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Inoculation Techniques of Crude Extract Toxin Produced by *Pyricularia oryzae* Cav

Ram Narayan Chaudhary[1], Narong Singburaudom[2], Tharmmasak Sommartya[2], and Ed Sarobol[3]

ABSTRACT

The mechanical wounding of leaves is necessary for symptom development when inoculated with crude extract toxin(CET) as well as the most suitable method of inoculation for detached leaf and whole plant conditions. The experimental results revealed that the CET could produce disease symptoms even without leaf wounding before inoculation. For detached leaf condition, inoculation by micropipette was found to be efficient and effective. While foliar spray method was the most suitable for whole plant inoculation, symptoms produced by crude extract toxin varied by methods of inoculation. A 30 microlitres drop of CET by micropipette inoculation produced the similar typical blast symptom whereas spray inoculation produced small brown necrosis spots. CET spraying on detached leaves and whole plants gave the same result indicating that CET could be applied for screening toxin resistance on the whole plant condition.

Key words: crude extract toxin, *Pyricularia oryzae* Cav., rice blast, inoculation techniques

INTRODUCTION

Rice blast is common in all rice growing areas and it is considered to be the most important disease in the temperate and tropic zone at the present. The economic importance of blast can be seen recently by panicle blast epidemic in Thailand. This was reported in 1992 affecting over 200,000 ha in 12 provinces of the northern part of Thailand. The result of disease diagnosis confirmed that about 60% of diseased plants had panicle blast caused by *P. oryzae* and the rest were infected by other fungi. Total yield loss of 60% was confirmed to be due to the disease, approximately equivalent to 65,000 tons of paddy rice (Disthaporn, 1994). Despite the magnitude of the problem, satisfactory control of blast is achieved in tropical irrigated rice primarily through the use of resistant varieties. The use of resistant cultivars has been recognized as the most practical and economical method by which the farmers could control the blast disease (Correa-Victoria *et al.*, 1994). But the varietal resistance broke down within a few years of its release due to high pathogenic variability among the isolates of the blast fungus (Ou, 1985). Hence, the effective and efficient screening techniques are essential to a successful breeding program for resistance to blast.

[1] Regional Agriculture Research Station, Tarakara, Sunsari, Nepal.
[2] Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
[3] Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

The fungus, *Pyricularia oryzae*, produces some effective toxins considered to be determinant of pathogenicity and ultimate causal agent of the disease. Toxins have been isolated from the culture filtrates of the fungus as well as from severely blasted rice plants (Tamari *et al.*, 1965, Umetsu *et al.*, 1972, Wang *et al.*, 1988, Singburaudom *et al.*, 1995 a, b, Chaudhary *et al.*, 1995). However, there is not much report available on screening for disease resistance in rice with phytotoxins. Umetsu *et al.* (1972) studied the phytotoxic effect of tenuazonic acid on rice varieties and found variation in growth inhibition of seedlings and remarkable differences in chlorotic spot formation among rice varieties. Leburn (1981) applied tenuazonic acid, a phytotoxin of *P. oryzae* to obtain resistant mutant through anther culture and proposed a protocol for selection by measuring blast resistance of the plants which are resistant to tenuazonic acid. Wang *et al.* (1990) reported that crude extract toxin of *P. oryzae* could be used for screening rice for resistance to blast. Leburn *et al.* (1985) applied tenuazonic acid on young leaves of rice varieties with general and specific resistance. Varieties with general resistance showed browning after 48 hrs while after 96 hrs a few small brown specks were observed on leaves of varieties with specific resistance. Selection of blast resistance in rice variety KDML 105 using culture filtrate and tissue culture is investigated. (Singburaudom *et al.*, 1992; Chuntarathin, 1994). The results of the studies indicated that resistance to blast in plants using tissue culture and culture filltrate was higher than plants which were not derived from this technique. Singburaudom *et al.* (1995) evaluated the response of rice varieties to culture filtrate and tenuazonic acid of *P. oryzae* and the result indicated that rice varieties exhibited different responses to culture filtrate and tenuazonic acid and it could be applied to differentiate resistant or susceptible genotypes of rice. They concluded that varieties producing small roundish brown lesion without halo and running lesion were resistant whereas those producing larger round lesion with gray center, halo and running lesion susceptible.

