FACTORS AFFECTING THE ISOLATION OF MICROORGANISMS ASSOCIATED WITH CONIFEROUS SEEDS[1]

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Introduction

Microorganisms associated with coniferous seeds have been extensively studied by Fisher (1941), Garbowski (1936), Sato (1955), Timonin (1964), and Vanine et al. (1932). Most of them were proved to incite both pre- and post-emergence damping-off of conifers (Fishers, 1941; Garbowski, 1936; Hartley, 1918; Rathbun-Gravatt, 1931; Vanino, 1931). Further evidence revealed that saprophytes of the coniferous seed-coat microflora could attack seedlings and produce symptoms of damping-off under favorable conditions (Timonin, 1964). Seed treatment was suggested to be more efficacious than soil treatment for the disease control (Berbee et al. 1953; Chen, 1961; Cockerill, 1961; Weihing, 1961). Gibson (1956) demonstrated that Granosan used in soil treatment indirectly assisted the spread of the damping-off pathogens through the soil by its selective action on the antagonistic microflora. Since seeds are feasibly conceivable to carry causal organisms of damping-off of conifers into the soil, an attempt to survey the microflora associated with seeds is very practical to understand the diseases incidence. With regard to this matter, attention was firstly directed to the factors affecting the isolation of microorganisms from coniferous seeds.

Materials and Methods

Seeds of slash pine (*Pinus elliottii*), Luchu pine (*P. luchuensis*), and Japanese black pine (*P. thunbergii*) were used for this series of experiments. Slash pine seeds, imported from the States on March 26, 1962, were secured by courtesy of the Taiwan Forestry Bureau. Luchu pine and black pine seeds, collected in the Grass Mountain in September of 1963, were purchased from a forester. They were stored in a cold room at 0° to 5°C, for a period of time perior to the determinations.

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Surface treatment was made by soaking seeds respectively in 0.1 per cent mercuric chloride, 0.1 per cent silver nitrate, 1 per cent sodium hypochlorite, and 50 per cent alcohol containing 0.1 per cent mercuric chloride for a given period of time. Potato-dextrose agar acidified with lactic acid, nutrient dextrose agar, Czapek's sucrose agar, Czapek's nitrate agar, and Czapek's sucrose nitrate agar diminished one of the following constituents: Potassium dibasic phosphate, magnesium sulphate, potassium chloride, or ferrous sulphate were used. The treated seeds were transfered aseptically to agar plate, 10 seeds a Petri-dish, and incubated at the temperature cencerned. Five or ten replications were made in each treatment.

The composition and initial pH values of media tested are listed as follows:

1. Czapek's sucrose agar (pH 4.6)

Distilled water	1,000.00 ml.
Potassium dibasic phosphate	1.00 g.
Magnesium sulphate	0.50 g.
Potassium chloride	0.50 g.
Ferrous sulphate	0.10 g.
Sucrose	30.00 g.
Agar	15.00 g.

2. Czapek's nitrate agar (pH 4.8)

Distilled water	1,000·00 ml.
Sodium nitrate	2.00 g.
Potassium dibasic phosphate	1.00 g.
Magnesium sulphate	0.50 g.
Potassium chloride	0.50 g.
Ferrous sulphate	0.10 g.
Agar	15.00 g.

3. Czapek's sucrose nitrate agar deficient in potassium dibasic phosphate (pH 4.6)
4. Czapek's sucrose nitrate agar deficient in magnesion sulphate (pH 5.0)
5. Czapek's sucrose nitrate agar deficient in potassium chloride (pH 4.6)
6. Czapek's sucrose nitrate agar deficient in ferrous sulphate (pH 5.2)
7. Nutrient dextrose agar (pH 6.6)

Distilled water	1,000.00 ml.
Beef extract	3.00 g.
Peptone	10.00 g.
Dextrose	10.00 g.
Agar	17.00 g.

8. Potato dextrose agar (pH 5.6)

Distilled water	1,000.00 ml.
Peeled and sliced potatoes	200.00 g.
Dextrose	20.00 g.
Agar	15.00 g.

