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Correlations Between *In Vitro* and *In Vivo* Methods for Screening Fungicides Against Corn Smut Disease

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옥수수 깜부기병균에 대한 살균제 활성검정시 실내와 생체검정 결과와의 상관

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ABSTRACT : To examine degree of correlations between *in vitro* and *in vivo* tests using seedlings for screening chemical compounds against corn smut caused by *Ustilago maydis*, five fungicides were compared for their inhibitory activities by three test methods in laboratory and seedling inoculation tests in the greenhouse. All of the three *in vitro* methods; agar diffusion test, sporidial germination test, and minimum inhibitory concentration test were found to be highly correlated for their results with those from the greenhouse seedling tests. However, the results of agar diffusion test reflected most accurately those from the greenhouse seedling test. This suggests that *in vitro* test such as agar diffusion test could be used practically as a simple method for mass screening of chemical compounds against the smut disease.

***Key words*: smut, *Ustilago maydis*, corn, screening, fungicide, MIC.**

Smut often causes considerable damages when improperly managed in cereals such as barley, wheat and maize (1, 2, 8, 9). Control of this disease heavily depends on seed dressing with systemic fungicides (3, 4). However, due to the reduced effectiveness of such systemic fungicides by development of resistant pathogen strains, demands for developing new fungicides effective against smut diseases have been increased.

Screening of chemical compounds against smut diseases has depended largely on field tests, and thus required much time and effort. In fact, lack of a simple screening technique hampered development of new fungicides effective to smut diseases. For this reason, we initiated a series of researches in 1994, and developed a screening technique with seedlings in the greenhouse (5).

This study was performed to select dependable *in vitro* methods for screening fungicides against a smut fungus, *Ustilago maydis*, for the purpose of mass screening of chemical compounds, prior to *in vivo* tests being performed. Parts of the research results have been published elsewhere (6, 7).

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MATERIALS AND METHODS

Inoculum preparation. Smut fungus, *Ustilago maydis* was grown in potato dextrose agar (PDA) at 28°C for 4 days. Sporidia were harvested by gently brushing the culture surface after adding sterile water. Sporidial suspension used as an inoculum was adjusted to various concentrations prior to inoculation.

Fungicides used and MIC tests. Five fungicides, benomyl 50 WP, triadimenol 5 WP, bitertanol 25 WP, CGA 173506, and metalaxyl 25 WP selected as a negative control were used in this study. CGA 173506, a 98% technical form was obtained from the Hannong Chemical Co., and others were purchased from a market. All of the fungicides are soluble forms in water.

The fungicides were dissolved in PDA to make their concentrations of 0.25, 0.5, 1, 2, 8, 16, 32, 64, and 128

μg/ml, and plated in Petri dishes. The Petri dishes were point-inoculated in the center with 0.1 ml of the sporidial inoculum of *Ustilago maydis* at the concentration of 10 sporidia/ml, and incubated at 28°C. Treatment was replicated fourteen times. Colony growth of the smut fungus was checked 48 hr after inoculation.

Agar diffusion test. PDA was mixed thoroughly with the sporidial inoculum at the concentration of 10^7 sporidia/ml and plated in Petri dishes. Sterilized stainless rings (0.7 cm diam.) were placed on the medium three rings each. Suspensions of each fungicide adjusted at the concentrations of 0.5 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml and 100 μg/ml were put inside the ring 0.2 ml each. Treatment was replicated five times. The Petri dishes were incubated at 28°C and inhibition zone appearing around the ring was measured 48 hr after incubation.

Sporidial germination test. Each fungicide suspension at the concentration of 5 μg/ml was thoroughly mixed with sporidial suspension at the concentration of 10^7 sporidia/ml. The mixed suspension was put 100 ml each into 250 ml flasks and placed in a shaking incubator with 120 rpm at 30°C. Treatment was replicated five times. Sporidial germination rate was examined at 2 hr intervals until 8 hr.

Greenhouse test with corn seedlings. Corn cultivar Jaerae was seeded two each in pots (10.5 cm diam., 10 cm high) containing standard soil (Flora gard, TKS 2) and grown in a greenhouse at 21~35°C until use. Seedlings at the 4th leaf stage were sprayed with each fungicide suspension at the concentrations of 10 μg/ml and 100 μg/ml using an atomizer in an inoculation rotary chamber, and left until air-dried. The seedlings were then inoculated similarly by spraying the sporidial inoculum at the concentration of 10^5 sporidia/ml. Inoculated corn seedlings were kept in a dew chamber at 28°C for 24 hr and placed in the greenhouse for smut development. Smut development on the inoculated seedlings was examined 10 days after inoculation based on the scale of 0 (healthy) to 5 (killed) described elsewhere (5).