In the past, screening of rice to blast has been carried out mostly by the use of pathogen inoculation, where effectiveness of screening depended on seasonal condition conducive for disease development. In order to improve efficiency and effectiveness of screening for resistance to blast, studies on toxin inoculation should be given due to consideration as one of the subtitutes for pathogen inoculation. Although there are several reports on culture filtrate and purified toxins of the blast fungus used *in vitro* screening, there is little information regarding the use of crude extract toxin *in vivo* screenging of rice resistance to blast. Therefore, the present study was carried out to gather more information about crude extract toxin inoculation and to develop techniques for screening rice genotypes for resistance to blast disease. Primary purpose of the research is to determine whether the crude extract toxin inoculation is equally efficient and effective as pathogen inoculation. The experiments involving the evaluation of crude extract toxin inoculation in rice included: (i) effect of leaf wounding on symptom development upon crude extract toxin inoculation, (ii) investigation on crude extract toxin inoculation techniques. The success and results of the study will help to accelerate breeding and screening programs for resistance to blast since it can be carried out at any time of the year.

MATERIALS AND METHODS

Effect of leaf wounding on symptom development upon crude extract inocutation

The experiment was carried out on detached leaves of rice varieties Dawk Payawm and KTH-17 to determine whether mechanical wounding to leaves before toxin inoculation was necessary for

symptom development or not. The second or third leaves from the top of 30 days were detached and prepared following the standard procedures mentioned by Chaudhary (1995). Treatments included two methods of leaf wounding, by needle and by carborundum and a nonwounded control. For needle wounding., the middle of the leaf blade was pressed lightly with the point of needle while in another method, carborundum was mixed in water (1 g/l), soaked in cottonwood and the cottonwood was rubbed over leaf blade surface. A drop of 30 microliters of CET at 25% concentration was dropped by micropipette on mechanically wounded lesion spots and on healthy leaf blades for wounded and non wounded treatments, respectively. Crude extract preparation, leaf samples preparation, inoculation, incubation and disease assessment were conducted following the same procedures by Chaudhary (1995). The experiment was carried out using a factorial in RCB design with 6 replications.

Study on inoculation methods of crude extract toxin on symptom development.

The experiment was carried out using a factorial in RCB design with 4 replications to find out the most suitable method for CET inoculation in substitution of pathogen inoculation. Detached leaves from two rice varieties, Dawk Payawm and KTH-17, were inoculated following five methods of inoculation; (i) spray inoculation without wounding, (ii) wounding leaves by needle and micropipette inoculation, (iii) wounding leaves by carborundum rubbing and spary inoculation, (iv) smear inoculation without wounding, (v) wounding leaves by carborundum rubbing and smear inoculation. CET at 25% concentration was inoculated on detached leaves. Inoculation by micropipette was done by dropping 30 microlitres of CET solution on wounded spot while in smear inoculation, cottonwood soaked in CET solution was smeared on leaf blade. Spray inoculation was done by a plastic hand sprayer to obtain very uniform and fine spray drift of CET. The incubation of inoculated materials and disease assessment were done following the methods described by Chaudhary (1995).

Comparative study of inoculation methods on detached leaves and whole plants.

