Studies on the White Root-Rot of Tea Bush. IV*

On the Toxicities of Cultural Filtrate of the Fungus

By

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In the previous work[1] (1955) we reported the toxicities of cultural filtrate of the fungus upon bean and tea seedlings. In the present experiment, relationship between hyphal growth of the causal fungus under different cultural conditions and the toxicity of filtrate, and the effect of dilution, H-ion concentration, heating and osmotic pressure upon toxicity of filtrate, were investigated.

I. Relations of hyphal growth of the causal fungus to different cultural conditions, and osmotic pressure to the toxicities of culture filtrates.

Components of cultural filtrate of fungi differed by kind and quantity of media used, culture period and other cultural conditions. As the leaves and stems of bean seedlings which adsorbed the culture filtrate showed symptoms of black spots, discolouration, wilting, curling and blackening etc., these phenomena were presumed to have been caused by the action of toxin in the filtrates. In the first step, we tried to clear whether these phenomena were caused by osmotic pressure of the filtrates.

Media used in this experiment were extracts of potato (sugar 2%) and thiamine medium (Glucose : 15g, KH_2PO_4 : 2g, $MgSO_4$: 0.2g, Asparagine : 2g, $Fecl_3$: trace, Thiamine : 100γ, Distilled water : 1l). The culture media were prepared by the usual way. Respectively 30cc. and 15cc., and 100cc. and 200cc. of the media were poured into 50cc. and 500cc. ERLENMEYER's flasks. They were sterilized in an autoclave held at 15 lbs. pressure for 15 minutes. By reason of its comparatively high pathogenicity, culture strain R1 of the causal fungus was used in the present experiment.

A 2 mm disk of mycelium cut from the peripheral parts of 6 days old plate culture was transfered to the flask containing the above media. The flasks inoculated with the fungus R1 were held for 10, 20, 30, 40, 50, 60, and 90 days under 24°C, and the quantity of absolute growth of the fungus was compared by measuring the dry weight of mycelia filtered from the media. Toxicity of the culture filtrates was investigated by the effect on the cuttings of bean seedlings. Osmotic pressure of original media (before culture) and the culture filtrate of thiamine media was calculated by the freezing point method using BECKMANN's apparatus. The experimental results are shown in Table 1.

From the results of Table 1, the hyphal growth of the fungus on potato extracts was more rapid than on thiamine media. Although the toxicity and mycelium weight varied with the variety and quantities of culture media in general, yet toxicity of culture filtrates had no direct relation with the mycelium weight under different cultural conditions. However, interdependence of the mycelium weight and toxicity of filtrate was recognized on the same thiamine medium. LUKE and WHELER[4] (1955) found that toxin produced by 3-isolate of *Helminthosporium victoriae* correspond with hyphal growth of the fungus. WHITE and WOLF[7] (1953) described that the oak wilt fungus, *Endoconidiophora fagacearum* in shake cultures has more rapid growth, glucose utilization, a higher economic coefficient, rise in pH, earlier toxin production, and higher titers than by stationary culture.

In the present examination by stationary culture, the mycelium weight was not uniform on respective flask, and the media in which

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Table 1. Toxicity of culture filtrates, hyphal growth under different cultural conditions, and relation between toxicity and osmotic pressure of the culture filtrates (average results of 7 flasks per set).

