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Resinous Canker Disease of Cupressaceae Caused by *Monochaetia unicornis* (Cke. & Ell.) Sacc. (II)

Physiologic characters of the causal fungus

By

Katsuhiko Sasaki[1] and Takao Kobayashi[2]

Summary: Conidia of the fungus germinate well on water agar, and their germ tubes normally arise from the two outer colored cells. The fungus germinates and grows at the temperatures ranging from 5° to 35°C. Optimum temperature for conidial germination is 30°C, whereas that for mycelial growth is 25° to 30°C. Conidial germination and mycelial growth are never observed at 0° and 40°C. Conidia can germinate at the range of pH regulated from 2.1 to 10.6, and optimum pH for their germination has a wide range from 3.9 to 8.2. On the other hand, good growth of mycelial colony was recorded at the relatively narrow range of pH from 3.6 to 4.1. The fungus grows well both on agar and in liquid media of potato dextrose, malt extract, V-8 juice and Waksman's media but poorly on and in corn meal, Czapek's, Hopkins' and Richards' media. Mycelial growth of the fungus is best in Waksman's medium containing soluble starch instead of glucose. Mannose, maltose, glucose, fructose, sucrose and galactose respectively seem to be better as a carbon source instead of glucose for growth of the fungus. As a nitrogen source instead of KNO_3 in Hopkins' medium containing 1 mg per liter of thiamine hydrochloric, aspartic acid gave best growth among amino acids tested. Conidia of the fungus are scantily produced on all agar media, but a large number of conidial masses are produced on steam sterilized twigs. Formation of the perfect stage could not be attained on all cultures.

Introduction

Monochaetia canker disease is one of the most important diseases of Monterey cypress, *Cupressus macrocarpa* Hartw., and is now widespread in the cypress plantations of the world. The disease is characterized by a heavy resin flow from the cankered parts on various hosts, that is *Cupressus*, *Juniperus* and *Chamaecyparis*. In Japan, the disease caused by a species of *Manochaetia* was recorded on four species of Cupressaceae including two exotics (Sasaki & Kobayashi 1973). In the previous paper (Sasaki & Kobayashi 1975), the fungus causing a resinous canker of Cupressaceae plants in Japan was identified as *Monochaetia unicornis* (Cke. & Ell.) Sacc. and its pathogenicity was demonstrated through several inoculation tests. The present paper chiefly deals with the physiology of the fungus.

Material and methods

Usually the fungus was isolated from conidia, though the causal fungus formed only a few acervuli on the lesions of canker. In the case of canker lesions without fructification,

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(1) (2) Laboratory of Forest Pathology, Forest Protection Division, Government Forest Experiment Station, Meguro, Tokyo

pieces of the inner bark were taken from the edge of the lesion and they were placed on PDA plates. Colonies grown from these pieces were transplanted to potato dextrose agar slants and they were stored until the start of the studies.

For all germination tests, conidia on potato dextrose agar media were used. They were suspended in sterilized distilled water. This conidial suspension was streaked on the surface of water agar plates, and the treated petri dishes were each kept at regulated temperature or H-ion concentration. Germination percentage and length of germ tubes were recorded after 6 hours of incubation. At the same time position of cells developing germ tube was examined.

Growth of the fungus was tested with solid or liquid cultures. In the case of solid culture, agar plates poured into petri dishes were used. Disk of inoculum, about 3 mm in diameter, was cut from the edge of colony and was transplanted to the centre of test plates. The inoculated plates were kept at each temperature. At 35° and 40°C, moist filter paper was fixed on the inside of lid to prevent the drying of agar plate. Diameter of colonies was measured at every week after incubation, and the formation of conidia on them was recorded. Large tubes, 20 cm in length and 3 cm in diameter were used for liquid cultures. Two milliliter of conidial suspension was inoculated into each tube having 40 cc of solution medium after sterilization, and they were shaken on reciprocating mini-shaker at 25°C. Dry weight of mycelia was measured after 10 days of incubation.