RESULTS

Minimum inhibitory concentration. Degree of growth inhibition differed greatly with fungicides tested (Table 1). CGA 173506 and bitertanol completely

Table 1. Minimum inhibitory concentration (MIC) of five fungicides as examined by the status of colony growth of *Ustilago maydis* on PDA after 48 hr incubation at 28°C

Fungicide	Degree of growth[a] at conc. (μg/ml) of										MIC (μg/ml)
	128	64	32	16	8	4	2	1	0.5	0.25	
Benomyl	−	−	−	−	−	−	+	+	++	++	4
Triadimenol	−	−	−	−	−	−	−	+	++	++	2
Bitertanol	−	−	−	−	−	−	−	−	−	+	0.5
CGA 173506	−	−	−	−	−	−	−	−	−	−	< 0.25
Metalaxyl	++	++	++	++	++	++	++	++	++	++	128 <
Control	++	++	++	++	++	++	++	++	++	++	−

[a] Based on observations of fourteen replications. − : no growth, + : reduced growth, ++ : vigorous growth.

Table 2. Inhibition of growth of *Ustilago maydis* by five different fungicides 48 hr after incubation at 28°C in an agar diffusion test

Fungicide	Diam. (mm) of inhibition zone at conc. (μg/ml) of					
	0.5	1	5	10	50	100
Benomyl	0[a]	9.4	15.5	17.7	24.5	27.4
Triadimenol	0	0	0	0	34.8	38.9
Bitertanol	16.2	18.8	24.9	26.5	23.7	25.1
CGA 173506	19.6	36.0	45.3	47.9	47.4	47.7
Metalaxyl	0	0	0	0	0	0
Control	0	0	0	0	0	0

[a] Values are means of five replications.

inhibited the growth of *U. maydis* with MIC of < 0.25 μg/ml and 0.5 μg/ml, respectively, whereas metalaxyl did not show any growth inhibition at the highest concentrations tested. Benomyl and triadimenol were intermediate with MIC of 4 μg/ml and 2 μg/ml, respectively.

Size of inhibition zone. Inhibitory effects of the fungicides shown by an agar diffusion test were generally similar to those of MIC test. CGA 173506 and bitertanol began to inhibit the growth of *U. maydis* at 0.5 μg/ml and triadimenol at 50 μg/ml (Table 2). By this test benomyl was found to be more effective than triadimenol showing the inhibition zone appeared from 1 μg/ml. Metalaxyl did not develop any inhibition zone.

Sporidial germination. Among five fungicides tested, CGA 173506 inhibited most effectively the sporidial germination (Table 3). Metalaxyl did not affect sporidial germination. Inhibition of sporidial germination by other three fungicides was similar with much lower degree compared with CGA 173506. However, degree of the inhibition varied significantly with time of examination.

Table 3. Temporal germination of sporidia of *Ustilago maydis* in sporidial suspension containing 5 μg/ml of each of five fungicides after shaking-incubation at 30°C

Fungicide	% sporidial germination after			
	2 hr	4 hr	6 hr	8 hr
Benomyl	3.5[a]	6.7	27.4	58.5
Triadimenol	1.4	5.4	28.4	77.7
Bitertanol	6.4	2.8	21.4	73.2
CGA 173506	0.5	1.1	13.4	18.0
Metalaxyl	3.1	7.5	49.1	70.5
Control	2.7	8.5	39.1	78.6

[a] Values are means of five replications.

Seedling tests in greenhouses. Smut development varied greatly with fungicide and concentration applied. Metalaxyl that did not show any *in vitro* activity also failed to inhibit smut development on the seedlings (Table 4). Both CGA 173506 and bitertanol effectively reduced smut development at 100 μg/ml with averaged severity index of 0.4 and 0.6, respectively. Benomyl was more effective than triadimenol at both application rates. CGA 173506 and triadimenol did not show any inhibition at 10 μg/ml. However, when application dosages of the fungicides raised 10 times higher, from 10 μg/ml to 100 μg/ml, their control efficacy became greater, 2.2 times for benomyl, 1.2 times for triadimenol, 4.7 times for bitertanol and 9.8 times for CGA 173506.