Media		Days of culture	Final pH of culture	Dry weight of mycelium (mg)	Toxicity of cultural filtrate to bean seedlings		Osmotic pressure (atom. at 20°C)
Kinds	Quantity				degree*	symptom on leaf	
Extract of potato	30cc to 50cc flask	10	6.2~6.4	107.36	±	a slight wilting	——
		20	7.0	118.46	+	wilting and discolouration	——
		30	7.4~7.6	130.04	++	black-spot, discolouration and wilting	——
Thiamine	30cc to 50cc flask	10	5.4~5.6	20.7	±	a slight black spot	3.876
		20	5.6~5.8	34.3	±	a slight discolouration, wilting and black spot	4.005
		30	6.2~6.6	68.1	+	black spot, blackening and wilting	4.005
		40	6.8~7.2	105.4	++	do	4.005
Thiamine	15cc to 50cc flask	10	5.4~5.6	22.3	−	health	4.005
		20	6.0~6.2	53.2	±	a slight discolouration	4.392
		30	6.8~7.0	64.7	+	a slight discolouration and wilting	3.617
		40	6.8~7.0	84.9	+	black spot and wilting	2.713
Thiamine	100cc to 500cc flask	30	4.8~5.0	52.9	±	a slight wilting and discolouration	——
		60	6.0~6.4	328.9	+	black spot and wilting	——
		90	5.8~6.2	439.3	++	wilting, discolouration, and curling	——
Thiamine	200cc to 500cc flask	30	4.8	82.9	±	discolouration and black spot	——
		40	6.8	424.9	++	wilting and black spot	3.488
		50	6.6~6.8	——	++	do	2.971
		60	6.0~6.4	600.6	++	curling, blackening and black spot	——
		90	5.6~6.2	944.5	+++	black spot, wilting and curling	——
Original media (thiamine)		0	4.6	0	−	health	——

* − : Health. ± : Slight toxic symptom.
\+ : Moderate toxic symptom.
++ : Heavy toxic symptom.
+++ : Heavy toxic symptom and rapid development of the symptom.

H-ion concentration was held about pH 5 before culture showed neutral or slightly alkaline after 40~60 days of culture, further, it changed again to slight acidic after 90 days. The filtrate from a longer culture (50 days) showed brown, dark brown and deep reddish brown colou r, and the

acid media changed to neutral or alkali. FOSTER[2] (1949) described that a number of changes in the medium accompany the autolytic process: the medium gradually assumes a colour similar to freshly prepared ordinary tea and at times may be a deep red brown; this intrinsic dark brown coloration is ascribed to accumulation of resistant N-containing humin, and it is especially characteristic of neutral or alkaline autolysates; METZ (1930) also observed the correlation between autolysis and the dark red brown color of the solution in a study of 13 different organisms. In present experiments, the H-ion concentration and colouration of the toxic filtrate appear to develop by the process of autolysis, though, we suppose the toxin of filtrates is not autolysate but metabolite since the fungi do not arrive at maximum growth in the experimental term.

Toxicity of filtrate to bean seedlings could not be compared quantitatively, but the filtrate was recognized to be toxic in the cultures that were more than 20 days old and the mycelial weight beyond 30 mg. Correlation between mycelial weight and toxicity of the filtrate existed on the same media, but was not shown on different media since the fungus metabolism in culture is supposed to have differed by kinds of media.

Osmotic pressure of the filtrate appeared to have no direct relation with toxicity of the filtrate.

II. Toxicity of culture filtrate to germination of rice and other seeds.

LUKE and WHELER[4] (1955) reported that the toxin produced by *Helminthosporium victoriae* seriously inhibited root growth of seedlings of oat, wheat, barley, rice, corn and vegetable crops, specially, *victoria* type of oat showed 50 percent inhibition in $^1/_{1200000}$ dilution of culture filtrate from the fungus.

HIROE and NISHIMURA[3] (1956) reported that wilt toxin by *Fusarium bulbigenum* var. *niveum* causing watermelon wilt indicated wilting in $^1/_{32}$ dilution of the cultural filtrate on sensitive variety of watermelon.

TAMARI and KAJI[6] (1954) descibed that $^1/_{50000}$ and $^1/_{200000}$ dilution of crude toxin produced by *Piricularia oryzae* CAV. showed a toxicity to rice seedling of susceptible varieties, and resistance to the disease correspond with resistance to the toxin.

Since the quantitative estimation of toxicity of the culture filtrate by symptoms on bean and tea seedlings are unreliable, the toxicity to various plants was compared again by measurements of root length of seedlings germinated on the culture filtrates.

Toxicity for rice seedlings (var. *Senbon asahi*) was investigated with 0, $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$, $^1/_{32}$ dilution of the culture filtrate.