In pH tests, melted culture media, after regulating their H-ion concentrations with NaOH or HCl, were poured into petri dishes. H-ion concentration was rechecked with glass electrode of pH meter, because pH value changed to acid side at highly alkaline concentrations after solidification of the agar media. Water agar was used for germination test of conidia and PDA for mycelial growth in the pH tests.

Twigs about 15 cm long of the following five kinds of conifers, namely *Chamaecyparis obtusa*, *Pinus densiflora*, *Larix leptolepis*, *Cryptomeria japonica* and *Abies firma*, were used for the formation of conidia. These twigs were washed clean and were soaked in tap-water for about an hour. They were autoclaved for 60~90 min. at 121°C in the test tubes. A piece of fresh mycelia of the fungus was inoculated on the upper end of the sterilized twig. The inoculated test tubes were kept at 20°C for about a month. Then they were placed under laboratory room conditions. Formation of conidia was examined at irregular intervals.

Results and discussion

1. Germination of conidia

Conidia of the fungus germinated well on 2% agar plate. Germination percentage and growth of germ tube seemed to be variable with the age of conidia used. One, two, or three germ tubes are put forth from a single conidium within 6 hours. These normally arise from the two outer colored cells, although the germ tubes are able to sprout from all colored cells (Fig. 1a). Germ tubes ordinarily begin to branch during from 6 to 24 hours. A quite small number of conidia can germinate from the hyaline cells or appendages in pH test (Fig. 1b) and extension of these germ tubes is soon inhibited. Abnormal swellings develop from conidia sowed on agar plate regulated at pH 2.1 (Fig. 1c). No growth of the normal hyphae from these swellings is observed.

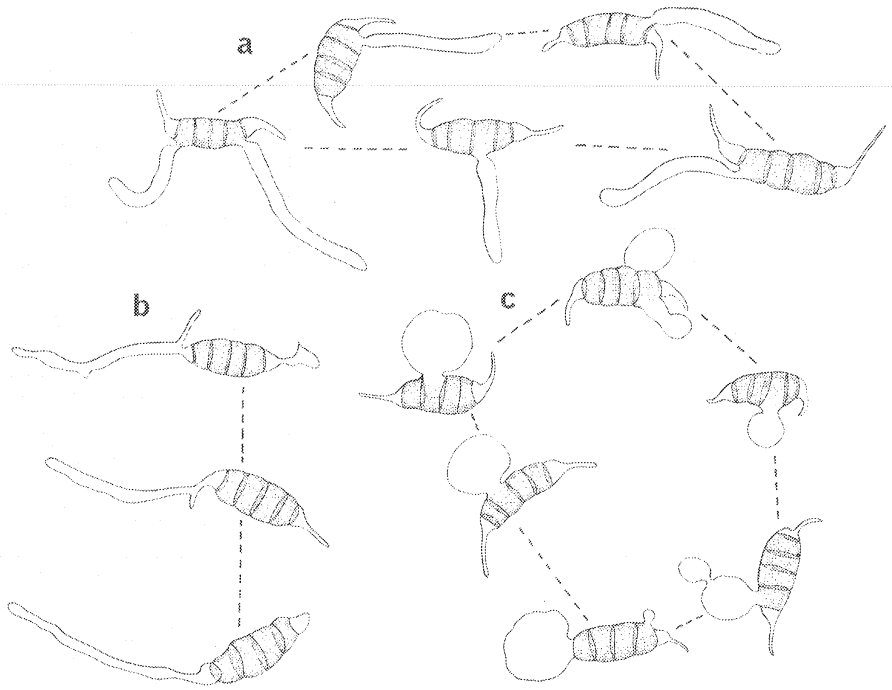

Fig. 1 Germinating conidia of *Monochaetia unicornis* (⟷ : 20μm)
a : Germination from colored cells
b : Germination from hyaline cells or appendages
c : Abnormal germination at pH 2.1

