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Pathogenicity Determination of Crude Extract Toxin Produced by the Fungus *Pyricularia oryzae* Cav. the Causal Agent of Rice Blast

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ABSTRACT

Series of experiments were carried out to determine the disease determinant factor of crode extract toxin produced by the rice blast fungus, *Pyricularia oryzae*, to determine an optimal concentration of crude extract toxin for screening against this toxin, and to obtain information on the phytotoxic effect of crude extract toxin on different varieties. The result indicated that crude extract toxin produced by *P. oryzae* was disease determinant factors. Leaf trated with this toxin produced spindle shaped to roundish lesion with gray centre and brown margin with or without halo and running lesion. The symptom was similar to those produced by the blast fungus infection. The capability of crude extract incited blast symptom increased when crude extract concentration increased. It was concluded that 25 percent concentration of the crude extract would be an appropriate concentration to inoculate rice plants. Genotypic variation was found among rice varieties against crude extract toxin inoculation. Varieties tested against crude extract toxin could be rated resistant, moderately resistant and susceptible to the toxin. The varieties produced small, roundish brown lesion with gray centre and without halo and running lesion were classified to be resistant whereas the varieties that produced larger round lesion with halo and running lesion were classified as susceptible. The result suggested that variety KTH-17 was considered to be susceptible whereas Usen was resistant.

Key words : crude extract toxin, pathogenicity, *Pyricularia oryzae*, resistance, rice blast.

INTRODUCTION

Rice blast caused by the fungus *Pyricularia oryzae* Cav. is the major disease of rice because of its wide distribution and its destructiveness under favourable environments. The disease occurs in almost all rice growing areas of the world and is the most serious in temperate and tropical area of non irrigated (upland) environments. In temperate region, the blast problem is perpetuated by the high pathogenic variability of the fungus while in the upland environments, blast exacerbated by drought is the major constraint to rice production. As rainfed rice production is becoming important, there is growing concern that seasonal drought and high level of fertilizer application will cause the crop susceptible to blast. Despite the magnitude of the problem, satisfactory control of blast has been achieved in tropical irrigated rice primalrily through the use of resistant varieties. Hence the effective and efficient screening techniques are essential to a successful breeding program for resistance to

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blast. Screening for resistance of rice to blast disease has mostly been based on pathogen inoculation in the field where effectiveness of selection depended on seasonal condition that favoured disease development. Disease severity may vary between geographic locations and seasons. Expression of resistance might also be expected to vary with prevalent races of the fungus at different locations.

In order to improve the efficiency and effectiveness of screening for resistance, toxin inoculation should be an alternative method for the screening. The purpose of the experiments was to determine the disease determinant and appropriate concentration of the crude extract toxin produced by the virulent Khon Kaen isolate of the fungus *Pyricularia oryzae*, to be used for inoculation on rice plants to screen rice plant resistance to blast disease.

Several investigations have been carried out on toxin produced by the blast fungus *Pyricularia oryzae* Cav. Tamari and Kaji (1954) reported that two toxins were isolated from the culture broth of the fungus *P. oryzae* as well as from severely diseased rice plant. One of the toxin was identified as α-picolinic acid and the other was designated as -piricularin. When 0.56 mcg of piricularin or 6 mcg of α-picolinic acid was applied to wounded rice leaves a necrotic spot similar to that of blast disease produced. Each toxin produced a characteristic spot. Umetsu *et al.* (1972) reviewed that three toxins piricularin (pir.), α-picolinic acid (P.A.) and pyriculol have been isolated from the culture broth of the blast fungus. They also isolated tenuazonic acid from some strains of this fungus. Narayana and Suryanarayana (1974) also reported the production of picolinic acid, pyriculol and tenuazonic acid by *Pyricularia oryzae*.