With a pipet 5cc. of diluted culture filtrate poured into a sterilized PETRI dish which contained a filter paper on bottom, then 25 grains of rice seed were set with a suitable arrangement on filter paper in each dish. Lengths of buds and roots were measured after keeping for 6 days at 28°C.

The culture filtrates were divided into two sets; namely: heat (heated for 10 minutes at 15 lbs. pressure) and filter (filtered by modified BERKEFELD type's bacterial filter) treatments. The original media (before culture) adjusted with NaOH to correspond to H-ion concentration of the culture filtrate were examined on germinability of rice seeds in the same dilution to the culture filtrate. And also influences of toxin to germination of cabbage, radish, barley, wheat and rye seeds were investigated after incubation for 6 days at 24°C. For comparison of toxicity, toxic index was calculated from rates of root-length on the filtrates and media relative to standard root length of the seedlings on sterilized distilled water.

$$\text{Toxic index} = \frac{A-B}{A} \times 100$$

A: root-length on sterilized distilled water.

B: root-length on the filtrate or original media.

Toxicity of diluted filtrates showed in Table 2 (illustrated on Plate II).

From the results of Table 2, the culture

Table 2. Toxicity of diluted culture filtrates (average length of buds and roots of 100 rice seedlings in centimeter after 6 days incubation at 28°C)

Dilution	Original media pH 5.4~5.6			Heated cultural filtrate pH 5.4~5.6			Original media pH 6.6~6.8			Filtered cultural filtrate pH 6.6~6.8		
	length		toxic	length		toxic	length		toxic	length		toxic
	bud	root	index	bud	root	index	bud	root	index	bud	root	index
0	2.35	7.79	0	—	—	—	—	—	—	—	—	—
1/32	2.82	8.29	−6.4	2.57	2.95	62.1	2.59	7.31	6.1	2.68	3.21	58.7
1/16	3.06	8.41	−7.9	2.58	2.64	66.1	—	—	—	2.58	2.71	65.2
1/8	2.58	7.67	1.5	2.45	2.07	73.4	—	—	—	2.60	2.58	66.8
1/4	2.40	7.11	8.7	2.32	1.37	82.4	2.55	6.86	9.8	2.61	1.54	80.2
1/2	2.02	6.99	10.2	2.13	0.92	88.1	2.92	7.18	11.5	2.04	1.42	81.7
1	2.02	6.80	12.7	1.85	0.93	88.0	2.47	7.88	11.8	1.81	0.85	89.0

filtrates were found to greatly inhibit root growth of rice seedling. Although the original media showed a growth inhibition at high concentration yet it showed a growth acceleration at low concentration, and 1/4 dilution of the media except glucose did not inhibit growth at all. Besides, the toxicity for bud growth of rice seedling is recognizable at high concentration (in 0 and 1/2 dilution), it showed severe symptom on root than the bud.

Toxic index at 1/32 dilution shows above 60, and it becomes greater on account of an increase in concentration, but rice seedling was not killed even at undiluted filtrate.

This toxicity inhibited not only development of root length but also number of roots and root hairs. Namely, then numbers of root were 10 in control, 4~5 in 1/32 dilution and only one in non-diluted culture filtrate, and the root on culture filtrate indicated brownish discolouration in various grades. As toxic index for rice seedling on the original media was about 10, and so in original media for seed germination there may contain some toxic substance. But germination of rice seed and its growth on the media excepted glucose and asparagine were normal, and also the composition of culture filtrate after 50 days culture is supposed to change from original media, so that glucose and asparagine in the original media probably may have disappared or decreased in the culture filtrate. According to above experimental results, culture filtrate of *Rosellinia* sp. contains toxic substances as metabolic product of the fungus and the toxic substances are supposed to be composed from more than 2 kinds, one of which inhibits root-growth in low concentration and the others inhibits bud growth in high concentration.

Experimental results on the toxicity of culture filtrate for cabbage, radish, wheat, barley and rye seeds are given in Table 3.

