INTERACTION OF SEED-BORNE AND SOIL-BORNE MICROORGANISMS IN RELATION TO DAMPING-OFF DISEASE OF CONIFERS[1]

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(Received Sept. 29, 1964)

Introduction

Damping-off disease in forest nursery is the universal problem causing the irregularity of losses as well as the severe reduction in seedling stands, especially of conifers. More than 30 different fungi inducing the discease have been recognized (Cockerill, 1951; Hartley et al., 1918; Padgett, 1958; Rathbun, 1923; Sato et al. 1955; Spaulding, 1914; Vaartaza, 1953; Vaartaza, et al., 1956). Most of them were indicated as soil-inhabiting fungi which were facultative parasites (Hartley et al, 1918; Shigeyasu, 1953). Nevertheless, a series of microflora associated with coniferous seeds were also found to be pathogenic to radicle and seedling of conifer (Fisher, 1941; Garbowski, 1936; Rathbun-Gravatt, 1931; Ten Houten, 1939; Timonin, 1964). These microorganisms not only existed in surface layers but also in the inner parts of seed structure. (Part III). The disease occurs under various conditions and associates with numerous species of fungi, thus no definite method can be applied for all cases. In this connection, further studies on interaction of seed-borne and soil-borne microorganisms seem to be useful to clarify the nature of damping-off disease in coniferous nurseries of Taiwan.

Materials and Methods

Eight samples of coniferous seeds comprised seven species of pines, e. g. slash pine (*Pinus elliottii*), Luchu pine (*P. luchuensis*). Japanese black pine (*P. thunbergii*), Taiwan armand pine (*P. armandi* var. *mastersiana*), Taiwan red pine (*P. taiwanensis*), Taiwan white pine (*P. morrisonicola*), and horsetail pine (*P. massoniana*), were used in the present experiments. All the seed samples were collected in this island except that one of slash pine samples was imported

(1) Research was supported in part by USDA grant No. FG-Ta-103. Paper No. 4, Important Epidemic Diseases of Forest Trees in Taiwan Journal Series.

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from the States. The samples of Luchu pine and Japanese black pine were purchased from a forestor. For the rest, the seed samples were secured by courtesy of the Taiwan Forestry Bureau. The soil used throughout the present experiments, was collected from a forest nursery located in Siaokotou, Taipei Hsien.

Unless otherwise specified, coniferous seeds were surface-sterilized with 0.1 per cent mercuric chloride for 2 minutes and soils were sterilized by an autoclave at 15 pounds per square inch for 15 minutes. Young seedlings were grown in Erlenmeyer flasks or ordinary clay pots under aseptical conditions.

Results

Experiment I.

Autoclaved and non-autoclaved soils were separately seeded with seven species of pines, 25 seeds a pot, then kept in a sterilized chamber at room temperature ranging from 16° to 26°C (Fig. 1, 2). In this experiment, seeds were not treated. Four replications were made for each. Percentage of seed germinated and number of injured seedlings were recorded 3 weeks after seeding. Isolation and identification of the causal organisms were made with those diseased seedlings.

From the results shown in Table 1, the diseased seedlings were mainly infected by 5 genera of fungi i. e. *Cylindrocladium, Diplodia, Fusarium, Rhizoctonia,* and *Verticillium.* Seedlings infected by *Cylindrocladium* were found only among the seedlings grown in the non-antoclaved soil, whereas *Diplodia* appeared only on the diseased seedlings grown in the autoclaved soil. *Fusarium, Rhizoctonia,* and *Verticillium* were isolated from diseased seedlings grown on both autoclaved and non-autoclaved soils. These facts indicated that *Cylindrocladium* and *Diplodia* were respectively derived from soil and seed while *Fusarium, Rhizoctonia,* and *Verticillium* were possibly from both sources. However, this might not be true since the seeds used were not sterilized in this experiment.

Under the present experimental conditions, cotyledon rot generally appeared to be more serious than basal stem rot. For instance, *Diplodia* caused more cotyledon rot than basal stem rot. On the other hand, *Cylindrocladium* showed

Explanation of Plate

Fig. 1. Damping-off appears on the seedlings of pines, i. e. *Pinus elliottii* (A, B), *P. luchuensis* (C), *P. taiwanensis* (D), *P. morrisonicola* (E), *P. massoniana* (F), *P. thunbergii* (G), and *P. armandi* var. *mastersiana* (H), grown in the non-autoclaved soil in a sterilized chamber.