Wang *et al.* (1988) reported the production of crude toxin extracts from spores or mycelia of *P. oryzae* which was highly toxic to rice plants. Leburn (1981) reported that in the infected leaves of rice, *P. oryzae* produced non specific toxins. Among these toxins, tenuazonic acid inhibited protein synthesis by acting on ribosomes.

Singburaudom *et al.* (1995a) reported that culture filtrate of *P. oryzae* incited symptoms which were quite similar to blast symptom. The capabilities of culture filtrates for inciting disease symptoms were different among isolates of the fungal pathogen. They suggested that the culture filtrate contained toxic substance which played as determinant of pathogenicity in blast disease development. Singburaudom *et al.* (1995b) purified tenuazonic acid from culture filtra.te of *P. oryzae*. They found that it could produce similar symptom on rice leaves as those produced by the blast fungus, severity increased as the concentration of purified toxin was increased. They concluded that the culture filtrate of *P. oryzae* contained tenuazonic acid which was classified as pathotoxin and played an important role as disease determinant of blast pathogen.

MATERIALS AND METHODS

Preparation of crude extract toxin (CET)

The modified Fries medium was used to culture *P. oryzae* for toxin production. The composition of medium was as follows: sucrose 30g, ammonium tartrate 5g, ammonium nitrate (NH_4NO_3) 1g, potassium dihydrogen phosphate (KH_2PO_4) 1g, magnesium sulphate ($MgSO_4.7H_2O$) 0.5g, sodium chloride (NaCl) 0.1 g, calcium chloride ($CaCl_2$) 0.1g, yeast extract 0.5 g and distilled water 1l. All the chemicals were thoroughly mixed and dissolved in distilled water and then 150 ml of medium was transfered to 250 ml flasks contained 1 g rice seeds. The flasks were plugged by cotton and the medium was sterilized by autoclaving.

The sterilized modified Fries medium was then inoculated by mycelia of Khon Kaen isloate of *Pyricularia oryzea*. The inoculated medium was incubated at 25°C on electrical shaker at 200 rpm for 2 weeks. After incubation the medium was filtered through three layers of cheese cloth to obtain culture filtrate. The volume of this filtrate was reduced to about ten percent of original volume by evaporation in the rotary evaporator. The concentrate culture filtrate was then deproteinized by adding equal volume (1:1) of methanol and kept overnight at 5°C. The sediment was filtered through Whatman No. 1 filter paper. The methanol and the water portions were evaporated by rotary evaporator. The viscous brown coloured crude extract toxin was obtained and preserved in refrigerator for fruther use.

Inoculation Procedures:

The detached leaf technique was employed for crude extract toxin inoculation. The second or third leaves from the top of 40 - 45 days old rice plants were detached. Tips and the lower portions of leaves were cut off to retain a 7 - 8 cm leaf segment from each leaf. The leaf segments were surface sterilized in clorox 10% for 1 to 2 minutes and washed three times in sterile water. Then, each leaf segment was placed on a slide glass with ends fixed by cellulose tape and placed in a moist petridish. On the middle of each leaf wound was made with a needle and 30 micro-litre of crude toxin was dropped over the wounded area by a micro-pipette. The inoculated leaves were incubated for 5 days at 25° - 28°C under light for symptom development.

Assessment of disease reaction

The response of rice leaves to crude extract toxin was assessed by measuring lesion width, length and running lesion. The lesion length along with lesion type (reaction) was considered as main criterion for assessing the degree of resistance or susceptibility. The five standard scales of measurement and reaction were modified by Singburaudom *et al.* (1995a) as follows:

Scale 1 = HR (Highly Resistant). Small brown lesion of needle head size, length of lesion 0.0 - 0.1 cm

Scale 2 = R (Resistant). Slightly larger brown lesion with gray centre. Length of lesion 0.2 - 0.4 cm.

Scale 3 = MR (Moderately Resistant). Brown to dark brown roundish lesion with gray centre and halo around the lesion. Length of lesion 0.5 - 0.7 cm.