2. **Germination of conidia at various temperatures**

Germination of conidia on 2% agar-agar at various temperatures is shown in Figure 2. Germination of conidia occurred at the range of temperatures from 5° to 35°C. High germination percentage more than 70% was recorded at from 10° to 35°C after 24 hours incubation. The most suitable temperature for germination of conidia seems to be at 30°C. No germination of conidia was observed at 0° and 40°C after 24 hours, but the conidia could germinate when the agar plates were transferred to the incubator regulated at 20°C for the following 24 hours. Conidia completely lost their germination ability after 48 hours at 40°C. Percentage of the conidia germinated from median two colored cells was somewhat higher at high temperatures than that at low temperatures, although the rate of these conidia among the germinated conidia was relatively low (Fig. 3).

3. **Germination of conidia under various H-ion concentrations**

Result after 6 hours at 25°C is given in Figure 4. Difference of pH did not influence germination percentage of the conidia except the extreme acidic side, and high germination percentage more than 90% was obtained at wide pH range from 3.9 to 8.2. Judged from the growth of germ tube, optimal pH for germination seems to be between 5.8 and 7.1. At pH

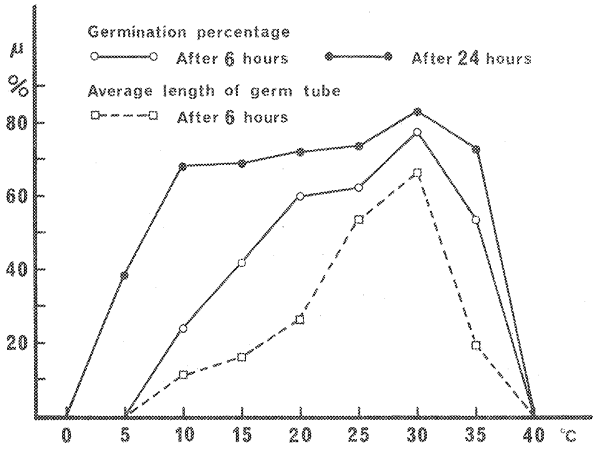

Fig. 2　Relation between temperature and germination of conidia in *Monochaetia unicornis*

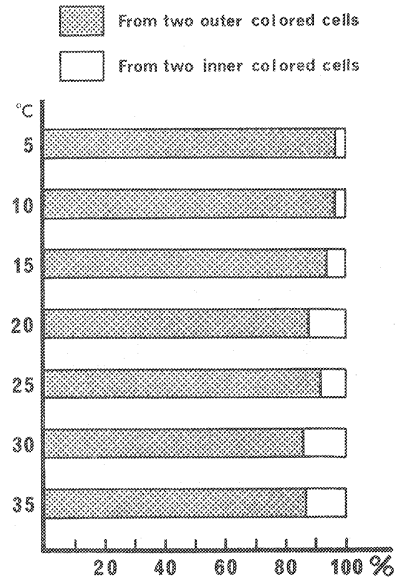

Fig. 3　Position of cell developing germ tube in conidia of *Monochaetia unicornis* (1)

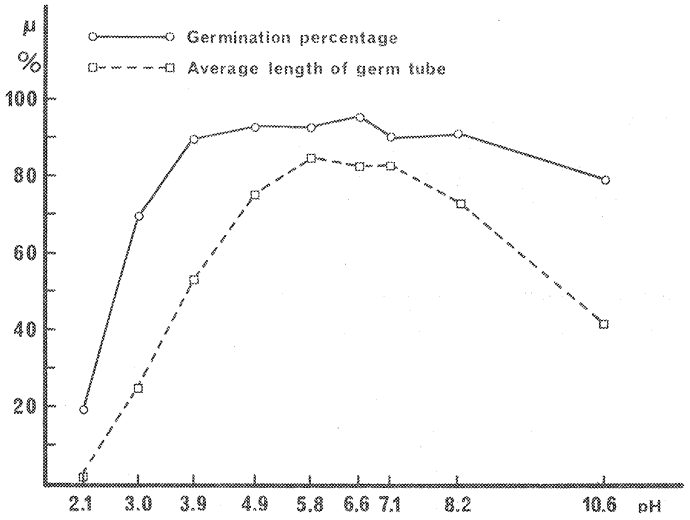

Fig. 4　Relation between H-ion concentration and germination of conidia in *Monochaetia unicornis* (after 6 hours at 25°C)