From Table 3, it is obvious that the culture filtrate inhibited root-growth of cabbage, radish, wheat barley and rye seedlings, but toxicity on these seedlings was shown to be lower than on rice seedling. In present examination must be remarked on toxicity of original media, but culture filtrate showed low toxic index to cabbage seedlings and also the original media were non-toxic to the same seedlings. From the above statement it may be said that the cabbage is resistant to white root-rot disease.

III. Effect of H-ion concentration for toxicity of culture filtrate.

In the present examination, symptoms on bean seedlings were observed on the culture filtrate, original media and distilled water, in which the pH levels were adjusted to 3~11. The results are given in Table 4.

Bean seedlings put into sterilized distilled water and original media remained healthy after 4 days, but serious symptoms such as black-spotting and discolouration of leaves appeared

Table 3. Effect of culture filtrate to germination and growth of seed of crop plants (average bud and root lengths of 100 seedlings in centimeter 6 days incubation at 24°C).

Sample solution	Cabbage			Radish			Wheat			Barley			Rye		
	length		toxic	length		toxic	length		toxic	length		toxic	length		toxic
	bud	root	index	bud	root	index	bud	root	index	bud	root	index	bud	root	index
Sterilized distilled water	2.80	2.56	0	6.16	7.10	0	5.37	11.69	0	3.34	11.04	0	8.28	10.43	0
¼ dilution of original media excepted glucose			* 2.28			* 19.84			* 3.74			* 21.3			* 3.17
¼ dilution of original media	2.94	2.77	−8.2	4.86	4.70	33.8	4.98	4.76	59.2	3.05	8.68	21.2	8.08	9.32	10.6
¼ dilution of cultural filtrate	2.94	1.66	35.1	5.98	2.45	65.4	4.80	4.05	65.3	2.86	5.07	54.0	5.43	3.69	64.6

* Results obtained from experiment carried at 26~30°C in summer.

Table 4. Effect of H-ion concentration on the toxicity of the filtrate to bean seedlings. (4 days after treatments)

pH	3.0	5.0	7.6	9.4	11.0
Sterilized distilled water	−	−	−	−	−
Original media	−	−	−	−	−
Culture-filtrate	++	++	++	+	±

Note : − : Health ± : Slight toxic symptom
+ : Strong toxic symptom but slow in development
++ : Strong toxic symptom and rapid in development

in culture filtrate except under alkaline reaction where only a slight symptoms appeared. From these results, toxins in the culture filtrate are supposed to be comparatively stable under acidic condition but unstable when alkaline. On the effect of H-ion concentration for toxicity of the filtrates, however, must be inspected since the sample solutions indicated weak buffer action. To make sure of above supposition we continued further experiment using rice seed.

The cultural filtrate (pH 5.8~6.0) and original media excepting glucose and asparagine (pH 4.8) were modified to pH-level 13, 10, 3 and 1. They were held for 24 hours, and then they were readjusted at pH 5.8–6.0. On the above solutions, effect of H-ion concentrations upon toxicity of the cultural filtrate were investigated by germination of rice seed. The results obtained are given in Table 5.

From the results of Table 5, the toxic substance of cultural filtrate held for 24 hours in acidic or alkaline condition appeared to be stable.

Table 5. Effects of H-ion concentration on toxicity of the filtrate (average length of buds and roots of 100 rice seedlings in centimeter after 6 days incubation at 28°C.)

Sample solution	pH controled for 24 hours	Length of bud	Length of root	Toxic index
Distilled water (pH 5.8~6.0)	—	1.48	5.58	0
Original media excepted glucose and asparagine (pH 4.8)	checks	1.46	6.05	− 8.42
	1	1.31	5.58	0
	3	1.83	6.76	−21.14
	10	1.53	6.67	−19.53
	13	1.53	5.21	6.63
Cultural filtrate (pH 5.8~6.0)	checks	1.52	1.09	80.46
	1	1.26	0.90	83.87
	3	1.43	0.78	86.02
	10	1.49	1.23	77.95
	13	1.17	1.09	80.46

Summary

Relation between toxicity of the culture filtrate and cultural conditions, dilution of culture filtrate, H-ion concentration, as well as heat treatment and osmotic pressure were investigated with regard to cultural filtrate of *Rosellinia* sp. (R1).