Scale 4 = S (Susceptible). Round large brown lesion with gray centre and dark brown margin and distinct yellow halo and running lesion. Length of lesion more than 1.0 cm.

Disease determination of crude extract toxin

The experiment was conducted to determine the capability of crude extract toxin to incite the blast symptom on leaves of rice variety KDML105. Leaf samples were prepared according to the procedures as mentioned above. Five concentrations of crude extract toxin at 0,25, 50, 75 and 100% were made by diluting in distilled water and then crude extract toxin was inoculatd by dropping 30 micro litre with micropipette at wounded site on the leaves. The inoculated laeves were incubatd in moist petridesh at 25°C for 5 days. Disease reactions were assessed by measuring the size of lesion and type of symptoms were described by the method as mentioned above.

Response of rice leaves to crude extract concentrations

The experiment was carried out in a factorial CRD with 4 replications. Four rice varieties, Hang yi 71, Dawk Payawm, Nang Mon S 4 and Khao Tah Hang 17 , classified as resistant, moderately resistant, susceptible and highly susceptible, respectively were used to determine the response of rice leaves to crude extract toxin. Crude extract toxin at 0,5,10,15,20 and 25 percent concentration (W/V) were prepared by diluting in distilled water. Leaf samples preparation, crude extract toxin inoculation and disease assessment were conducted as mentioned above.

Response of rice varieties to crude extract toxin

To assess the varietal responses of crude extract toxin, twenty rice varieties were transplanted in clay pots and grown for 21 days. Second and third leaves from the top were detached and used for crude extract toxin inoculation. Leaf samples were prepared as mentioned earlier. Crude extract was prepared at 25 percent concentration and 30 micro-litre was dropped on the leaves at wounded site made by the needle. The inoculated leaves were incubated at 25°C for 5 days under continuous light. The disease reaction was assessed by measuring the lesion size and length of running lesion. The experiment was carried out in CRD with four replications.

RESULTS

Disease determinant of crude extract toxin

The capability of crude extract toxin to incite blast sympton was determined on leaves of rice variety KDML 105 crude extract toxin obtained from culture filtrate of *P. oryzae* was diluted by distilled water to obtain 5 concentration levels and used for inoculation on rice leaves. The result of determination indicated that crude extract could produce quite similar symptom as produced by the infection of the fungus (Figure 1 B). Typical symptom produced by crude extract was brown round to spindle shape lesion with grey at the middle and dark brown border. Beside the chlorosis, area expands along with the lesion called running lesion occurred at higher concentration of crude extact (Figure 1 C and D). Disease severity was also assessed by measuring the size of lesions and running lesion. The result revealed that the severity of lesion produced tended to increase by increasing crude extract concentrations (Table 1). At concentration of 25 % to 100 %, the width and length of lesions and length of running lesions were different ($P>0.01$). This preliminary study on the response of rice leaves to concentration of crude extract suggested that the concentration of 25% would probably be appropriate for inciting blast symptom.

Response of rice leaves to crude extract toxin

The experiment on determination of appropriate concentration of crude extract to incite blast symptom was carried out by inoculating six concentrations of crude extract toxin on leaves of four rice varieties. The results of determination are presented in Table 2 and 3. Among the levels of crude extract toxin concentrations tested, the 25% concentration produced the highest width, length and length of running lesion of 0.5, 0.76 and 1.12 cm, respectively (Table 2).

The response of rice varieties to crude extract toxin concentrations was analyzed. The result indicated that the susceptible variety Khao Tah Hang 17 produced the highest mean lesion width, length and running lesion of 0.40 . 0.60 and 0.75 cm, respectively while resistant variety Hang Yi 71 produced the lowest mean lesion width, length and running lesion of 0.27, 0.41 and 0.58 cm, respectively (Table 3). The result could suggest that KTH 17 was the most susceptible where as Hy 71 was the most resistant variety to crude extract toxin of *P. oryzae*.