Results

Effect of medium

Seeds of slash pine and Luchu pine were separately surface-treated for one minute with 4 chemicals aforementioned before rinsing in five changes of sterile water. The treated seeds were transfered aseptically to agar plate,

Table. 1. *Comparison of different media in relation to number of microorganisms yielded by coniferous seeds incubated at 25°C for 4-day.*

Medium	Treatment*	No. of bacterial** colonies from		No. of fungous** colonies from	
		P. elliottii	*P. luchuensis*	*P. elliottii*	*P. luchuensis*
Potato dextrose agar	CK	50	50	50	6
	1	0	1	9	0
	2	6	1	0	0
	3	21	50	21	21
	4	0	0	11	0
Nutrient dextrose agar	CK	50	50	36	0
	1	1	0	0	0
	2	2	0	0	0
	3	50	50	2	0
	4	0	3	1	0
Czapek's sucrose agar	CK	3	50	14	9
	1	0	0	1	0
	2	1	0	0	0
	3	3	50	0	2
	4	0	0	1	1
Czapek's nirate agar	CK	4	0	6	0
	1	0	0	0	0
	2	2	0	0	0
	3	3	0	0	0
	4	0	0	0	0
Czapek's sucrose nitrate agar (without K_2HPO_4)	CK	3	4	49	50
	1	2	0	1	1
	2	0	0	0	0
	3	14	0	18	21
	4	0	0	1	0

* Seeds, rinsed in five changes of sterile water (CK): surface-treated for 1 minute with 0.1% mercuric chloride (1), 0.1% silver nitrate (2), 1% sodium hypochlorite (3), or 0.1% mercuric chloride in 50% ethanol (4) before rinsing in five changes of sterile water.

** Total number of colonies from 50 seeds.

10 seeds a Petri-dish, and incubated at 25°C for appropriate length of time. Five replications were made in each treatment. Seeds rinsed in five changes of sterile water were served as control.

From the results shown in Table 1, the number of microorganisms yielded by coniferous seeds was largely affected by the medium used. The presence of carbon source, such as dextrose and sucrose, was required for the growth of microorganisms on the coniferous seeds. However, seed yielding microorganism was not considerably changed for lack of nitrogen source or minerals in the media tested. Among the media examined, potato dextrose agar was found to be suitable for the isolation of both bacteria and fungi.

Eight genera of fungi, i. e. *Aspergillus, Curvularia, Diplodia, Fusarium, Glomerella, Mucor, Penicillium,* and *Rhizopus* appeared frequently on the seed

Table. 2. *Effect of hydrogen-ion concentrations on the number of microorganisms yielded by coniferous seeds incubated on potato dextrose agar at 25°C for 4-day.*

pH value*	Treatment**	No. of bacterial*** colonies from		No. of fungous*** colonies from	
		P. elliottii	*P. luchuensis*	*P. elliottii*	*P. luchuensis*
5.6	CK	50	50	50	6
	1	0	1	9	0
	2	6	1	0	0
	3	21	50	21	21
	4	0	0	11	0
4.8	CK	0	50	50	8
	1	0	0	3	2
	2	0	0	3	0
	3	3	50	40	6
	4	0	0	2	0
4.0	CK	0	0	50	17
	1	0	0	3	0
	2	0	0	1	0
	3	0	0	45	9
	4	0	0	3	0
3.2	CK	0	0	50	28
	1	0	0	1	0
	2	0	0	7	0
	3	0	0	37	6
	4	0	0	0	0

* pH value was adjusted by adding lactic acid.
** The same as those mentioned in the foot-note of Table 1.
*** Total number of colonies from 50 seeds.

served as control. *Fusarium* and *Diplodia* were mostly isolated from the seeds of slash pine, whereas *Glomerella* was often from those of Luchu pine.

Effect of hydrogen ion concentration

The methods and materials were almost the same as that already mentioned in the previous section. Lactic acid was added to potato dextrose agar (PDA) for the adjustment of pH value to a given value of hydrogen ion concentration. The results were shown in Table 2.

Hydrogen ion concentration of pH 5.6 was found to be suitable for growth of both bacteria and fungi. However, the growth of bacteria was greatly diminished by change of pH value below 4.8, particularly below pH 4.0 there was no bacteria yielded by coniferous seeds. On the other hand, luxuriant growth of fungi was observed at any concentrations of hydrogen ion tested.

Effect of temperature

From the results obtained in the foregoing experiments, coniferous seeds were treated with 1 per cent sodium hypochlorite for 1 minute followed by rinsing in 5 changes of sterile water. The treated seeds were plated on potato dextrose agar (pH 5.6) and incubated at 13°, 16°, 19°, 22°, 25°, 28°, 31°, and 34°C, respectively.