Fig. 2. Damping-off appears on the seedlings of pines, i. e. *Pinns elliottii* (A, B), *P. luchuensis* (C), *P. taiwanensis* (D), *P. moroisonicola* (E), *P. massoniana* (F), *P. thunbergii* (G), and *P. armandi* var. *mastersiana* (H), grown in the autoclaved soil in a sterilized chamber.

Table 1. *Occurrence of cotyledon rot and basal stem rot of coniferous seedlings grown in soil.**

Species of conifer	Treatment**	Percentage of seeds germinated	No. of cotyledon rot caused by					No. of basal stem rot caused by				
			Cylindrocladium	*Diplodia*	*Fusarium*	*Rhizoctonia*	*Verticillium*	*Cylindrocladium*	*Diplodia*	*Fusarium*	*Rhizoctonia*	*Verticillium*
Pinus armandi var. *mastersiana*	a	2	—	—	—	—	—	—	—	—	—	—
	b	1	—	—	—	—	—	—	—	—	—	—
*P. elliottii**** A	a	69	—	25	2	2	—	—	—	1	1	1
	b	81	29	—	2	2	—	—	—	3	—	—
*P. elliottii**** B	a	66	—	12	—	—	1	—	6	—	1	2
	b	61	—	—	—	—	—	—	—	—	—	—
P. luchuensis	a	81	—	—	—	—	22	—	—	—	—	—
	b	66	14	—	—	—	—	6	—	—	—	—
P. massoniana	a	73	—	2	—	—	3	—	—	—	—	—
	b	46	—	—	—	—	4	—	—	—	—	—
P. morrisonicola	a	21	—	—	—	—	—	—	—	—	—	—
	b	16	—	—	—	—	—	—	—	—	—	—
P. taiwanensis	a	58	—	2	—	14	1	—	—	—	14	—
	b	52	3	—	—	—	—	34	—	—	—	—
P. thunbergii	a	79	—	—	—	—	—	—	—	—	—	—
	b	59	15	—	2	—	—	14	—	—	—	—

* Record was made 3 weeks after seeding, 100 seeds for each.
** Seedlings were grown in autoclaved(a) and non-autoclaved(b) soils.
*** Seeds including these imported from the States(A) and native to Taiwan(B).

rather vague results. This fungus incited more cotyledon rot of slash pine and Luchu pine while less cotyledon rot was found in the case of Taiwan red pine; and about the same numbers of cotyledon rot and basal stem rot were detected among the seedlings of Japanese black pine. This might not be just a matter of host susceptibility per se. The interaction between seed-borne and soil-borne microorganisms may be suspected. In connection with this matter, germination of coniferous seeds in both autoclaved and non-autoclaved soils should be compared. Autoclaved soil seemed to induce augmented germinability of the seeds except one case, i. e. slash pine seeds imported from the States.

Nematodes were detected abundantly in *Cylindrocladium* infected seedlings grown on non-autoclaved soil, but not in the seedlings infected by the other fungi. Again, this indicated the complexity of the disease incidence.

Further evidence obtained from the isolation of microorganisms associated with non-germinated seeds showed that *Cylindrocladium* was soil-borne in nature. Non-germinated seeds, obtained in autoclaved soil, were surface-sterilized with 4 per cent sodium hypochlorite for 1 minute followed by rinsing in five changes of sterilized distilled water. The treated seeds were transferred aseptically to potato dextrose agar plate, 10 seeds per Petri-dish, and incubated at 28°C. for 4 days to yield the microorganisms.

Four genera of fungi, e. g. *Diplodia*, *Fusarium*, *Rhizoctonia*, *Verticillium*, and a large number of bacteria were isolated (Table 2). With one exception, Taiwan white pine, all the seeds yielded bacteria. Among the fungi mentioned above, *Verticillium* was most frequently isolated and from the seedlings grown in autoclaved soil mostly. Less seeds, obtained in non-autoclaved soil, yielded *Rhizoctonia* might indicate the inferiority of this fungus in counterpart of the other soil fungi. In contrast, *Fusarium* appeared more on the seeds obtained in non-autoclaved soil than those in autoclaved.

