solani* mycelium (fresh weight) at the base of the plant and then covered with transparent plastic bottles to maintain high humidity after inoculation. The resistance of R_1 was enhanced than their progenitors, the disease index of R_1 of Kasalath was 2.27 which was higher than that of IR64, 9311, TeQing and Gan72, while the disease index of R_1 of Lemont was 3.14 which was higher than that of 9311, TeQing and Gan72. According to the nine-grade disease classification, the R_1 of Lemont was moderately resistant, while the R_1 of Kasalath was resistant.

Key words: *Rhizoctonia solani* Kühn, toxin, micro-chamber method, disease index, stem lesion heights, highly resistant cultivars.

INTRODUCTION

Sheath blight disease caused by the soil-borne necrotrophic fungal pathogen, *Rhizoctonia solani* Kühn (*R. solani*) is a serious problem in all rice-growing countries and one of the most destructive rice diseases in the world (Srinivasachary and Serge, 2011). Over the years, many researchers have worked to develop sheath blight-resistant cultivars through conventional breeding and achieved only marginal success due to the lack of resistant germplasms and the limited efficiency and effectiveness of the available screening methods (Willocquet et al., 2012). For breeding purposes, replicated inoculated field nurseries are the most common means of evaluating overall responses to a pathogen. The field screening method requires a large amount of inoculum, high humidity conditions, and long observation periods to complete field evaluations and summarize results. Disease ratings in the field are labor intensive and involve multiple assessments of disease severity from where the infections are initiated (the flood water level) up to the top of the canopy. Plot ratings are a visual average of the number of plants observed in a plot. Results are often confounded by many variables, including fluctuations in environmental conditions and the distribution of inocula (Jia et al., 2007). When compared with the field screening method, plant tissue culture techniques offer the advantages of simplicity, rapid completion and convenience. Some resistant plants hav been obtained. Carlson (1973) used the toxin medium of a tobacco wildfire pathogen as a selection agent and screened stable genetic disease mutants. Other reports

*Correcponding author. E-mail: aipingzh@163.com. Fax: 028-86290903.

have focused on the *Helminthosporium* toxin screening of maize and sugar cane, black shank toxin screening of grapes and *Phytophthora* toxin screening of potatoes (Vanden, 1991). Since then, scholars from many countries have also screened disease-resistant mutants, such as those of corn, canola, wheat, tomato and rice (Margaret, 1986; Vanden, 1991).

Ao et al. (2006) used the callus and suspension cells of mature rice embryos as screening materials and the crude toxin produced by *R. solani* as a selection pressure to obtain regenerated seedlings. The crude toxin screening can strongly enhance plant resistance (Carlson, 1973). Vidhyaseharan et al. (1990) reported that screened plants exhibited some form of resistance that was heritable and stability resistance was observed in the R_1, R_2, and R_3 gene-rations. Researchers have screened some resistant varieties from semi-dwarf rice, such as LSBR-33, LSBR-5, ZH5, YSBR1, Jasmine85, and TeQing using the field screening method (Zuo et al., 2009). The micro-chamber method is a new, widely used method to evaluate rice resistance (Deshpande et al., 2006; Jia et al., 2011; Jia et al., 2012). This method was initially conceived and developed by Ali at the Bangladesh Rice Research Institute, Jia used this method to evaluate 1794 accessions from 114 countries (Jia et al., 2011).

When compared with field detection, the micro-chamber assay reduces environmental effects, has a short experimental period, takes three to five weeks per cycle, requires a small space for experimental operations, presents unlimited experimental time, all year round applicability, and reliable statistics, and applies numerical measurements to replace traditional visual measurements (Jia et al., 2007). These advantages allow filtration of a large number of resistant germplasm resources. The greenhouse testing method used in sheath blight studies is one application of these advantages. The micro-chamber screening method for resistance testing has been widely used to determine disease-resistant rice varieties and provide valuable resources for breeding.

The objectives of this study are (i) to use the crude toxins of *R. solani* for screening of the callus of susceptible rice cultivars and breeding sheath blight-resistant rice and (ii) to develop a standardized robust screening method in the greenhouse scale to search for germplasm lines resistant to sheath blight by *R. solani*.

MATERIALS AND METHODS

Plant materials

Rice cultivars, Tetep, Kang60, Lemont, Kasalath, Gan72, IR64, TP309, 9311 and TeQing were used in this study. Rice plants were grown in pots in a greenhouse or under field conditions. Rice plants at three or four leaves stage were used for inoculation of *R. solani*.