The experiment was carried out using factorial in RCB design with 5 replications, in greenhouse and in laboratory for whole plant and detached leaf inoculations, respectively. Forty- day-old plants of rice varieties HY71, Dawk Payawm, NMS-4 and KTH-17 were used for inoculation. Inoculation methods included (i) wounding leaves by needle and spray inoculation (ii) spray inoculation without leaf wounding (iii) wounding leaves by carborundum rubbing and spray inoculation (iv) carborundum mixed with CET solution and spray inoculation. CET at 25% concentration was used for inoculation. Leaf sample preparation, incubation of inoculated material and disease assessment were done using the procedures mentioned above.

RESULTS AND DISCUSSION

Effect of leaf wounding on symptom development upon CET inoculation.

The results on lesion width, length and running lesion length produced by CET inoculation with different wounding methods are in Table 1, 2, and 3. Analysis of variance on lesion width, length and running lesion length produced by different methods of wounding showed a non-significant difference between two varieties. A non-significant interaction effect between wounding method and varieties was found. However, significant differences in lesion length and running lesion length were found among wounding methods. Leaves wounded by rubbing carborundum on leaf blade surface before CET inoculation produced highest mean lesion width, length and running

Table 1 Mean lesion width (cm) produced by crude extract toxin inoculation with different methods of leaf wounding on two rice varieties.

Wounding method	Varieties		Mean wounding method
	D.P	KTH-17	
No wounding (control)	0.42	0.47	0.44
Needle wounding	0.48	0.44	0.46
Carborundum wounding	0.52	0.56	0.54
Mean variety	0.47	0.49	

CV = 23.2%

F-tests

Variety = ns

Wounding method = ns

Interaction = ns

ns = non-significant

Table 2 Mean lesion length (cm) produced by crude extract toxin with different methods of leaf wounding on two rice varieties.

Wounding method	Varieties		Mean wounding method
	D.P	KTH-17	
No wounding (control)	0.76	0.67	0.71 b[1]
Needle wounding	0.79	0.67	0.73 b
Carborundum wounding	0.93	0.95	0.94 a
Mean variety	0.83	0.76	

CV = 24.67%

F-tests

Variety = ns

Wounding method = *

Interaction = ns

ns = non-significant

* = significantly different at 5% level of probability

[1] Any two means with in column followed by the same letter are not significantly different at 5% level of probability.

Table 3 Mean running lesion length (cm) produced by crude extract-toxin inoculation with different methods of leaf wounding on two rice varieties.

Wounding method	Varieties		Mean wounding method
	D.P	KTH-17	
No wounding (control)	0.95	0.97	0.96 b[1]
Needle wounding	0.98	1.13	1.05 ab
Carborundum wounding	1.17	1.93	1.55 a
Mean variety	1.03	1.34	

CV = 44.84%

F-tests

Variety = ns

Wounding method = *

Interaction = ns

ns = non-significant

* = significantly different at 5% level of probability

[1] Any two means within column followed by the same letter are not significantly different at 5% level of probability.

Table 4 Mean lesion width (cm) produced by different methods of CET inoculation on two rice varieties.

Inoculation methods	Varieties		Mean method
	Dawk Payawm	KTH-17	
1. Micropipette(control)	0.4	0.44	0.42
2. Spray (without wounding)	0.29	0.33	0.31
3. Carborundum wounding and spray	0.44	0.44	0.44
4. Smear (without wounding)	0.29	0.29	0.29
5. Carborundum wounding and smear	0.38	0.36	0.37
Mean variety	0.36	0.37	

CV = 29.13%

F-tests

Variety = ns

Method = ns

Interaction = ns

ns = non-significant difference

lesion length of 0.54 , 0.94 and 1.55 cm, respectively, whereas these of the non-wounded control produced the shortest means of 0.44 , 0.71 and 0.96 cm, respectively. Rice variety KTH-17 produced higher mean lesion width of 0.49 cm and running lesion length of 1.34 cm than variety Dawk Payawm. It was noted that CET could produce symptom on non-wounded leaves as well (Figure 1) and lesion length and length of running lesion would be an appopriate criteria to use for differentiateing toxin reaction on rice plants.