1) On the different media toxicity of the culture filtrates and mycelium weight showed no direct correlation but on the same media former was responsive to later. This toxin probably may not be autolysis products but metabolites.

2) Osmotic pressure of the culture filtrate indicated no correlation with toxicity of culture filtrate.

3) It was observed that the culture filtrate is toxic for the germination of rice seeds.

Because of the toxicity of $^1/_{32}$ dilution of the culture filtrate, which was either autoclaved for 10 minutes at 15 lbs. pressure or filtered through modified BERKEFELD type's bacterial filter, showed toxic index of above 60, the toxic substance in culture filtrate was assumed to be thermostable.

4) Inhibition of root growth of cabbage, radish, wheat, barley and rye seedlings was similar to that obtained on rice seedling. Cabbage showed the lowest toxic index of 35.1 in $^1/_4$ dilution. Inhibition on bud growth was recognizable only in high concentration of culture filtrate. Toxin of the culture filtrate was supposed to contain more than 2 components.

5) No difference in the toxicity of the culture filtrate to rice seedling was shown at pH levels ranging between 5~7, but on bean seedling it decreased in alkaline and increased in acidic. However, from further experimental result on rice seed, the toxic substance of cultural filtrate held for 24 hours in acidic or alkaline condition appeared to be stable.

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Explanation of Plates

Plate I

A. Tea seedlings in original media (before culture) and water.
1. $\frac{1}{2}$ dilution, 2. $^1/_8$ dilution, 3. $^1/_{32}$ dilution, 4. sterilized distilled water.

B. Tea seedlings in cultural filtrates.
1. $\frac{1}{2}$ dilution, 2. $^1/_8$ dilution, 3. $^1/_{32}$ dilution.

C. Bean seedlings in
1. sterilized distilled water, 2. original media, 3. cultural filtrates.

Plate II

A. Germinations of rice seedlings in
1. cultural filtrates,
2. sterilized distilled water.

B. Germinations of various seedlings in sterilized distilled water (above) and $^1/_4$ dilutions of cultural filtrate (below).
1. barley, 2. wheat, 3. rye, 4. radish, 5. cabbage.

摘　　要

安部卓爾・河野又四 ： 茶白紋羽病に関する研究（第4報）
茶白紋羽病菌培養濾液の毒性について

茶白紋羽病菌の培養濾液の毒性について，前報に引続いて培養条件，培養濾液の稀釈，加熱，水素イオン濃度及び滲透圧等が濾液毒性に及ぼす影響並びに各種作物の種子に対する毒性の差異を調査した．

1. 異種の培地上菌体重と濾液の毒性とは直接の関連を示さないが，同一培地上では菌体重の大なる培養から得られた濾液ほどその毒性が高い．そしてこの毒性は自己消化によつて生産される物質に基因するものではなく，代謝産物に基くものと考えられる．