Culture medium

The culture media used in the experiments were as follows: potato sugar agar (PSA, 200 g/L potato, 20 g/L sugar, 15 g/L agar, pH 7.0), potato sucrose broth (PSB, 200 g/L potato, 20 g/L sucrose, pH 7.0), and Richard's medium (10 g/L potassium nitrate, 5 g/L potassium dihydrogen phosphate, 2.5 g/L magnesium sulfate·$7H_2O$, 0.25 g/L ferric sulfate, 50 g/L sugar, pH 7.0). The medium for callus induction from rice seeds was composed of NB (N6 salts and B5 vitamins, supplemented with 30 g/L sucrose, 500 mg/L proline, 300 mg/L casein hydrolate, 500 mg/L glutamine) and 2 mg/L 2,4-dichlorophenoxyacetic acid (pH 5.8). The medium for callus growth was composed of NB and 2 mg/L 1-naphthaleneacetic acid (NAA) (pH 5.8). The medium for bud differentiation from the callus was composed of NB, 10 mg/L kinetin and 0.4 mg/L NAA (pH 5.8). The medium for bud growth was composed of NB with 0.1 mg/L NAA. The medium for rooting was composed of 1/2 Murashige and Skoog salts (MS), 30 g/L sucrose and 0.25 mg/L NAA (pH 5.8). The pH of all of the solutions was adjusted with KOH.

All the media were autoclaved for 20 min at 0.10 to 0.15 MPa at 121°C and inoculated after attaining room temperature.

Fungal culture and toxin production

R. solani AG1-IA strain was isolated and preserved by the laboratory of the Rice Research Institute of Sichuan Agricultural University. The virulent isolate of *R. solani* AG1-IA was grown on PDA at 28°C for two days. Five mycelium plugs (6 mm diameter) from PDA cultures were transferred into a 150 mL flask containing 50 ml of PSB. The cultures were then incubated at 28°C on a rotary shaker (180 rpm) for two days and then stored at 4°C.

For toxin production, four mycelium groups from the PSB cultures were transferred into a 500 ml flask containing 200 ml of Richard's medium; *R. solani* produces toxins in this medium. The cultures were incubated under stationary conditions at 28°C on artificial vibration once a day for 15 to 20 days. Crude toxins were obtained by passing the liquid through four layers of cheesecloth and Whatman No. 1 filter paper, then concentrated to a quarter of the original volume. The biological activities of the crude toxins were determined by the leaf-necrosis assay on the rice cultivar (9311).

Callus cultivation

Lemont and Kasalath, which are highly susceptible and moderate resistant to *R. solani* AG1-IA respectively, were stored in the Rice Research Institution of Sichuan Agricultural University. The callus cultivation method proposed by Vidhyasekaran et al. (1990) was followed with some modifications. The toxin medium was calculated by volume (v/v). Only the first- or second-passage calli were used in the toxin treatment. Toxin medium screening included callus pre-culture, crude toxin treatment and toxin-free media culture. To bring as many calli as possible into contact with the toxin, the calli were crushed into pieces as small as possible (about 4 mm in size) with an inoculating spoon. The callus pieces were cultivated in different concentrations of toxin medium.

During toxin medium processing, well-growing calli were selected from pre-cultured calli inoculated with 5, 10, and 15% toxin medium for 10 to 15 days for inoculation calli inoculated in toxin-free medium and served as control; those that exhibited poor growth or were dead were discarded. The calli were cultivated in toxin-free medium at an interval of two inoculations of toxin medium to restore growth for 12 to 15 days. After the proliferation culture, callus mutants were derived. Every 2 weeks, the calli were transferred to a new batch of toxic-free medium and bright-yellow calli were transferred to a differentiation medium. After two weeks, the calli began to turn green. After three weeks, shoots began to sprout and roots began to grow. The seedlings were cultured in rooting medium. When their roots had fully grown, the seedlings were planted in a field in Lingshui, Hainan Province, south of China, where rice is planted all year round.