Study on inoculation methods of crude extract toxin on symptom development

The study was conducted to compare the efficiency and effectiveness of five methods of inoculation to symptom development by CET. The spray method of inoculation combined with wounding leaf by rubbing carborundum produced the largest mean lesion width of 0.44 cm while smearing method of inoculation without leaf wounding produced shortest mean lesion width of 0.29 cm (Table 4 and Figure 2). Rice variety KTH-17 produced mean lesion width of 0.37 cm while variety Dawk Payawm produced mean lesion width of

Table 5 Mean lesion length (cm) produced by different methods of CET inoculation on two rice varieties.

Inoculation methods	Varieties		Mean method
	Dawk Payawm	KTH-17	
1. Micropipette (control)	0.63	0.81	0.72 a[1]
2. Spray without wounding	0.40	0.63	0.51 b
3. Carborundum wounding and spray	0.58	0.79	0.68 ab
4. Smear (without wounding)	0.72	0.71	0.71 a
5. Carborundum wounding and smear	0.71	0.81	0.76 a
Mean variety	0.61 b[2]	0.75a	

CV = 21.3%

F-tests

Variety = **

Method = *

Interaction = ns

ns = non-Significant difference

* = significantly difference at 5% level of probability

** = significantly different at 1% level of probability

[1] Any two means in the same column of method mean followed by a common letter are not significantly different at 5% level of probability.

[2] Any two means in the same column of variety mean followed by a common letter are not significantly different at 5% level of probability

0.36 cm. Analysis of variance showed non-significant difference in lesion width among inoculation methods, varieties and interaction effect between varieties and methods of inoculation.

The inoculation method, smearing CET after leaf wounding by rubbing carborudum produced largest mean lesion length of 0.76 cm whereas spray inoculation without leaf wounding produced shortest mean lesion length of 0.51 cm. Rice variety KTH-17 produced larger mean lesion length of 0.75 cm compared with 0.61 cm of variety Dawk Payawm (Table 5). Analysis of variance of lesion length showed significant differences among methods of inoculation and between varieties while a non-signficant interaction effect was found.

The spray method of inoculation combined with leaf wounding by carborundum rubbing produced the largest mean running lesion length of 2.24 cm while smear method of inoculation without leaf wounding produced the shortest one of 0.8 cm. The rice variety KTH-17 produced larger mean running lesion of 1.33 as compared with 1.22 cm of variety Dawk Payawm (Table 6). The analysis of variance showed significant differences on running lesion length among methods of inoculation while non significant differences were found between varieties and interaction effect between methods and varieties.

Comparative study of inoculation methods on detached leaves and whole plants

The result of the previous experiment on inoculation methods indicated that CET could produce symptoms on detached leaves whether they

Table 6 Mean running lesion length (cm) produced by different methods of CET inoculation on two rice varieties.

Inoculation methods	Varieties		Mean method
	Dawk Payawm	KTH-17	
1. Micropipette (control)	1.57	1.25	1.41 b[1]
2. Spray (without wounding)	0.63	1.05	0.84 b
3. Carborundum wounding and spray	2.38	2.1	2.24 a
4. Smear (without wounding)	0.6	1.0	0.8 b
5. Carborundum wounding and smear	0.93	1.23	1.08 b
Mean variety	1.22	1.33	

CV = 54.6%

F-tests

Variety = ns

Method = **

Interaction = n s

ns = non-significant difference

** = significantly different at 1% level of probability

[1] Any two means within column followed by the same letter are not significantly different at 5% level of probability.

were wounded by mechanical means, before inoculation. It was hypothesized that CET might produce the same effect when applied on the whole plant with different methods of inoculation. To gain more understanding of the inoculation methods on detached leaf and whole plant conditions this experiment was conducted.