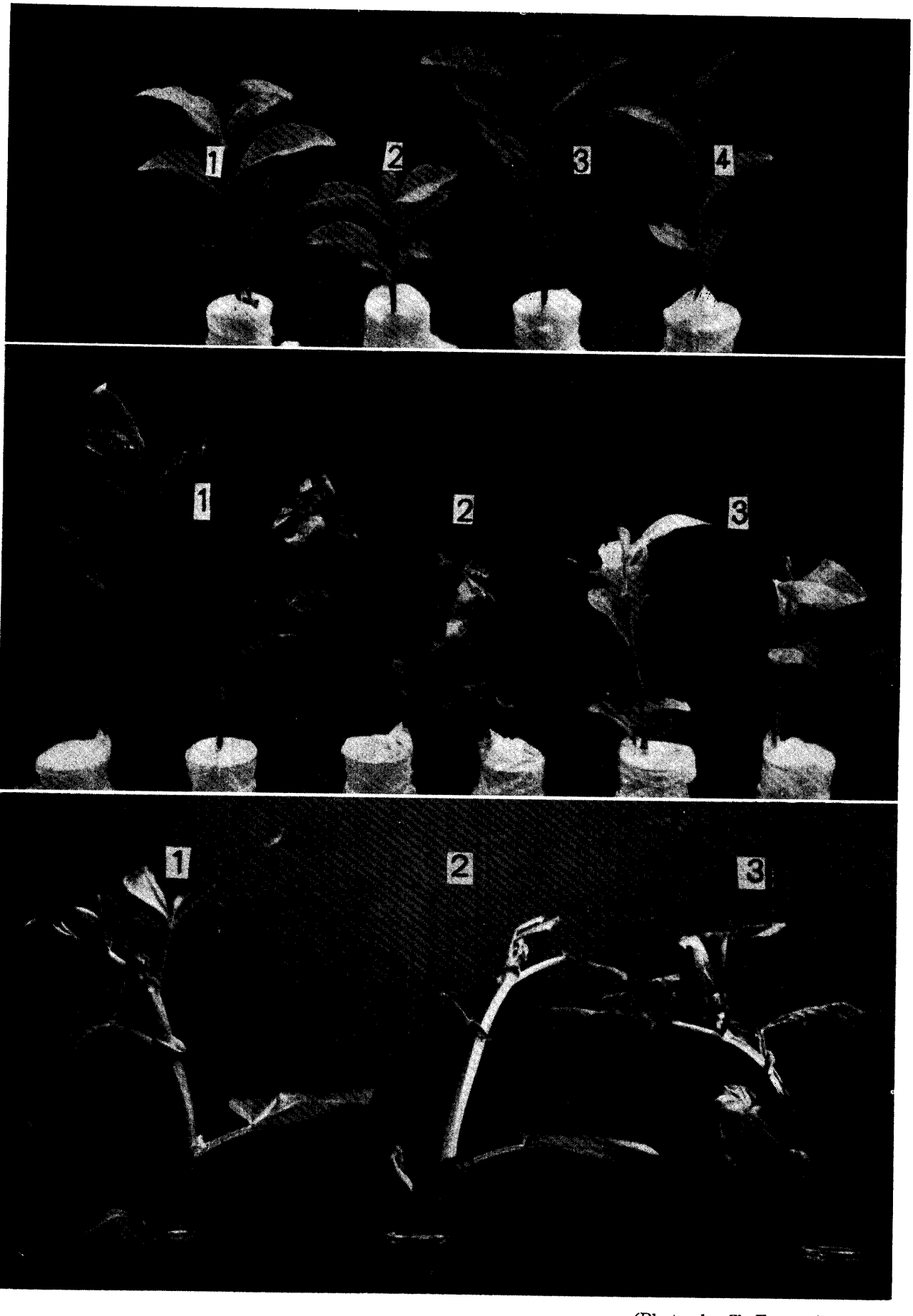
2. 濾液の滲透圧はその毒性と直接の関係はない．

3. Thiamine 培地上50日間培養から得られた濾液の毒性を水稲種子の発根生長によつて検定したが，濾液を15封度圧10分間加熱区及び細菌沪過管による沪過区に分つたものでは，兩者共 $^1/_{32}$ 稀釈まで毒性指数60以上を示し，毒性物質は熱に対し安定と考えられる．

4. 甘藍，大根，小麦，稗麦及びライ麦に対しても水稲の場合と同様に根の伸長を阻害するが，芽の生長に対しては沪液濃度が高い場合に毒性を示す．これらの作物の中，甘藍の発根に対する阻害度が最も低いことから，甘藍は白紋羽病に対し抵抗性の高い作物ではないかと考えられるが尚検討を要する．