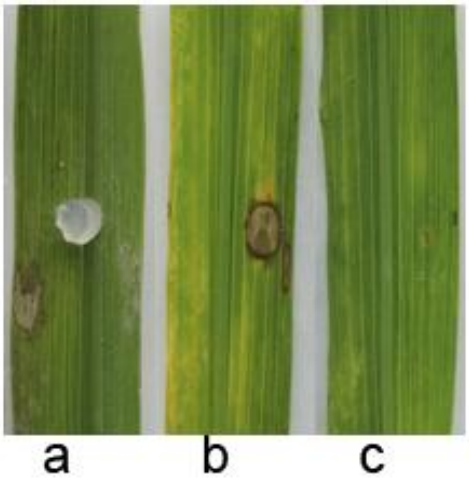

Figure 1. The virulence of mycelium and culture filtrates of *R. solani* AG1 IA to rice leaves. “a” was infected by the mycelium of AG1 IA, “b” was inoculated by culture filtrate of AG1 IA, “c” was treated with sterile water.

In vitro resistance evaluation

The sheath blight resistance of the regenerated plants (R_0) was determined by leaf necrosis assay. The R_0 were screened for their efficacy in suppressing sheath blight symptoms in a detached leaf assay. Plants at the three- to four-leaf stage in the field were cut and brought to the laboratory in an ice box for surface sterilization. They were then cut into uniform lengths of 8 cm and placed in 15 cm diameter sterilized glass Petri dishes containing moistened filter paper according to the method of Vijay et al. (2011) with some modifications. Three pieces of leaf per Petri dish was replicated once to ensure the result was reliable. In each Petri dish, sterilized filter paper was placed on the edges of the leaf pieces to prevent inward rolling. A mycelium block (6 mm diameter) was placed on the surface of leaf pieces in the Petri dish.

Leaves sprayed with sterile distilled water served as the control treatment in each assay. The Petri dishes were incubated in a growth chamber at 28 ± 1°C and 16 h light. About 48 h after incubation, the leaves were rated for sheath blight disease lesions. Sheath blight severity was rated by the relative lesion area calculated as the total lesion area divided by the total leaf area. Plants in which the lesion area was less than 25% were classified as resistant, whereas those with a lesion area of over 25% were classified as susceptible.

Micro-chamber method

Rice cultivars Tetep, Kang60, Lemont and Gan72 were used as standards to evaluate the sheath blight resistance of the regenerated plants. IR64, TP309, 9311 and TeQing were used to compare the sheath blight resistance of the regenerated plants. The micro-chamber method was performed by removing the screw caps and bottoms of 2 or 3 L soft drink bottles. Before planting in pots, rice seeds were germinated on moist filter paper in Petri dishes for 2 days at 28°C. Three seedlings of similar germination rate were selected and planted at least 3 cm apart in a 15 cm diameter plastic pot filled with soil obtained from the field. Soil was first steam sterilized to ensure that the results will not confound with the presence of soil-borne *R. solani* inocula. Pots were placed in a greenhouse until the three- to four-leaf stage before inoculation. Pots were arranged in a completely randomized design with the susceptible Lemont and Gan72 and the resistant Kang60 and Tetep served as standards.

Each cultivar was represented by three replicate plants per pot per replication. Each seedling was inoculated with 0.25 g of mycelium (fresh weight) placed and pressed up to the base of the stem, ensuring that the mycelium was in contact with the plant. After inoculation, each pot was covered with a soft drink bottle. The screw caps and bottoms of the 2 or 3 L bottles that had been pushed down into the soil were removed to create a seal that allowed the bottle to serve as micro-humidity chambers. The cap was removed from the bottle. Day and night greenhouse temperatures averaged 30 ± 2°C and 26 ± 2°C, respectively. Inoculated pots were maintained in trays containing water in the greenhouse. The relative humidity was always above 80 ± 2% within the bottles.

Lesion development was monitored starting three days post inoculation (dpi). Plants were evaluated for disease reactions approximately 7 to 10 dpi or when Lemont and Gan72 exhibited highly susceptible reactions. Diseased seedlings were cut at the ground line and the length of the lesion was measured along with the length of the stem using a ruler. The disease index was calculated as the stem lesion length divided by the plant height and multiplied by 9 (Marchetti and Bollich, 1991). Micro-chamber inoculations were repeated twice for each cultivar.

Data analysis

Data of the leaf necrosis assay and the micro-chamber test were subjected to ANOVA. When significant treatment differences were found, means were compared by the test of least significant difference (LSD, $P<0.5$).