So far as the experimental results (Table 3) were concerned, 25°C seemed to be preferable for the isolation of pathogenic fungi such as *Diplodia, Fusarium, Glomerella* etc.. A few saprophytes *e. g. Aspergillus, Penicillium* and

Table. 3. *Effect of temperature on the isolation of microorganisms associated with coniferous seeds**

Species		*P. elliottii*								*P. luchuensis*							
Temperature (°C)		13	16	19	22	25	28	31	34	13	16	19	22	25	28	31	34
No. of seeds yielding	Bacteria	0	9	21	35	36	36	42	50	0	50	50	50	50	50	50	50
	Fungi	0	4	10	13	14	15	13	8	0	1	0	10	14	14	14	14
Aspergillus		0	0	0	0	2	4	11	8	0	0	0	0	0	0	6	4
Curvularia		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Diplodia		0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0
Fusarium		0	0	2	2	2	4	2	0	0	0	0	2	2	6	2	0
Glomerella		0	0	0	0	0	0	0	0	0	0	0	0	9	5	4	0
Mucor		0	0	8	0	1	0	0	0	0	0	0	0	0	1	0	0
Penicillium		0	4	0	3	4	0	0	0	0	1	0	0	0	0	0	0
Rhizopus		0	0	0	8	0	3	2	4	0	0	0	8	4	1	0	0
Unidentified**		0	0	0	1	0	1	0	0	0	0	0	1	2	1	2	0

* Isolation of microorganisms in each treatment was made from 50 seeds incubated on potato dextrose agar (pH 5.6) for 4 days.

** No spore formation was observed when the identification was made.

Rhizopus were still found at the same temperatures aforementioned, however, the number of isolates were much less than that at the other temperatures.

Effect of chemical treatment

Seeds treated with different chemicals showed that microflora of coniferous seeds varied with the chemical applied (Table 1). Among the chemicals tested, sodium hypochlorite was found to be very mild as a disinfectant, whereas mercuric chloride was rather effective for seed disinfection without visible phytotoxicity. Therefore, sodium hypochlorite and mercuric chloride were selected for the determination of microflora and disinfection or seed, respectively.

In order to assure the experimental data closely related to the natural microflora of coniferous seeds, concentration and time effect of sodium hypochlorite were taken into consideration. Experimental results showed that changes in the number of seeds yielding *Aspergillus, Diplodia, Fusarium, Glomerella,* and *Penicillium* were not appreciable at different concentrations of sodium hypochlorite, *i.e.* 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0 per cent (Table 4). Contrarily, the growth of *Rhizopus* was significantly inhabited by the same concentrations examined.

Table. 4. *Effect of sodium hypochlorite on the isolation of microorganisms associated with coniferous seeds at different concentrations**

Species		*P. ellisttii*						*P. luchuensis*					
Concentration (%)		0.0	0.5	1.0	2.0	4.0	8.0	0.0	0.5	1.0	2.0	4.0	8.0
No. of seeds yielding	Bacteria	50	50	47	44	21	27	50	50	47	50	43	48
	Fungi	50	7	8	13	11	6	9	11	7	9	18	6
Aspergillus		0	0	3	5	1	0	0	0	0	0	0	0
Diplodia		0	3	2	3	1	2	0	0	0	1	7	1
Fusarium		2	1	0	1	2	2	1	0	4	0	0	0
Glomerella		0	0	0	0	0	0	7	9	1	6	1	5
Penicillium		0	3	2	4	4	0	0	0	0	0	0	0
Rhizopus		50	0	1	0	3	2	0	0	0	0	1	0
Unidentified**		0	0	1	0	3	2	1	2	2	2	10	0

* Isolation of microorganism in each treatment was made from 50 seeds incubated for 4 days.

** No spore formation was observed when the identification was made.

With regard to time effect of sodium hypochlorite, 4.0 per cent aqueous solution of sodium hypochlorite was used to treat coniferous seeds for various length of time, i.e. 1, 2, 4, 8, and 16 minutes. From the results shown in Table 5, time effected on the microorganism yielded by coniferous seeds was not definite though the rapidly growing bacteria were hindered. In addition

to the fact mentioned above, there was no indication of phytotoxic effect on both slash pine and luchu pine.