Table 2. *Isolation of microorganisms from non-germinated seeds obtained in autoclaved and non-autoclaved soil*

Species of conifer	*Pinus armandi* var. *mastersiana*	*P. clliottii** A	*P. clliottii* B	*P. luchuensis*	*P. massoniana*	*P. morrisonicola*	*P. taiwanensis*	*P. thunbergii*
Soil autoclaved:								
No. of seeds tested	93	24	37	18	23	27	26	20
Bacteria	93	24	37	18	23	—	26	20
Diplodia	—	16	2	1	—	—	1	—
Fusarium	2	—	—	—	—	1	—	—
Rhizoctonia	—	17	6	1	—	—	—	—
Verticillium	93	15	32	15	22	15	19	20
Soil non-autoclaved:								
No. of seeds tested	91	17	42	27	31	45	50	40
Bacteria	91	17	42	27	31	—	50	40
Diplodia	—	7	3	—	5	4	—	2
Fusarium	9	3	6	—	5	—	4	2
Rhizoctonia	—	1	—	1	—	2	—	—
Verticillium	91	15	8	5	3	40	19	—

* Seeds including those imported from the States (A) and native to Taiwan (B).

Experiment II.

Surface-sterilized and non-sterilized seeds were respectively seeded in 500–ml Erlenmeyer flask containing autoclaved and non-autoclaved soils to make the combinations of autoclaved soils with surface-sterilized and non-sterilized seeds, non-autoclaved soils with surface-sterilized and untreated seeds. The results are shown in Table 3.

Table 3. *Occurrence of cotyledon rot and basal stem rot of coniferous seedlings grown in flask with soil**

Species of conifer	Treatment**	Percentage of seeds germinated	No. of cotyledon rot caused by						No. of basal stem rot caused by						
			Aspergillus	*Cylindrocladium*	*Diplodia*	*Fusarium*	*Penicillium*	*Pestalotia*	*Aspergillus*	*Cylindrocladium*	*Diplodia*	*Fusarium*	*Penicillium*	*Pestalotia*	*Pythium*
Pinus armandi var. *mastersiana*	a	0	—	—	—	—	—	—	—	—	—	—	—	—	—
	b	0	—	—	—	—	—	—	—	—	—	—	—	—	—
	c	2	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	1	—	—	—	—	—	—	—	—	—	—	—	—	—
*P. elliottii**** A	a	41	—	—	2	—	—	—	—	—	4	—	—	—	—
	b	68	—	—	—	25	—	—	—	—	—	7	—	—	—
	c	54	—	—	—	2	—	—	—	—	—	—	—	—	—
	d	82	—	—	—	8	—	—	—	—	—	3	—	—	2
P. elliottii B	a	7	—	—	2	—	—	—	—	—	—	—	—	—	—
	b	41	—	—	—	2	—	—	—	—	—	—	—	—	—
	c	37	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	70	—	17	—	5	—	—	—	18	—	2	—	—	—
P. luchuensis	a	58	—	—	3	—	—	—	—	—	3	—	—	—	—
	b	77	—	—	—	—	4	—	—	—	—	—	—	—	—
	c	79	3	—	—	—	—	—	1	2	—	—	—	—	—
	d	73	—	18	—	—	—	—	—	7	—	—	—	—	—
P. massoniana	a	22	—	—	—	—	—	—	—	—	—	—	—	—	—
	b	64	—	—	10	4	—	—	—	—	—	5	—	—	—
	c	77	—	14	—	1	—	—	—	14	—	—	—	—	—
	d	44	—	3	—	5	—	—	—	6	14	—	—	—	—
P. morrisonicola	a	33	—	—	—	—	—	—	—	—	—	—	—	—	—
	b	38	—	—	—	—	—	—	—	—	—	—	—	—	—
	c	34	—	—	—	—	—	—	—	1	—	—	—	—	—
	d	37	—	2	—	—	—	—	—	—	—	—	—	—	—
P. taiwanensis	a	40	—	—	—	—	—	—	—	—	—	—	—	—	—
	b	64	—	—	4	—	—	—	—	—	5	—	—	—	—
	c	37	—	—	—	—	—	—	—	6	—	—	—	—	—
	d	34	—	3	—	—	—	—	5	21	—	—	—	—	—
P. thunbergii	a	49	—	—	—	—	1	—	—	—	—	—	—	—	—
	b	50	1	—	—	—	1	7	1	—	—	—	1	7	—
	c	60	—	—	—	—	—	—	—	18	—	—	—	—	—
	d	55	—	28	—	—	—	—	—	6	—	—	—	—	—