RESULTS

The crude toxins of *R. solani* grown on Richad’s media can induce leaf necrosis and the symptoms was similar to those produced by pathogenic *R. solani* (Figure 1), this means that the crude toxins contain pathogenic factors and can be used to screen rice callus. The crude toxins concentration used in the callus screening is very important. The calli in the differentiation medium were green but slowly became chlorotic and would not form plantlets when the toxin concentration was greater than 20% (Figure 2); thus, to favor callus regeneration, a maximum toxin concentration of 15% was adopted.

In leaf bio-assay of Lemont, the relative lesion area of R_0 screened from different toxin concentration was 24, 28 and 32%, respectively, and no difference was found among the resistance of R_0 screened from different toxin concentrations ($P<0.05$), while the relative lesion area of the control was 70 and 72%, respectively, which had significant difference ($P>0.05$) with the resistance of the R_0. The relative lesion area of R_1 was 33, 35 and 40%, respectively, no difference was found in the resistance of R_1 screened from different toxin concentrations ($P<0.05$), but they had difference with the control (Table 1). The relative lesion area of R_0 and R_1 had no difference that is to say that the resistance of R_0 was transmitted to R_1. These results show the toxin screening callus method

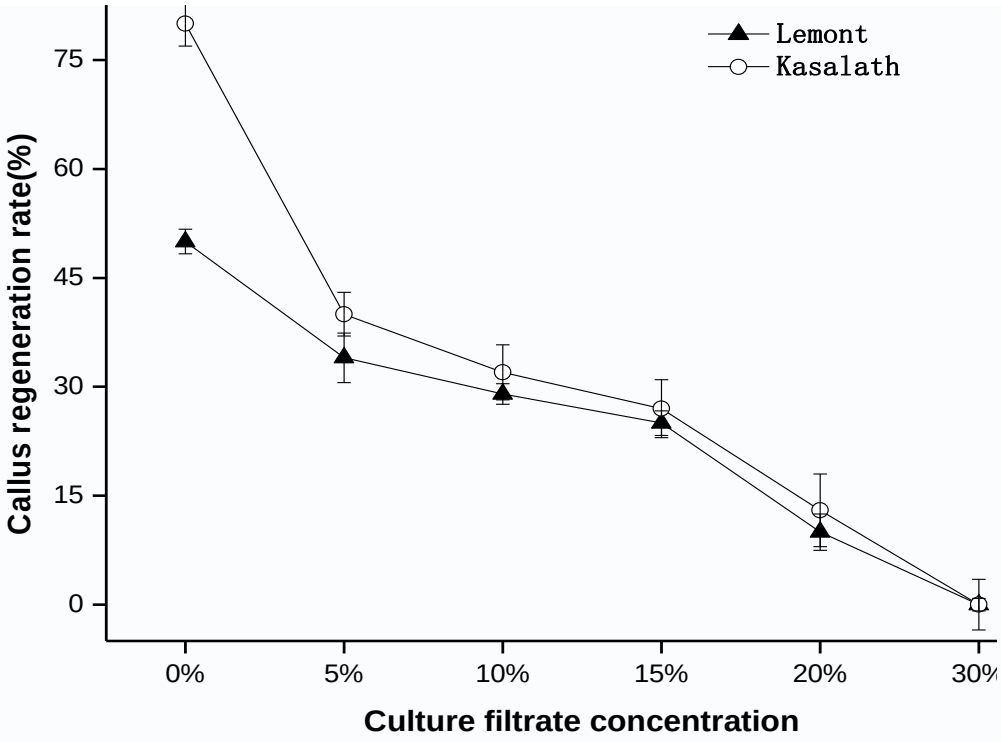

Figure 2. Effect of pathogen culture filtrates on rice callus regeneration.

Table 1. The relative lesion area of Lemont in the leaf bio-assay in different toxin concentration medium.

Culture filtrate concentration (%)	Relative lesion area (%)	
	R_0	R_1
0	70[a]	72[a]
5	24[b]	33[b]
10	28[b]	35[b]
15	32[b]	40[b]
Lemont	72[a]	79[a]

is an effective way to change the resistance of Lemont and the traits can be transmitted in the next generation. In micro-chamber assay, the disease index of R_1 that screened from 5% of toxin concentration was 3.14 which was 57.91% higher than the control, had significant difference with the control (Figure 3). That is to say that the resistance of R_1 was also higher than that of the control.