The results on effects of detached leaves and whole plants inoculated by CET are presented in tables 7 and 8. The inoculation method of spraying CET mixed with carborundum produced the highest mean disease severity of 42.3% on detached leaves and the same method produced highest mean disease severity of 36.1% on whole plant. The inoculation method of spraying CET after wounding leaves by carborundum rubbing produced lowest mean disease severity of 37% in detached leaves whereas the lowest mean disease severity of 31% in whole plant was produced by spraying method of inoculation after needle wounding.

The rice variety KTH - 17 produced the high est mean disease severity on detached leaves as well as on whole plants of 48.3% and 36.8%, respectively. The rice variety HY 71 produced the lowest mean disease severity on both detached leaf and whole plant inoculations of 28% and 29%, respectively. However, the analysis of variance of data on disease severity among inoculation methods showed non significant differences, while significant difference on disease severity were found between rice varieties in both conditions. Non-significant interaction effects between varieties and inoculation methods were found in detached leaf inoculation as well as in whole plant inoculation.

The co-efficient of correlation was calcu-

Table 7 Mean percent disease severity produced by different methods of crude extract toxin inoculation on detached leaves from four rice varieties on detached leaves.

Inoculation methods	Varieties				Mean method
	HY - 71	D.P.	NMS-4	KTH-17	
Carborundum rubbing and CET spray	26	32	46	44	37.0
CET spray	28	32	44	48	38.3
CET mixed with carborundum spray	30	37	50	49	42.3
Needle wounding and CET spray	28	31	43	48	37.5
Mean variety	28.0 b[1]	33.0 b	45.8 a	48.3 a	

CV = 24.38%

F - tests

Variety = **

Inoculation method = ns

Interaction effect = ns

ns = non-significant difference

** = significantly different at 1% level of probability

[1] Mean variety followed by the same letter are not significantly different at 5% level of probability.

lated between disease severity produced by detached leaf inoculation and whole plant inoculation, which was highly positive (r = 0.88) and significant at 1% level of probability.

It is known that blast fungus penetrates into host by penetration peg that makes mechanical injury to host surface before penetrating through epidermal cell. In the case of toxin inoculation, it was hypothesized that toxin might penetrate into host through some sort of mechanical wounds combined with inoculation methods. The result of the experiment on the effect of mechanical wounding indicated that CET could produce symptoms on host plant without wounding leaf surface prior to inoculation. The CET solution probably entered into host tissue through natural openings such as stomata and hydratodes. Symptoms produced by CET with and without wounding were similar (Figure 1) and they were in agreement with Umetsu *et al.* (1972), Singburaudom *et al.* (1995) and Chaudhary *et al.* (1995). Typical symptom produced by CET on rice leaves as circular or spindle chlorotic spots. They were consisted of three parts; the central greenish gray necrotic area, the brown discolored necrotic area and the yellow poisoned area which surrounded the necrotic area.

Data on effect of inoculation methods showed significant differences on lesion length and running lesion length produced by various methods of inoculation. Round to spindle shaped lesions with gray centres and brown margins were produced by micropipette inoculation while small brown necrotic spots were produced by spraying. (Figure 2). The differences on typical symptoms probably due to the difference in CET quantity that deposited on leaf surface. Spray method gave very

Table 8 Mean percents disease severity produced by different methods of crude extract toxin inoculation on whole plant of four rice varieties.

Inoculation methods	Varieties				Mean method
	HY - 71	D.P.	NMS-4	KTH-17	
Carborundum rubbing and CET spray	28.4	30.1	38.2	37.6	33.6
CET spray	26.8	32.6	35.6	34.8	34.2
CET mixed with carborundum spray	33.7	32.2	38.6	40	36.1
Needle wounding and CET spray	27.2	27.4	34.6	35	31
Variety mean	29.0 b[1]	30.6 b	36.7 a	36.8 a	

CV = 17.85%

F - tests

Variety = **

Inoculation method = ns

Interaction effect = ns

ns = non-significant difference

** = significantly different at 1% level of probability

[1] Mean variety followd by the same letter are not significantly different at 1% level of probability.