Table. 5. *Effect of time in treatment with 4% sodium hypochlorite on the isolation of microorganisms associated with coniferous seeds**

Pine		*P. elliottii*						*P. luchuensis*					
Length of time (min.)		0	1	2	4	8	16	0	1	2	4	8	16
No. of seeds yielding	Bacteria	100	35	28	26	17	8	100	100	96	86	100	84
	Fungi	45	14	17	12	13	13	8	2	4	4	14	3
Aspergillus		0	1	1	3	1	0	0	0	0	0	0	0
Cephalosporium		0	0	0	0	2	1	0	0	0	0	0	0
Curvularia		3	0	3	0	0	0	0	0	1	1	1	0
Diplodia		0	5	4	2	2	3	3	2	1	0	2	0
Fusarium		0	0	1	0	0	0	1	0	0	2	6	0
Monilia		0	0	0	0	0	0	0	0	0	0	1	0
Mucor		0	1	0	0	0	0	1	0	0	0	0	0
Penicillium		0	4	4	4	8	9	0	0	0	0	0	0
phoma		0	0	0	0	0	0	1	0	0	0	3	0
Rhizoctonia		0	0	0	1	0	0	0	0	0	0	0	1
Rhizopus		45	2	1	1	0	0	0	0	1	0	1	0
Unidentified**		0	1	4	1	1	0	3	0	1	1	0	2

* Isolation of microorganisms in each treatment was made from 100 seeds incubated for 4 days.

** No spore formation was observed when the identification was made.

In order to obtain a healthy young plant, 0.1 per cent aqueous solution of mercuric chloride was used in the treatment of coniferous seeds for various length of time, *i. e.* 0, 1, 2, 4, 8, and 16 minutes. In Table 6, it was shown that the germinability of seeds was improved by the treatment of coniferous seeds with mercuric chloride, however, the percentage of germination slightly decreased with the lapse of time treated. Nevertheless, *Aspergillus*, *Diplodia*, and *Penicillium* were not entirely killed by 0.1 per cent mercuric chloride. Since these fungi were found to reduce the germination of seeds and to attack the seedlings of conifers (Timonin, 1964; Vanin and Kotchkina, 1931), efficacy of fungicide on these fungi should not ignored. These fast growing fungi appeared after the seed germination might indicate the fact that they were present in the inner part of seeds. Further evidence was shown by treating seeds with 0.5 per cent mercuric chloride, crushing, and mixing the fragments of seeds with potato dextrose agar of 43°C in a Petri-dish. More fungi as well as bacteria were secured from these plates incubated at 28°C for 12 days.

Table. 6. *Comparison of sterilities of coniferous seeds treated with 0.1% mercuric chloride for various length of time**

Pine		*P. elliottii*						*P. luchuensis*						*P. thunbergii*					
Length of time (min.)		0	1	2	4	8	16	0	1	2	4	8	16	0	1	2	4	8	16
Percentage of germination		4	4	5	10	8	5	9	60	62	48	56	42	10	60	48	45	44	42
No. of seeds yielding	Bacteria	100	2	0	0	0	0	100	2	2	3	2	2	100	0	0	0	0	0
	Fungi	100	52	32	13	8	5	69	8	8	4	2	2	100	12	10	2	3	0
Alternaria		0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Aspergillus		12	16	1	7	4	0	14	1	0	1	1	1	17	3	4	1	0	0
Cephalosporium		0	0	40	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Curvularia		0	0	0	0	0	0	3	0	0	0	0	0	5	0	0	0	0	0
Diplodia		10	8	4	3	4	4	8	4	1	3	1	1	1	0	1	1	1	0
Fusarium		4	0	0	0	0	0	14	0	0	0	0	0	5	0	0	0	0	0
Monilia		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mucor		1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Penicillium		28	27	14	2	0	1	9	1	6	0	0	0	19	9	3	0	0	0
Phoma		0	0	0	0	0	0	3	0	0	0	0	0	5	0	0	0	0	0
Rhizoctonia		0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0
Rhizopus		70	0	0	0	0	0	2	0	0	0	0	0	36	0	1	0	0	0
Unidentified**		0	0	1	1	0	0	16	2	1	0	0	0	6	0	1	0	2	0

* Isolation of microorganisms in each treatment was made from 100 seeds incubated for 12 days.