* Record was made 3 weeks after seeding, 100 seeds for each.

** Combinations inclding autoclaved soil with surface-sterilized (a) and non-sterilized (b) seeds, non-autoclaved soil with surface-sterilized (c) and non-sterized (d) seeds.

*** Seeds inclding those imported from the States (A) and native to Taiwan (B).

Apparently, the data indicated that *Cylindrocladium* and *Diplodia* were respectively derived from soils and seeds since the former appeared merely on the seedlings grown in non-autoclaved soil while the latter was found only on the seedlings grown on autoclaved soil. An interaction between the soil- and seed-borne microorganisms might result in the disappearance of *Diplodia* on the seedlings grown on non-autoclaved soil. Association of nematodes with the seedlings infected by *Cylindrocladium* was also significant.

Under experimental conditions, 7 genera of fungi were indentified, i. e. *Aspergillus, Cylindrocladium, Diplodia, Fusarium, Penicillium, Pestalotia,* and *Pythium.* Although the number of seedlings infected by *Aspergillus, Penicillium, Pestalotia,* and *Pythium* was not many, this might indicate the further evidence for the interaction among microorganisms either soil-or seed-borne in nature since these fungi were not found in the same seed samples in the foregoing experiment. In this respect, the disappearance of *Rhizoctonia* and *Verticillium* was also worthy to note. Experimental conditions seemed to cause this random fluctuations.

In regard to that germinability of the coniferous seeds, no definite pattern could be drawn among the 7 species of pines studied. Lower germinability was generally observed when surface-sterilized seeds were sown in autoclaved soils while the surface-sterilized seeds sown in non-autoclaved soils revealed the conflicting data. However, phytotoxic effect of mercuric chloride might not be ruled out in these cases. Two cases showed that germinability was higher with non-sterilized seeds germinated in non-autoclaved soil. The growth vigour of the seeds may cause the afterementioned result.

Experiment III.

Further investigation concerning the effect of microflora of coniferous seeds on seedling diseases were carried out with seed samples of slash pine and Luchu pine. The seeds surface-sterilized with 0.1 per cent mercuric chloride were separately sown in clay pot with autoclaved soil or in large test tubes containing corn meal agar, 10 seeds per pot or per tube. The pots were kept in a sterilized chamber to assure aseptical conditions and the test tubes were kept on a bench (Fig. 3, 4, 5, 6).

From the resultant data (Table 4) obtained from seedlings grown in soil, 5 genera of fungi, e. g. *Aspergillus, Diplodia, Fusarium, Penicillium,* and *Stemphylium,* were isolated. *Diplodia* and *Stemphylium* were respectively isolated from slash pine and Luchu pine. Different rates of germination and disease incidence between slash pine and Luchu pine might result from the microflora in inner structure of coniferous seeds examined.

On agar, *Aspergillus, Chactomium, Chaetomclla, Diplodia, Fusarium,* and *Penicillium* were isolated from both samples except that *Fusarium* was not obtained from Luchu pine. *Aspergillus, Diplodia,* and *Fusarium* apparently influenced the number of the survival of the seedlings. *Diplodia* appeared to be more destructive than the others.

Discussion

Gibson (1956) demonstrated that Granosan used in soil treatment indirectly assisted the spread of damping-off pathogens through the soil by its selective

Fig. 3. *Diplodia* sp. derived from inner layers of the slash pine seeds causes basal stem rot (A) and cotyledon rot (B) of the seedlings.