Correlation between *in vitro* tests and greenhouse seedling tests. Ranks in inhibitory effect by the five fungicides in each screening test were shown in order of the greatest effect (rank 1) to the least (rank 5) in Table 5. The fungicide with the greatest effect or with the least effect was consistent in all methods tested.

Table 4. Inhibition of smut development on corn seedlings 10 days after applications of five fungicides at two different rates in the greenhouse

Fungicide	Dosage applied (μg/ml)	Smut development index (0~5)					Average
		I[a]	II	III	IV	V	
Benlate	10	1.5[b]	2	4	1	2.5	2.2±1.2[c]
	100	0	0.5	2	0.5	2	1.0±0.8
Triadimenol	10	4	4	4	3.5	4	3.9±0.2
	100	3.5	2	3.5	4	3.5	3.3±0.8
Bitertanol	10	1	1.5	3	4.5	4	2.8±1.5
	100	1	0	1.5	0.5	0	0.6±0.7
CGA 173506	10	4.5	4	4.5	4	2.5	3.9±0.8
	100	0	0.5	0.5	1	0	0.4±0.4
Metalaxyl[d]	10	4	4	4	3.5	4	3.9±0.2
	100	4	3.5	4	4	4	3.9±0.2
Control	–	3.5	4	4	4	4	3.9±0.2

[a] Replication.

[b] Average of two plants. 0: healthy, 1: leaf discoloration, 2: leaf discoloration with mild distortion, 3: severe distortion and minor gall formation, 4: severe distortion and numerous gall formation, 5: death of whole plant.

[c] Standard deviation.

[d] Used as a negative control.

Table 5. Comparisons of the results of *in vitro* tests with that of the greenhouse seedling test shown by relative ranks for inhibitory effect by five fungicides against corn smut disease

Fungicide	Relative rank[a] of inhibitory effect			
	Seedling test	MIC[b] test	Agar diffusion test	Sporidial germination test
Benomyl	3	4	3	3
Triadimenol	4	3	4	4
Bitertanol	2	2	2	2
CGA 173506	1	1	1	1
Metalaxyl	5	5	5	5
Control	5	5	5	5

[a] In the order of the greatest inhibitory effect (rank 1) to the least (rank 5).
[b] Minimum inhibitory concentration.

The second effective fungicide detected by each method was also identical. Only varying ranking is shown by MIC test with benomyl and triadimenol on ranks 3 and 4.

Degree of the inhibition in sporidial germination by the five fungicides varied significantly with time of examination (Table 3). Among the three methods employed, agar diffusion test corresponded most accurately to the greenhouse seedling test.

DISCUSSION

In the greenhouse seedling test, the evaluation of control efficacy of some fungicides was difficult due to large variations among replications, particularly when the application dosage was low. One of the main reasons for the variation might be variability of successful colonization of host tissues by smut fungus itself irrespective of fungicides applied as observed in a study (4), because infections occur only by the fusion of two compatible haploid sporidia which gives rise to binucleate infection hyphae of *U. maydis*.

This study suggests that to lessen this intrinsic variations, the application dosage of fungicides has to be increased or the number of replications should be increased. High application concentrations as 100 μg/ml, compared to 10 μg/ml was much more effective to differentiate the disease suppression by the fungicides.

Among three methods employed, agar diffusion test reflected most accurately the results from the greenhouse seedling tests. Other two methods were also significantly correlated with the seedling tests, but failed to differentiate the degree of disease suppression between benomyl and triadimenol.

In sporidial germination tests, the results obtained varied considerably with time of examination. Most suitable time to examine sporidial germination was 6 hr after incubation. Thereafter, the differentiating ability was significantly reduced as the time prolonged.

Agar diffusion test that appeared most accurate in this study is simple, fast and easy to perform in a limited space without significant efforts. Thus, this method may be used practically in primary evaluation tests as a simple method for the mass screening of chemical compounds against smut disease prior to *in vivo* tests being performed.

요 약

옥수수 깜부기병균에 대한 살균제 활성검정시 실내검정과 온실 유묘검정과의 상관정도를 조사하기 위하여 5가지 살균제를 공시하여 실내와 온실에서 깜부기병균에 대한 억제효과를 비교하였다. 실내검정방법으로 사용한 최소생육억제농도법, 저지원법, 소생자발아검정법의 결과는 모두 온실유묘검정에서 얻어진 결과와 상관이 높았으며 특히 저지원법은 유묘검정결과와 매우 높은 상관관계를 보였다. 따라서 저지원법과 같은 실내검정방법은 대량의 활성검정시 간이검정방법으로 유용하게 이용될 수 있으리라 생각된다.

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