Response of rice varieties to crude extract toxin

Varietal response to crude extract toxin was determined by inoculating crude esxtract toxin at concentration of 25% on leaves of 20 rice varieties. There was significant difference on degree of disease responsibility among rice varieties (Table 4). The largest lesion width of 0.55 cm was produced

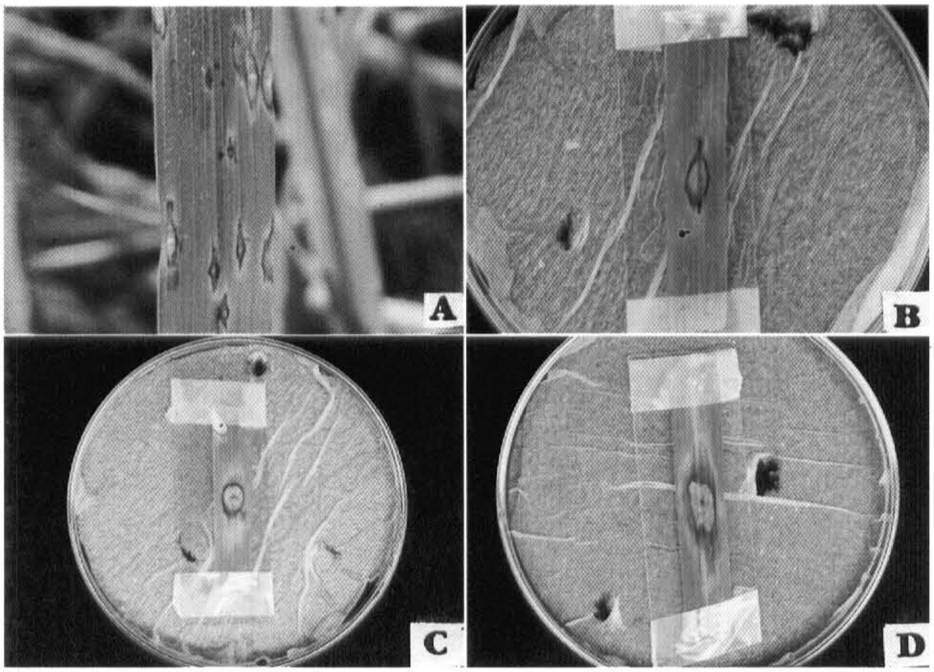

Figure 1 Characteristics of lesion produced by the fungus *P. oryzae* and its crude extract toxin.
A. Typlical blast lesion produced by the fungus.
B. Typical blast lesion produced by crude toxin.
C. Blast lesion with halo produced by higher cancentration of crude toxin.
D. Lesion with running lesion produced by higher concentration of crude toxin.

Table 1 Mean value of width, length and running lesion produced by crude extract toxin on rice leaves variety KDML 105.

Crude extract concentration (%)	Width (cm)	Length (cm)	Running lesion (cm)
0	0.0^{c1}	0.0^{c}	0.0^{c}
25	0.42^{b}	0.77^{ab}	1.48^{b}
50	0.68^{a}	0.95^{a}	3.02^{a}
75	0.52^{b}	0.80^{ab}	1.99^{b}
100	0.51^{b}	0.78^{ab}	1.55^{b}
CV%	28.18	28.17	42.14
F-Test[2]	**	**	**

1 In a column, means followed by a common letter are not significantly different by DMRT.

2 ** = significant at $p < 0.01$

Table 2 Mean values of width, length and length of running lesion produced by 6 concentrations of crude extract toxin on 4 rice varieties.