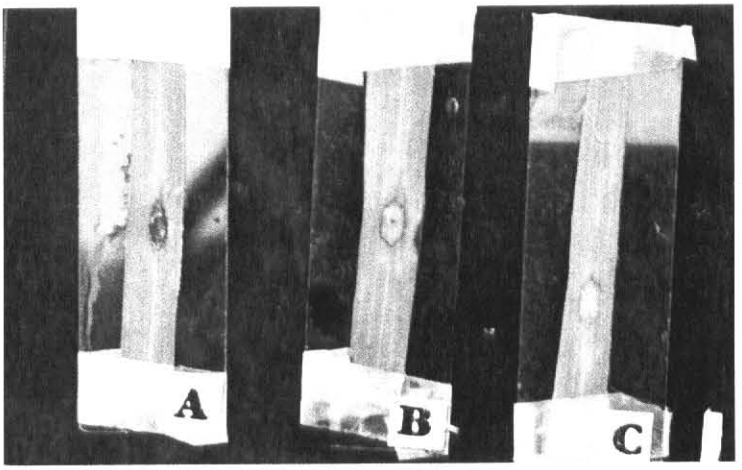

Figure 1 Symptoms on detached leaves produced by CET inoculation with different methods of leaf wounding .

A. No wounding (Control)

B. Wounding by needle

C. Wounding by carborumdum rubbing

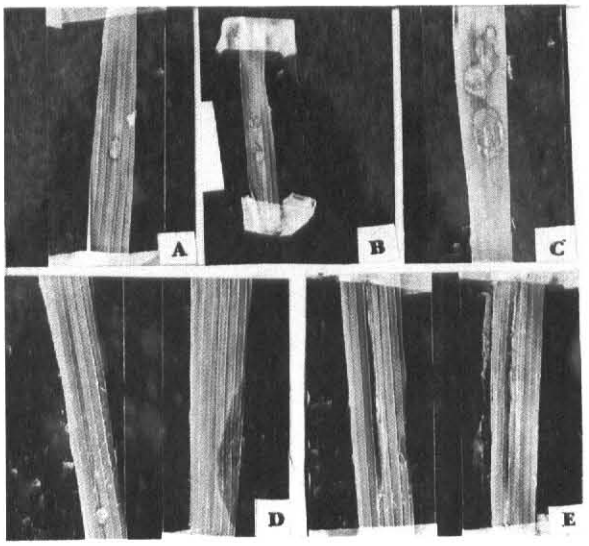

Figure 2 Symptoms produced by different methods of CET inoculation.

A . Needle wounding and micropipette inoculation.

B. Spray inoculation without wounding.

C. Carborundum wounding and spray inoculation.

D. Smear inoculation without wounding.

E. Carborundum wounding and smear inoculation.

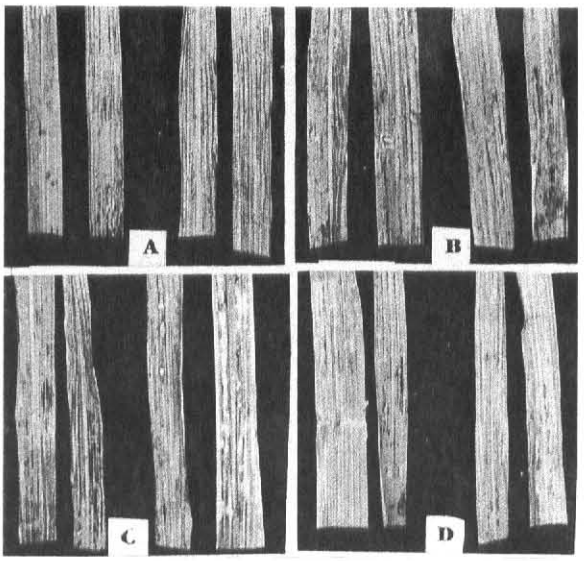

Figure 3 Symptoms produced on detached leaves by different methods of CET inoculation.
A. Symptoms produced by spraying CET after wounding leaves by carborundum rubbing
B. Symptoms produced by spraying CET without leaf wounding.
C. Symptoms produced by spraying CET mixed with carborundum.
D. Symptoms produced by spraying CET after wounding leaves by needle.