2.1, germ tubes did not develop as normal elongate hyphae, but the abnormal swellings sprouted from conidia (Fig. 1 c). In this test 2% agar-agar plates without any addition of HCl and NaOH (pH 6.2) were examined as control. On the control plate, 95% of conidia germinated and average length of germ tubes reached 102 μm after 6 hours.

Three types of germination were observed in the experimental series under various H-ion concentrations (Fig. 5). Usually, conidia bore their germ tubes from both ends of colored cells. A small numbers of conidia, 20 to 30% among germinated conidia, sprouted their germ

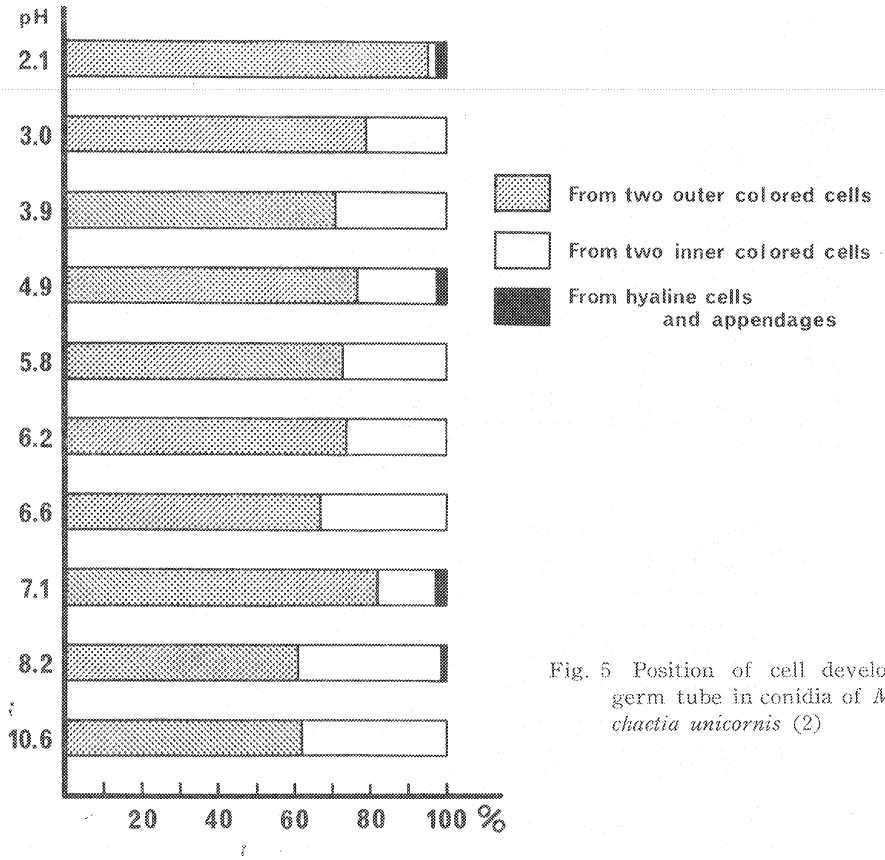

Fig. 5 Position of cell developing germ tube in conidia of *Monochactia unicornis* (2)

tubes from two median colored cells. Ratio of this type in germination somewhat increased at extreme alkaline side of H-ion concentration. Germination from hyaline end cells or appendages was rarely observed in this series.