Crude extract Concentrations(%)	Lesion width (cm)	Lesion length (cm)	Length of running lesion (cm)
0	0.00[e1]	0.00[f]	0.00[d]
5	0.20[cd]	0.38[e]	0.40[bc]
10	0.30[bc]	0.51[bcd]	0.72[ab]
15	0.40[ab]	0.58[bc]	0.94[ab]
20	0.40[ab]	0.63[b]	0.96[ab]
25	0.50[a]	0.76[a]	1.12[a]
CV(%)	23.5	21.8	40.6
F-test[2]	**	**	**

1 In a column, means followed by a common letter are not significantly different by DMRT.

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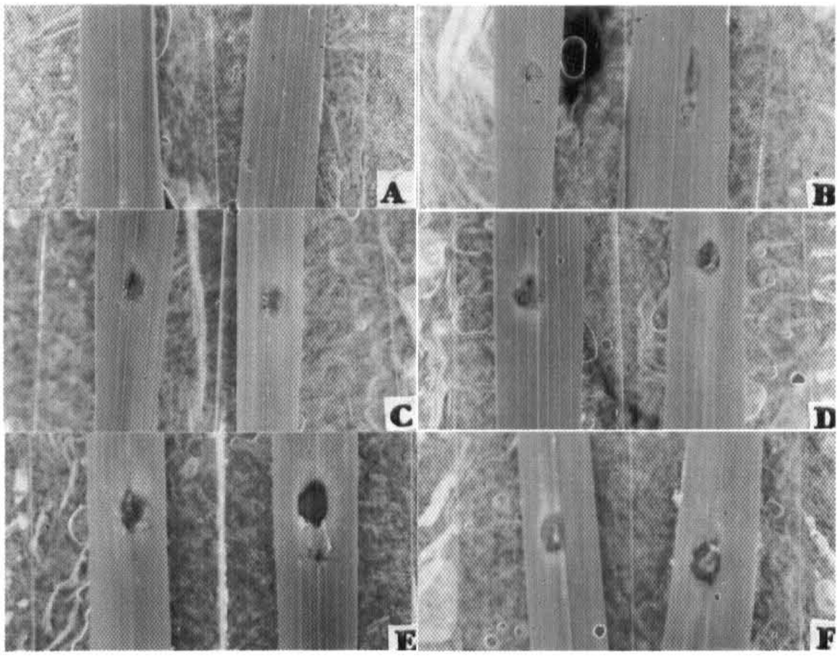

Figure 2 Effect of concentration on lesion size produced on rice variety KTH-17

A. Control, No lesion produced.
B. Lesion produced by 5 % concentration.
C. Lesion produced by 10 % concentration.
D. Lesion produced by 15 % concentration.
E. Lesion produced by 20 % concentration.
F. Lesion produced by 25 % concentration.

Table 3 Mean values of width, length and length of running lesion produced by 6 crude extract toxin concentraion on 4 rice varities.

Varieties	Lesion width (cm)	Lesion length (cm)	length of running lesion (cm)
Hang yi-71	0.27[bc1]	0.41[bc]	0.58[ab]
Dawkprayawm	0.27[bc]	0.44[bc]	0.71[ab]
Nang Mon S-4	0.30[ab]	0.47[ab]	0.71[ab]
Khao Tah Hang 17	0.40[a]	0.60[a]	0.75[a]
CV(%)	23.5	21.8	40.6
F-test2	**	**	**

1 In a column, means followed by a common letter are not significantly different by DMRT.
2 ** = significant at $p < 0.01$