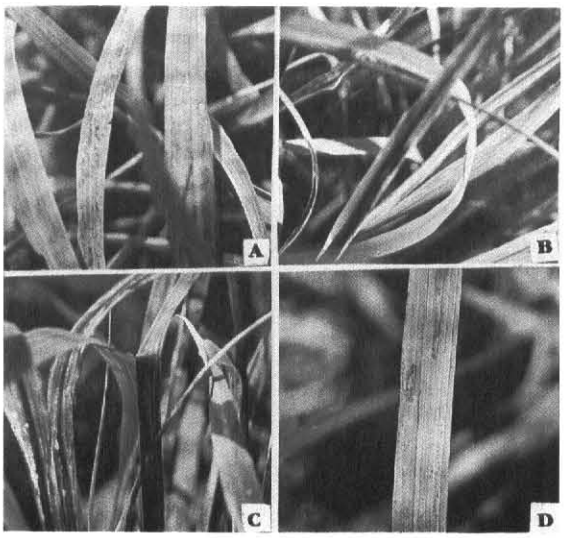

Figure 4 Symptoms on whole plants produced by different methods of CET inoculation.
A. Symptoms produced by spraying CET after wounding leave by carborundum rubbing
B. Symptoms produced by spraying CET without leaf wounding.
C. Symptoms produced by spraying CET mixed with carborundum.
D. Symptoms produced by spraying CET after wounding leaves by needle.

small and fine spray drifts as compared with a drop of 30 microlitres of CET by micropipette inoculation. The result of experiment suggested that CET mixed with or without carborundum followed by foliar spray inoculation was the most suitable method for whole plant inoculation because it was easy to carry out and effective as well. It might be also noted that symptoms produced by CET spraying were different from the typical blast symptoms produced by CET inoculated with micropipette and fungus infection.

A comparative study on detached leaf and whole plant inoculation was carried out. The results of the study showed non significant difference between methods of inoculation on detached leaves and whole plant. Foliar spray of CET without mechanical wounding produced the same level of disease severity, indicating leaf wounding caused no significant effect on symptom development by CET inoculation. Significant variation in symptom severity between rice varieties indicated their degree of sensitivity to CET. Higher disease severity in rice varieties KTH-17 and NMS-4 indicated their susceptibility as compared with varieties HY-71 and Dawk Payawm. In case of detached leaf inoculation, symptom was produced as brown chlorotic spots (Figure 3) whereas in whole plant inoculation symptoms appeared as brown chlorotic, necrotic spots and yellowing or drying of leaves suggesting the higher sensitivity to CET (Figure 4).

There are several reports that described on symptoms and effects on rice plant produced by toxin of *P.oryzae*. Phytotoxin, piricularin, produced brown necrotic spot and inhibited seedling growth (Tamari and Kaji, 1959). Tenuazonic acid at the concentration of 50 ppm completely inhibited growth of rice seedlings while produced blast symptom on rice leaves (Umetsu *et al.* 1972). Tenuazonic at 200 ppm inhibited shoot and root growth (Iwasaki, *et al* 1972). Tenuazonic acid produced small brown spots (Kozaka, *et al.* 1985) and produced similar typical blast symptom after inoculation by micropipette (Singburaudom *et al* 1995). Hence the study suggested that CET inoculation could be done successfully by spray method on whole plants as well as on detached leaves. A highly positive correlation coefficient was found on disease severity between detached leaves and whole plant suggesting their close association .

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