4. **Germination of conidia under various conditions of osmotic pressure**

Several different conditions in the osmotic pressure were arranged by adding sugar or salt into the media. Sucrose or sodium chloride was respectively added to 2% agar-agar at each five level, namely 0.05 M, 0.15 M, 0.30 M, 0.45 M and 0.60 M, before autoclaving. Germination of conidia on these agar plates was examined after 6 hours at 25°C and result is given in Figures 6 and 7. At the range of osmotic pressure from 0.05 M to 0.60 M made from sucrose, conidia of the fungus germinated nearly 100%, whereas germination ability of the conidia was affected obstructively at 0.60 M of the osmotic pressure made from NaCl. This experimental result shows similar tendencies to that obtained by Jones (1953) with *Monochactia unicornis* in Kenya.

5. **Mycelial growth on different kinds of media**

Mycelial growth of the fungus was examined on solid cultures and in liquid cultures. Kinds of cultural media used and their constituents are shown in Table 1. Results obtained are shown in Figure 8. Mycelial growth of the fungus showed similar tendency on solid and in liquid cultures. Good growth of the colony was recorded on and in V-8 juice, Waksman's,

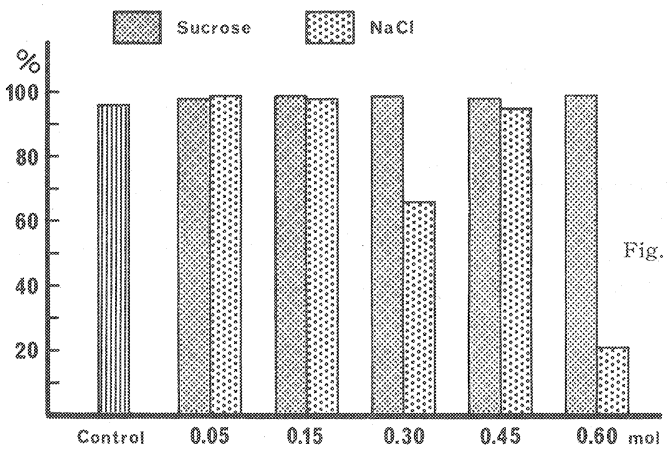

Fig. 6. Effect of adding sucrose and NaCl on germination of conidia in *Monochaetia unicornis* (after 6 hours at 25°C)

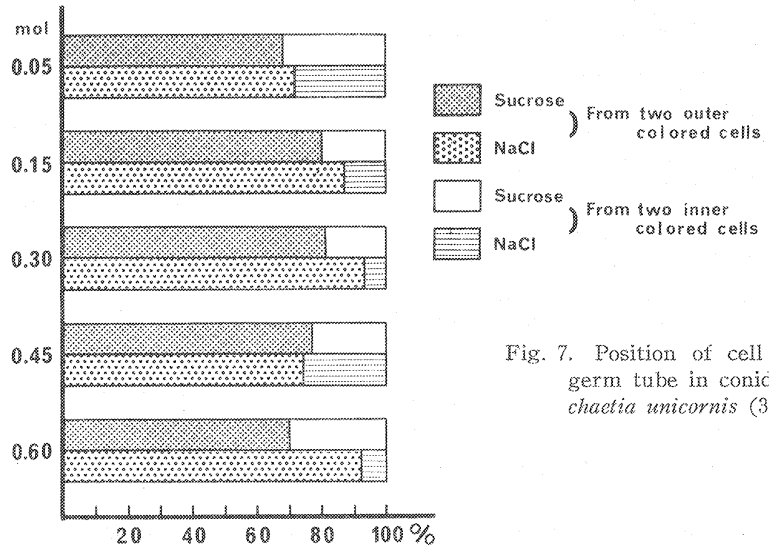

Fig. 7. Position of cell developing germ tube in conidia of *Monochaetia unicornis* (3)