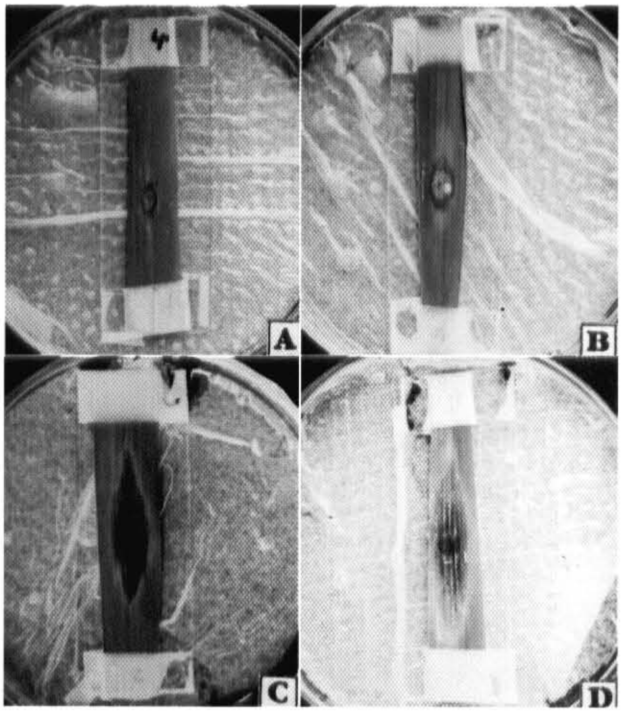

Figure 3 Responses of rice varieties to crude toxin.
A. Group I. Resistant type lesion.
B. Group II. Moderately resistant type lesion.
C. Group III. Susceptible type lesion
D. Group III. Susceptible type lesion.

Table 4 Mean value of lesion width, length and running lesion produced by crude extract toxin on rice varieties.

Varieties	Lesion width (cm)	Lesion length (cm)	Running lesion (cm)
Raminad	0.45^{abc} [1]	0.52^{cde}	1.85^{abc}
Usen	0.40^{bc}	0.47^{e}	1.60^{abcd}
Dular	0.52^{ab}	0.66^{bcde}	1.12^{cde}
Zenith	0.35^{c}	0.65^{bcde}	1.42^{bcde}
Hang yi-71	0.47^{abc}	0.62^{bcde}	1.15^{cde}
RD 13	0.47^{abc}	0.57^{bcde}	1.27^{bcde}
RD 6	0.52^{ab}	0.65^{bcde}	1.75^{abcd}
Suphan Buri 60	0.55^{a}	0.72^{bcde}	1.82^{abc}
Dawk payawm	0.54^{ab}	0.82^{ab}	0.90^{de}
RD 27	0.45^{abc}	0.77^{abcd}	1.92^{abc}
Pitsanulok 60	0.52^{ab}	0.80^{abc}	1.82^{abc}
RD 5	0.35^{c}	0.50^{de}	0.70^{e}
RD 7	0.47^{abc}	0.55^{bcde}	1.27^{bcde}
RD 10	0.55^{a}	0.72^{abcde}	1.80^{abc}
KTH - 17	0.55^{a}	0.97^{a}	1.32^{bcde}
Pathumthani 60	0.47^{abc}	0.70^{bcde}	2.37^{a}
NMS - 4	0.45^{abc}	0.82^{abc}	2.07^{ab}
KDML 105	0.42^{abc}	0.95^{ab}	1.92^{abc}
IR 42	0.45^{abc}	0.95^{ab}	1.92^{abc}
RD 23	0.47^{abc}	0.60^{bcde}	1.55^{abcde}
CV%	18.22	26.95	34.86
F-Test [2]	**	**	**

1 In a column, mea[illegible]lowed by a common letter are not significantly different by DMRT.
2 ** = significant at $p < 0.01$

on varieties KTH-17, RD-10 and Suphanburi 60, while the smallest lesion width of 0.35 cm was produced on varieties RD-5 and Zenith. The longest lesion length of 0.97 cm was produced on variety KTH-17, followed by KDML 105 and IR-42 which produced the lesion length of 0.95 cm. The shortest lesion was produced on variety Usen, and RD-5 at 0.47 cm. and 0.50 cm. respectively.