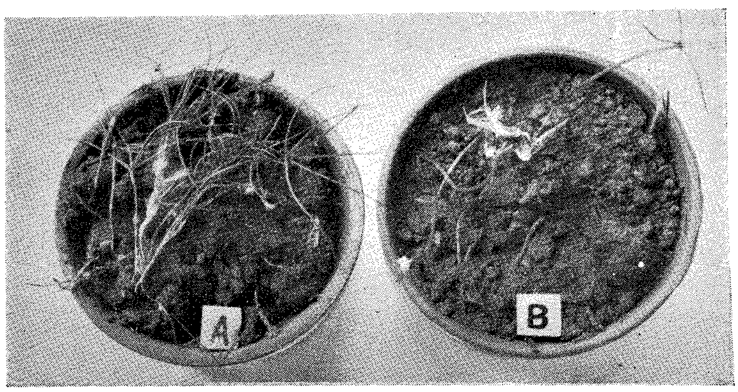

Fig. 4. *Fusarium* sp. derived from inner layers of the slash pine seeds causes basal stem rot (A) and cotyledon rot (B) of the seedlings.

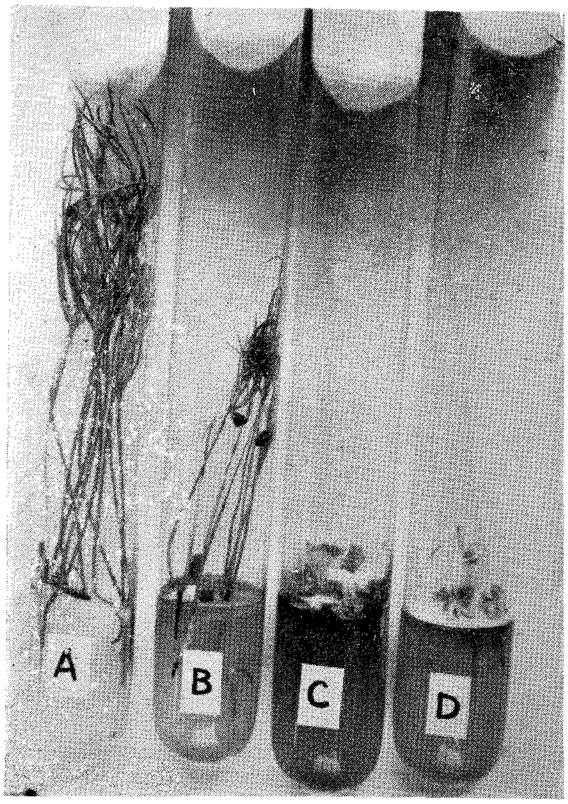

Fig. 5. *Aspergillus* (B), *Diplodia* (C) and *Fusarium* (D) derived from the inner layers of the slash pine seeds influence the survival of the seedlings. (A) shows no inner microorganisms existed in any seeds.