Table 1. Kinds of media and their constituents used for the mycelial growth

Kinds of media	Constituents of media[1)]
Potato	Potato extract (Eiken) 4 g, glucose 20 g, agar 15 g, distilled water 1 *l*
Malt	Malt extract (Difco) 4 g, glucose 20 g, agar 15 g, distilled water 1 *l*
Corn meal	Corn meal agar (Difco) 17 g, glucose 20 g, distilled water 1 *l*
V-8 juice	V-8 juice (twice filtration through filter papers) 200 cc, glucose 20 g, agar 20 g, distilled water 800 cc
Waksman's solution	Peptone 5 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, glucose 10 g, agar 20 g, distilled water 1 *l*
Czapek's solution	K_2HPO_4 1 g, KCl 0.5 g, $NaNO_3$ 2 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, sucrose 30 g, agar 20 g, distilled water 1 *l*
Hopkins' solution	KNO_3 2 g, $MgSO_4$ 0.5 g, KH_2PO_4 0.1 g, glucose 10 g, agar 20 g, distilled water 1 *l*
Richards' solution	KNO_3 10 g, $MgSO_4$ 2.5 g, KH_2PO_4 5 g, $FeCl_3$ 0.002 g, sucrose 50 g, distilled water 1 *l*

1) In the case of liquid culture these constituents without agar are applied.

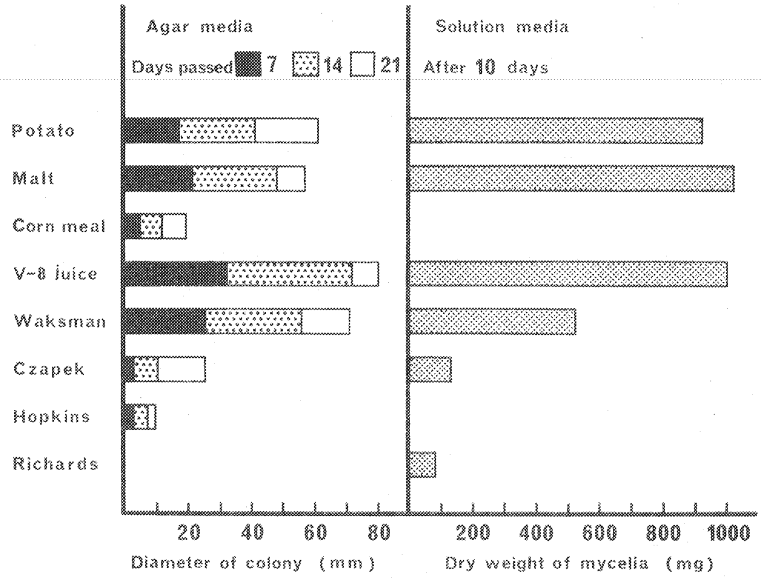

Fig. 8 Mycelial growth of *Monochaetia unicornis* on and in various media (at 25°C)

potato and malt media, but the colony often developed irregularly on potato and malt agar. Mycelial growth was quite poor in solution and on agar culture of corn meal, CZAPEK's, HOPKINS' and RICHARDS' media. Among the agar media tested, V-8 juice was the best for mycelial growth, and on this medium the fungus developed soft floccose colonies with woolly aerial mycelia. On potato dextrose and malt extract agars it formed a dense velvet colony. On WAKSMAN's agar the fungus grew as well as on V-8 juice, but the colony was thin and flat without aerial mycelia. Conidia of the fungus were produced on all kinds of agar media tested. Those on potato dextrose and malt extract agar were scatteredly produced on mycelial mat of the colony, whereas on the other media they were produced only around the inoculum disk.