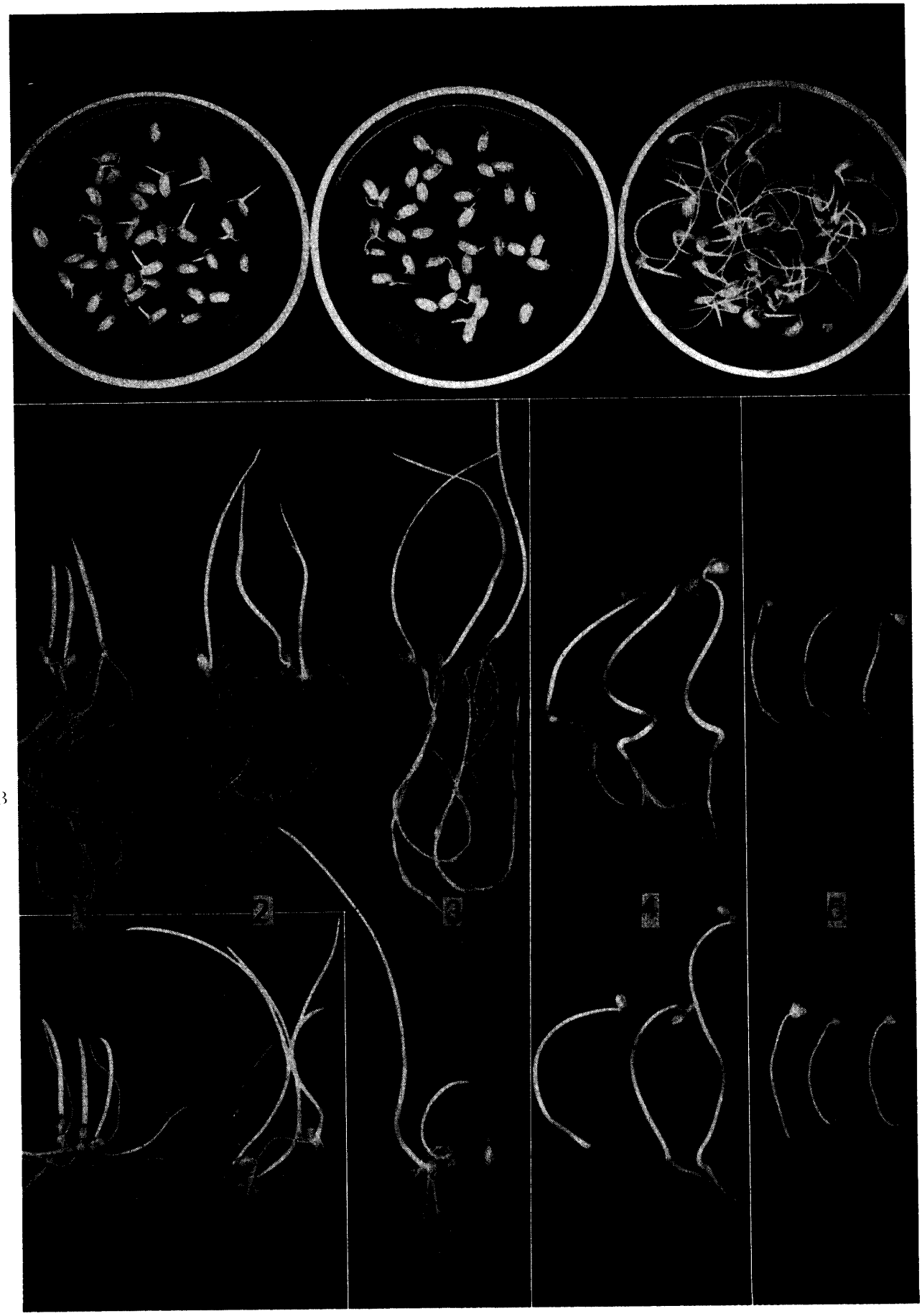
5. 水稲種子に対する培養沪液の毒性は pH 5～7では差異を認めないが，そらまめ苗に対しては強アルカリ性では毒性物質は稍不安定で，酸性では安定なものと推定された．併しながら酸性又はアルカリ条件で24時間保存した培養沪液を用いての水稲種子によるその後の実験の結果では毒性物質は反応を変えることによつて特別の影響を受けないものと考えることが出来る．

Plate I



(Photo. by T. FURUYA)

Plate II



(Photo. by T. Furuya)