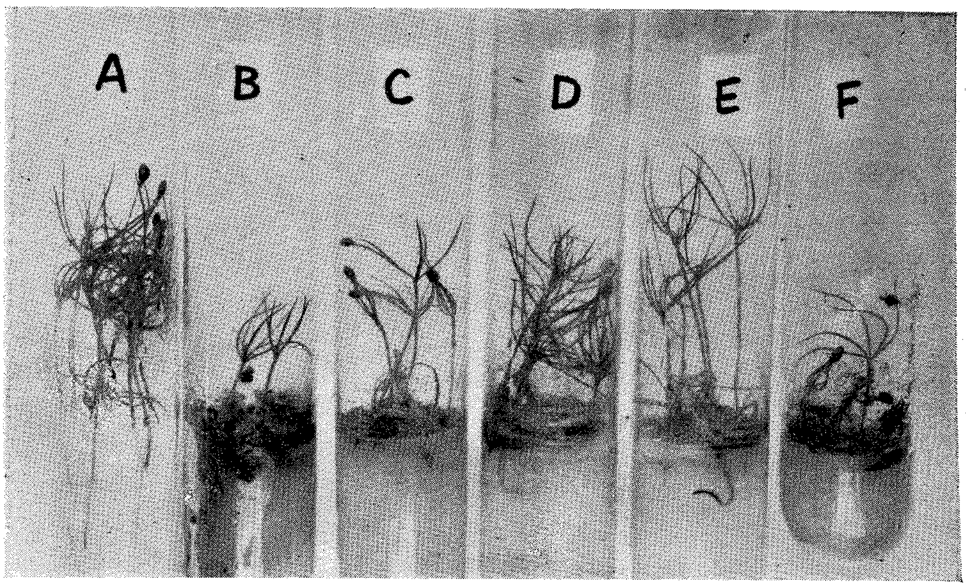

Fig. 6. *Diplodia* (B), *Aspergillus* spp. (C, D, E), and *Aspergillus niger* (G) derived from the inner layers of the Luchu pine seeds influence the survival of the seedlings. (A) shows no inner microorganisms existed in any seeds.

Table 4. *Occurrence of cotyledon rot and basal stem rot of coniferous seedlings grown in soil under aseptical condition**

Species of pines	No. of seeds emerged	No. of healthy seedlings	No. of cotyledon rot caused by				No. of basal stem rot caused by			
			Aspergillus	*Diplodia*	*Fusarium*	*Penicillium*	*Diplodia*	*Fusarium*	*Penicillium*	*Stemphylium*
Pinus elliottii**	63	10	3	3	1	12	38	7	1	—
P. luchuensis	88	56	1	—	1	13	—	8	6	3

* Record was made 40 days after seeding, 100 seeds for each.

** The seed sample of slash pine was imported from the States and Luchu pine seeds were native to Taiwan.

action on the antagonistic microflora. On the other hand, the control of damping-off was suggested by Simkover et al. (1951) by applying benzene hexachoride to stimulate mycorrhizal growth in the seedlings. In connection with these facts, further investigation on interaction between seed and soil microfloras is important to understand the natural incidence of damping-off in coniferous nurseries before the establishment of definite rules for seed treatment.

Symptoms of the disease were usually divided into pre- and postemergence damping-off (Hartley et al., 1918; Spaulding, 1914). The former consists of reduced emergence and decay of radicle just emerged from seed-coat (Hartley et al., 1918; Fisher, 1941); the latter was also subdivided into root rot, cotyledon rot, and basal stem rot after the seedling emerged from soil (Chen, 1962; Chen et al., 1963; Ito, 1962). In this series of experiments, reduced emergence, cotyledon rot and basal stem rot were mainly concerned as a criterion for clarifying the effect of interaction between seed and soil microfloras on occurrence of damping-off of conifers.

So far as the 8 seed samples were concerned, varied results obtained in Tables 1 and 3 might be due to the different microflora of each seed sample since the same soils were tested in each treatment. However, lower germinability was generally observed when surface-sterilized seeds were sown in autoclaved soil. The microorganisms present in the inner parts of seed might become active owing to the disappearance of antagonistic microflora from both soil and surface layers of seed (Simkover et al., 1951). On the other hand, relatively high percentage of seed germination was found in slash pine when the treated and untreated seeds were sown in non-autoclaved soil. The reason might be that slash pine seeds were mainly infested with *Diplodia* (Part III) which was easily hindered by soil-borne microorganisms.

Germination of coniferous seeds was proved to be inhibited from widespread contaminators. In this respect it was similar to the results obtained by Fisher (1941), Garbowski (1936), Rathbun-Gravatt (1931), Ten Houten (1939), and Timonin (1964). In view of the present experimental results, it was notable that either seed surface-sterilized or soil autoclaved seemed to induce augmented germinability of seeds except one case, i. e. slash pine seeds. Because the slash pine seeds were most abundantly infected by *Diplodia*. Under the conditions mentioned above, the antagonistic competition with *Diplodia* was there by reduced.

Isolation of the microorganisms associated with the non-germinated seed obtained in autoclaved soil indicated that the failures of germination seemed to be chiefly affected by seed-borne microorganisms. However, the soil-borne microorganisms could only exert the inhibitory action to the seed emergence upon the favorable microflora of the seeds mentioned above.