In leaf bio-assay of Kasalath, the relative lesion area of R_0 screened from 5% of toxin medium was 22%, and the resistance of R_0 had no significant difference among the regenerated plants screened from different toxin concentration medium, that result is similar to Lemont (Table 2). In the low-concentration screening, no significant difference was found in the R_0. The relative lesion area of the R_1 was 28, 31 and 33%, respectively, and no difference was found among the resistance of R_1 screened from different toxin concentrations ($P<0.05$). The resistance of R_1 had difference with the control ($P>0.05$), the resistance of Kasalath also can be transmitted from R_0 to R_1 (Table 2). In micro-chamber assay, the disease index of R_1 was 2.27 which was higher than that of the control (Figure 4).

In leaf bio-assay of Lemont and Kasalath, the R_0 resistance of Lemont and Kasalath was enhanced when compared with the control in terms of overall resistance level ($P>0.05$), respectively. The resistance of R_1 was weaker than that of R_0. Weakening of offspring resistance agreed with the results of Buiatti and Ingram (1991) and Brooks (2007). No significant difference was found in R_0

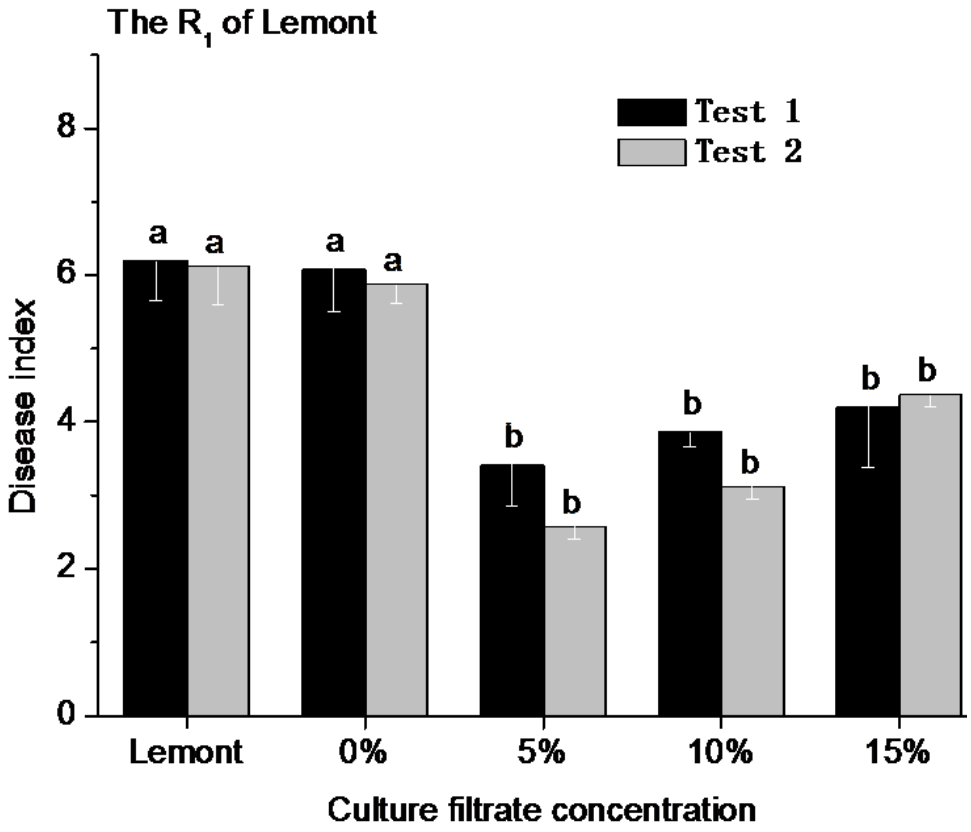

Figure 3. Sheath blight disease index of R_1 of Lemont inoculated with *R. shlani* inocula in micro-chamber assay. Test 2 is a complete experimental repeat of test 1, the bar means topped with the same letters do not significantly differ at 5% level in a test. S = Susceptible, R = resistance. Lemont were controls.

Table 2. The relative lesion area of Kasalath in the leaf bio-assay in different toxin concentration medium.

Culture filtrate concentration (%)	Relative lesion area(%)	
	R_0	R_1
0	59[a]	58 [a]
5	22[b]	28 [b]
10	25[b]	31 [b]
15	28[b]	33 [b]
Kasalath	61[a]	65 [a]

obtained with different toxin concentrations, and no significant difference was observed during low-concentration screening. During micro-chamber screening, the R_1 of Lemont and Kasalath showed more resistance than the control, which is consistent with the results of the leaf bio-assay.