The affected area expanding along with the leaves known as running lesion was also considered as the criterion for determining varietal response to crude extract toxin inoculation. Pathumthani 60 produced the longest running lesion at the length of 2.37 cm. followed by NMS-4 which produced running lesion at the length of 2.07 cm, while shortest running lesion length of 0.70 cm was produced on variety RD-5. Disease reaction of twenty rice varieties were classified into three groups by the criterion of lesion length and typical symptom produced are as follow:-

Group I (R-group): Small to slightly larger roundish brown lesion with gray centre, without halo and running lesion, length of lesion 0.2-0.4 cm. This group comprised of variety Usen. (Figure 3A)

Group II (MR-group): Round, brown lesion with gray centre, with distinct halo and with or without running lesion, lesion length of 0.5-0.7 cm. This group comprised of varieties Dular, Hang Yi-71, RD-5, RD-7, RD-23, Raminad, Zenith,Pathum thani 60, RD 13 and RD 6 (Figure 3B)

Group III (S-group): Large round brown lesion with halo and running lesion, lesion legnth was more than 0.8 cm. This group comprised of the varieties-Suphanburi 60, KTH-17, RD-10, KDML 105, IR-42, NMS-4,RD-27, Dawk payawm and Pitsanuloke 60. (Figure 3 C and D)

DISCUSSION

Results obtained in the series of experiments supported the findings of Tamari *et al.* (1954), Umetsu *et al.* (1977), Wang *et al.* (1988), Leburn (1981) and Singburaudom *et al.* (1955a) that blast fungus *Pyricularia oryzae* Cav. produced some toxins which played important role as disease determinants on rice plants. The result of this experiment indicated that the capabilities of crude extract to produce blast symptom corresponded to the previous reports on symptom produced by culture filtrate (Singburaudom *et al.* 1995a). Lesion type produced by culture filtrate was divided into three parts, they were grayish green in the middle of lesion, brown at the border of lesion and yellow area around the lesion, called halo, (Umetsu *et al*; 1972). However, typical lesion produced depended on the concentration of culture filtrate. Singburaudom *et al.* (1995a) characterized the lesions produced by culture filtrate into three types. First, spindle shaped lesion with greenish gray in the center, second, spindle shape lesion with greenish gray in the center and brown discoloured necrotic area surrounded with yellow area, third, spindle shape lesion surrounded with large yellow area called running lesion.

From the result of this experiment, all the levels of crude extract toxin produced lesions of various sizes except the control treatment which produced no symnptom at all. The lesion sizes increased when concentrations of the crude extract toxin was increased. Lesions produced by the crude extract toxin were not exacly typical to those produced by the blast fungus, probably due to error in making wound, selecting leaves, dropping toxin and in the process of toxin diffusion. Nevertheless, the symptoms produced by the crude extract toxin in some replicates were found more or less similar to those produced by the blast fungrs.

The effect of blast toxin concentration on symptom development and severity of reaction has been investigated by several investigators. They summarized that size of lesion increased by increaing toxin concentration, (Lokeshwari and Suryanarayanan, 1992; Singburaudom *et al.*, 1995b).

Data obtained in this experiment clearly indicated that among six levels of crude extract toxin concentration inoculated, the concentration of 25 percent was found appropriate concentration for screening rice plants against this toxin. Variation in lesion length,width and the size of running lesion was found among concentrations tested on different varieties. When concentration was decreased below 25 percent the lesion size also decreased. It was found that 25 pereent crude toxin could produce lesion size equivalent to susceptible score in the variety KTH-17 which is a highly susceptible variety to blast disease, while the variety Hangyi-71 and Dawk payawm produced lesion length equivalent to moderately resistant teaction at the same concentration. Data of the experiment on the varietal responses to crude extract toxin exhibited considerable variation in lesion sizes produced on varieties inoculated. The varieties were classified as resistant, moderately resistant and susceptible groups on the basis of lesion size, lesion type, running lesion and halo around the lesion produced on each variety. Variation in varietal reaction to crude extract toxin inoculation indicated that this could be used to screen rice plants against the blast disease as a substitute for pathogen inoculation.

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