Occurrence of cotyledon rot is chiefly caused by means of seed carrying pathogens from soil or on seed itself. Microflora existing in the inner layers of seed seemed to be more significant in this case. Some saprophytes, e. g. *Aspergillus* and *Penicillium*, under the present experiments, could infect the seedlings to cause damping-off. This confirmed the result obtained by Timonin (1964).

Basal stem rot of seedlings was brought about directly by soil-borne microorganisms and indirectly by seed-borne fungi. In this respect soilinhabiting fungi, e. g. *Fusarium*, *Rhizoctonia*, *Verticillium*, and *Cylindrocladium*, seemed to be very destructive. The first 3 fungi were found to be in soil and also frequently found in seeds. The disappearance of *Diplodia* on the seedlings grown on non-autoclaved soil might be resulted from the interaction between the soil- and seed-borne microorganisms since *Diplodia* was proved to be a seed-borne microorganism and easily affected by antagonistic soilmicroflora. In this connection, the infectivity of *Diplodia* seemed to be not so important when the nursery bed was not sterilized.

Cylindrocladium-nematode complex disease was frequently found in the soil non-autoclaved. Further evidence obtained from the isolation of microorganisms associated with non-germinated seeds indicated that the causal agents of complex disease was soil-borne in nature. Studies of nematodes in relation to damping-off disease are essential.

To sum up, occurrence of damping-off of conifers is closely related to the interaction of seed- and soil-borne microflora. The destruction of slash pine seedling caused by *Diplodia* in autoclaved soil is a good example. Both seed- and soil-borne microfloras were apparently varied with different conifers and soils as well as environmental conditions. With regard to this matter, to search a special treatment for controlling damping-off of a given species of conifer in a certain nursery bed is desired since it is unlikely true that any one treatment will be entirely suitable for all the forest nurseries (Gibson, 1955). In order to obtain reliable rules to maintain a nursery stock of a conifer, survey of both seed and soil microfloras is required in the first place. From the knowledge obtained from the interaction between seed- and soil-microflora, to establish a practical method for controlling damping-off disease should be possible. Particularly, applying a specific chemical to stimulate antagonistic microflora seems to be promising (Simkover et al., 1951).

Summary

So far as the seed samples tested were concerned, varied results obtained might be due to the different microflora of each seed sample. Germination of coniferous seeds was proved to be inhibited from widespread contaminators. However, lower germinability was generally observed when surface-sterilized seeds were sown in autoclaved soil, it might be that the internal microorganisms of seed were activated by the disappearance of antagonistic microflora from both soil and surface layers of seed.

Aspergillus and *Penicillium*, under the present experiments, could also infect the seedlings to cause damping-off. Occurrence of cotyledon rot was chiefly caused by microorganisms existing in the inner layers of seed. The soil-inhabiting fungi, e. g. *Fusarium*, *Rhizoctonia*, *Verticillium*, and *Cylindrocladium*, easily caused basal stem rot of seedlings. The disapppearance of *Diplodia* on the seedlings grown on non-autoclaved soil might be resulted from the interaction between the soil- and seed-borne microorganisms since *Diplodia* was proved to be seed-borne microorganisms and easily affected by antagonistic soil-microorganisms.

種子棲生菌與土壤棲生菌的相互關係對於猝倒病的影響

陳 其 昌　鍾 順 昌

就本文所測定的種子樣品而言，因各種子上的微生物羣不同，會產生各種不同的結果。已可證明的是松種子的發芽會受到廣泛存在的雜菌所抑制。然而，在消過毒的土壤中，種植經表面消毒的種子，發芽率反而低，係因爲，原存在於土壤中或種子表面，對種子內部微生物有拮抗作用的微生物羣消失之故。

本實驗顯示 *Aspergillus*, *Penicillium* 菌亦能致使種苗猝倒。子葉腐爛主要因爲種子內部微生物所引起。土壤永棲菌如，*Fusarium*, *Rhizoctonia*, *Verticillium*及*Cylindrocladium* 等菌，容易引起種苗莖基之腐爛。在未經消毒的土壤中，種苗上的 *Diplodia* 苗會因爲土壤及種子上微生物的拮抗作用而消失，故可證明 *Diplodia* 菌爲一種很容易受到拮抗的土壤傳染病原菌。

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