** No spore formation was observed when the identification was made.

Discussion and Conclusion

The frequent recurrence of germination losses in nursery beds has baffled nursery stock in Taiwan year after year. The contaminations of fungi have been proved to be inhibitory to the seed germination and inciting the decay of radicle emerged from the seed coat (Fisher, 1941; Garbowski, 1936; Hartley, 1918; Huss, 1952; Rathbun-Gravatt, 1931; Ten Houten, 1936; Vanin, 1931; Vanine et al., 1932). In order to clarify the primary causes of reduced emergence in nursery beds, survey of seed microflora was intended in the first place. For this purpose, the present experimental data are indispensable.

Although a reliable method for the isolation of pathogenic microorganisms from soybean seeds has been worked out by Wu *et al.* (1964), the factors affecting determination of microflora in coniferous seeds are rather complicated. The nature of coniferous seeds is distinctly different from that of soybean seeds, for instance, hard and thick seed-coat may cause slow emergence.

From the data obtained in the studies of effect of media, hydrogen ion concentration, temperatures and chemical treatments on the microflora of coniferous

seeds revealed that seeds treated with 4.0 per cent sodium hypochlorite for one minute before rinsing in five changes of sterilized water were found to be applicable to isolate the microorganisms present in the surface layers of the seeds, probably seed-coat. On the other hand, seeds completely surface-sterilized with 0.5 per cent mercuric chloride for one minute followed by washing five times with the sterile water were useful to determine the microflora of deeper layers of the seeds by grinding and mixing them with melted PDA in Petri-dish. These cultures were incubated at 28°C.

It is worthy to note that the seeds treated with 0.1 per cent mercuric chloride seemed to improve the germination of coniferous seeds, however, *Aspergillus*, *Diplodia*, and *Penicillium* appeared after the sprouting of seed. This revealed that the treatment was not able to eliminate the microorganisms inhabiting deeper layers of the seed treated. The complete disinfection of coniferous seeds seems to be very difficult so far as the experimental results are concerned.

Summary

For isolating the microflora from coniferous seeds, the present experimental data are indispensable. Seeds partially surface-sterilized with sodium hypochlorite were found to be applicable to isolate the microorganisms present in the surface layers of the seeds, probably seedcoat. On the other hand, seeds completely surface-sterilized with mercuric chloride were useful to determine the microflora existed in the deeper layers of the seeds by grinding and mixing these seeds with melted agar media. The treated seeds were incubated on potato dextrose agar at 28°C for an appropriate length of time. The complete disinfection of coniferous seeds seems to be very difficult so far as the experimental results are concerned.

分離針葉樹種子附生微生物之影響因子

陳 其 昌 鍾 順 昌

針葉樹種子能携帶微生物傳播病害，已爲林業者之一般常識。然此附生於種子上之微生物亦能拮抗其他土棲微生物之侵襲，而抑制土壤傳染性病害之發生。針葉樹幼苗立枯病之發生與嚴重性受種子附生微生物及土棲微生物之支配，尤爲顯著。各地區由於土壤微生物相及種子來源之差異，常有不同結果之試驗報告。例如應用種子消毒、土壤消毒、或二者兼用及土壤酸化劑等防治幼苗立枯病之不同方法，已各別被各地區之研究者所推薦。茲爲瞭解針葉樹種子挾帶幼苗立枯病病原菌之種類及苗圃微生物拮抗作用之機構，調查各樹種種子之微生物相乃爲先決之問題。

一般微生物不僅付着於種子表面，亦能潛伏於內種皮及種仁內。欲分離此等微生物時，所使用之培養基種類、培養基酸度、培養溫度及藥劑之處理方法等均能左右種子內外層微生物之出現機會。本試驗結果顯示，針葉樹種子浸漬於百分之四濃度之次氯酸鈉溶液一分鐘，經無菌水洗滌五次，置於培養皿內之馬鈴薯培養基平板上，孵育於 28°C 定溫箱下 4 天，則適合於分離種子外表之微生物羣。若浸漬於千分之五濃度之昇汞溶液一分鐘，經無菌水洗滌五次，再以無菌之乳鉢磨碎，每一種子分別置於已溶化且冷却至 43°C 馬鈴薯培養基之試管內，混合之，然後傾注於滅菌培養皿內，放置於 28°C 定溫箱下12天，則適用於分離種子內層之微生物羣。將已出現之微生物菌落分別移植於新培養基上，以便供鑑定之用。如在次代培養（Subculture）時不愼有二種以上之菌類存在，應設法分離，以得純培養（Pure culture）。

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