To confirm that the results of the micro-chamber screening method are reliable, we used four rice cultivars as standards, Kang60 and Tetep (resistant cultivars), as well as Gan72 and Lemont (susceptible cultivars), which produced consistent resistance and highly susceptible reactions, respectively. The resistance of R_1 was significantly enhanced and the lesion height of R_1 was significantly reduced. The resistance of R_1 was compared with the other known resistance cultivars. The resistance of R_1 was enhanced than that of their progenitors, the disease index of R_1 of Kasalath was 2.27 which was higher than that of IR64, 9311, TeQing and Gan72, while

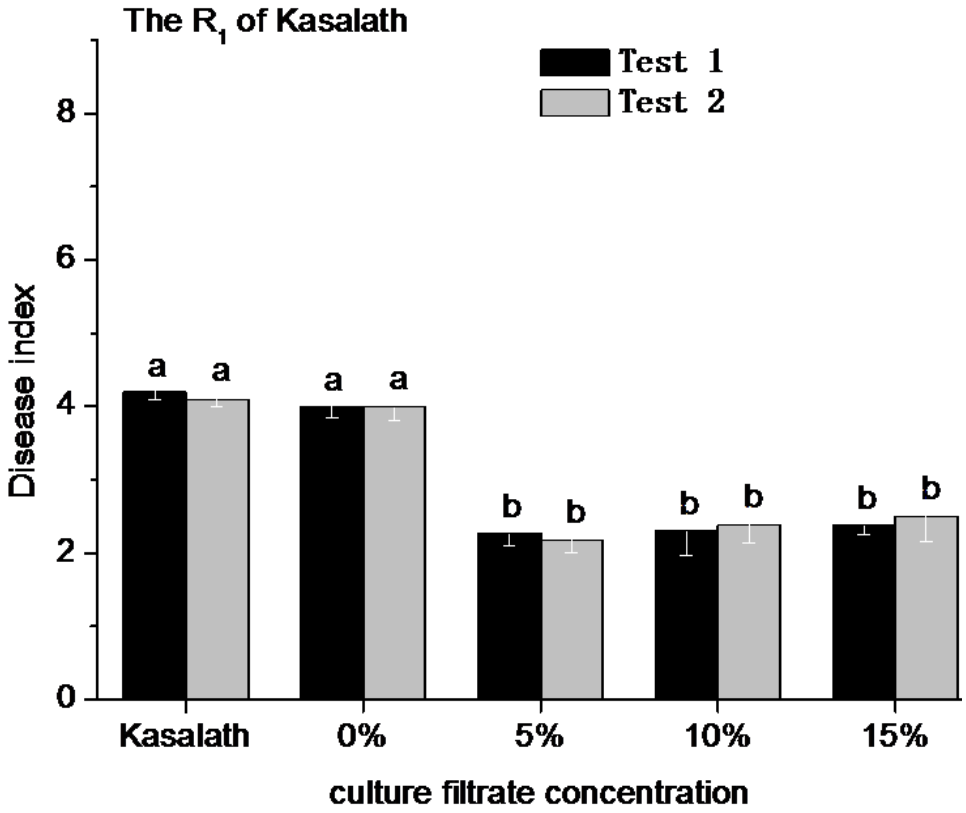

Figure 4. Sheath blight disease index of R_1 of Kasalath inoculated with *R. shlani* inocula in micro-chamber assay. Test 2 is a complete experimental repeat of test 1, the bar means topped with the same letters do not significantly differ at 5% level in a test. S=Susceptible, R = resistance. Kasalath were controls.

Table 3. The disease resistance of the regenerated plant of R_1 when compared with other resistant or susceptible cultivar and lines using micro-chamber method.

Cultivar and line	Disease index	Disease grade
Tetep	1.50±0.10	HR
TP309	1.77±0.50	R
Kang 60	1.83±1.20	R
IR64	3.14±0.67	MR
Kasalath	4.16±0.48	MR
R_1 of Kasalath (5%)	2.27±0. 17	R
9311	4.27±0.51	MS
TeQing	5.09±0.76	MS
Lemont	6.08±0.78	S
R_1 of Lemont (5%)	3.14±0.55	MR
Gan 72	8.68±0.80	HS

Both R_1 of Lemont and Kasalath are the offspring of their R_0 respectively, treated with 5% of *R. shlani* AG1 IA crude toxins. HR represents high resistant, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, HS = high susceptible.

the disease index of R_1 of Lemont was 3.14 which was higher than that of 9311, TeQing and Gan72 (Table 3). According to the nine-grade disease classification, the R_1 of Lemont was moderately resistant, while the R_1 of

Kasalath was resistant.

DISCUSSION

An efficient callus screening method is reported for the first time to improve the resistance of rice cultivars using crude toxins of *R. solani* AG1 IA. The ability of the method to evaluate disease reactions to *R. solani in vitro* or *in vivo* showed significant improvement over past field methods in terms of developing efficient response data for breeding. During *in vivo* test, two kinds of statistical methods were used to analyze the experimental data to ensure accurate experiment results. Using the stem lesion height for disease classification, the results excluded the effect of autoimmune responses and plant height, which is favorable for evaluating the resistance of cultivars and lines. This study is the first to evaluate the resistance of R_1. In the *in vitro* and *in vivo* bio-assay, the resistance of R_1 was weaker than the R_0, this result is similar to the findings of Mohan (2001) and Wang (2012). The crude toxin concentration and the processing time have significant effects on the resistance of regenerated seedlings (Zhou et al., 2006).

The callus is sensitive to the external environment; thus, if the toxin concentration is too high, the callus would easily lose the ability to differentiate. The screen concentration must be effective and the lowest effective concentration must be used. Lemont is highly susceptible to sheath blight, while Kasalath, which is widely used for genetic modification and seedling, is fairly susceptible to the disease. In this experiment, susceptible cultivars were screened in terms of calli to form a selection pressure, and the disease resistance levels of the regenerated plants varied. The leaf bio-assay and micro-chamber screening methods were used to evaluate plant resistance, with the results showing significant differences between the treatment group and the control group. This finding shows that toxin screening is an efficient method for breeding resistant plants. Yoder (1980) believed that a necessary condition for screening resistant plants is that the toxin must be a pathogenic factor. When calli are treated with toxins, the external environment creates a selection pressure that causes the resistance of the calli to change. These changes are not directed; the resistance of some plants is strengthened and some are weakened. After several treatments, the resistant calli were retained and eventually grew into resistant plants. However, the resistance of the progeny of these plants was unstable, and, with the passage of time, it slowly subsided. Thus, the toxin screening method can be used to study the toxin function and if we could get the stable disease resistance plant in the field, then this method will be particularly applicable in breeding.

In micro-chamber assay, the resistance of some R_1 showed significant difference when compared with the control; in some plants, disease resistance became weaker than that of the control. This phenomenon may because resistance of R_1 was unstable, similar to the findings obtained by Buiatti and Ingram (1991) in the culture filtrate of pathogens or ill-defined toxins as a means of selecting genotypes of plants with increased disease resistance. Richard (2007) posited that the variation usually present in cultures of plant pathogens, which is difficult to control, may lead to variable results; thus, the use of pure compounds would seem to be preferable. We obtained fewer Lemont R_0 plants than Kasalath ones because the latter are easier to differentiate into seedlings, which may be related to their germplasm and genetic background. Many researchers have performed experiments to screen calli by toxins but these studies merely evaluated the resistance of R_0 generation and forecasted related information on their offspring; no experimental data were offered to validate the conclusions of the studies. In the present study, we evaluated the resistance of R_0 and detected the resistance of their offspring to provide experimental basis for follow-up experiments.

The micro-chamber assay created a high-humidity environment and suitable temperature for infection to occur. Mycelium did not infect the plants when the greenhouse humidity was above 90%. In the winter, disease progress was delayed by three to five days so disease reactions were evaluated three to five days later. Comparing the results of the leaf bio-assay and micro-chamber assay, the cultivars and lines showed more susceptibility to rice sheath blight in the leaf bio-assay. Many factors affect the resistance of the leaf *in vitro* so the leaf produces a series of autoimmune reactions by itself and its resistance is weakened. The micro-chamber assay involves *in vivo* detection of plants as they complete their mechanism to resist external pathogens. The results of this of type of detection are more reliable than those of the leaf bio-assay. In the leaf bio-assay, the organ quickly turned yellow *in vitro*. When the leaves of a plant are in a state of stress, their metabolism is disrupted and their defense mechanisms may be initiated.

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