6. **Mycelial growth and conidial formation at various temperatures**

Radial growth of the fungus on potato dextrose agar plates at various temperatures is presented in Figure 9. Growth of colony occurred at the range of temperatures between 5° and 35°C, the optimal temperature being between 25° and 30°C. No growth was recorded at 0°C for 3 weeks, but the fungus could grow its colony when the plates were incubated at 20°C for the following 2 weeks. The test plates kept at 40°C dried up quickly, nevertheless, the moist filter paper method was applied to prevent the drying of agar. Therefore, the experiment at this temperature could

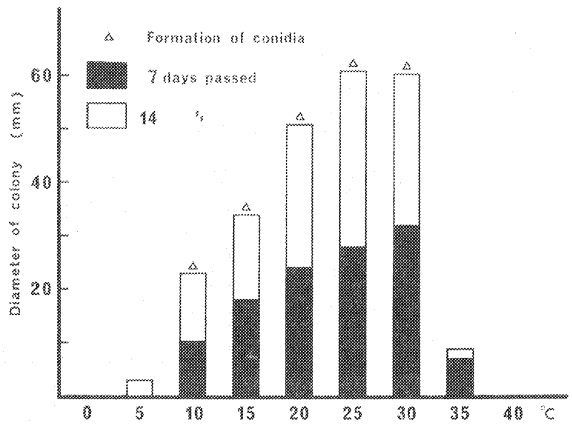

Fig. 9 Relation between temperature and mycelial growth of *Monochaetia unicornis*

not be continued after a week. Conidia were produced at the range of temperatures between 10° and 30°C within 3 weeks.

Ciccarone (1949) reported that optimal temperature for the growth of *Monochaetia unicornis* was 23°C with the maximum from 30°C to 35°C and the minimum at 5°C. Jones (1954) obtained a similar result. Optimal temperature for the growth in Japanese isolates seems to be somewhat higher than those reported from Africa.

7. Mycelial growth and conidial formation under various H-ion concentrations

Growth on potato dextrose agar plate regulated at different H-ion concentrations was examined after 1, 2 and 3 weeks of incubation at 25°C. Result obtained is shown Figure 10. Optimum pH range for the mycelial growth was rather narrow and more acidic than that for germination of conidia, although the growth occurred on all plates tested. Best growth was recorded at pH 3.6 and 4.5. The fungus formed conidia on the plates regulated at the

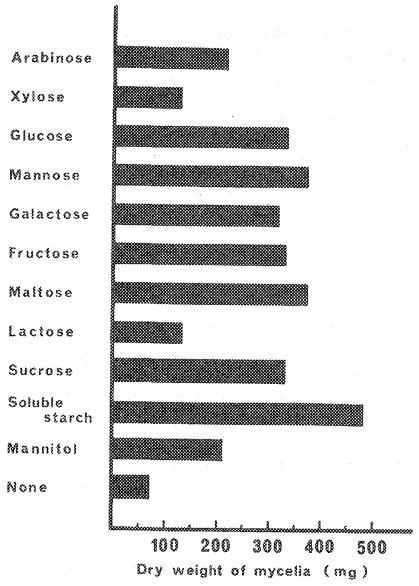

Fig. 11 Mycelial growth of *Monochaetia unicornis* dependent on different kinds of carbon sources (after 10 days at 25°C)

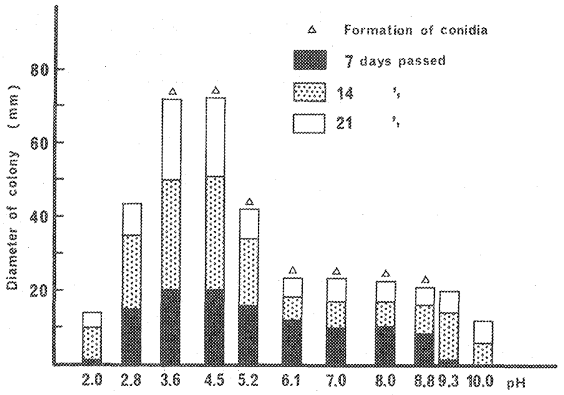

Fig. 10 Relation between H-ion concentration and mycelial growth of *Monochaetia unicornis* (at 25°C)

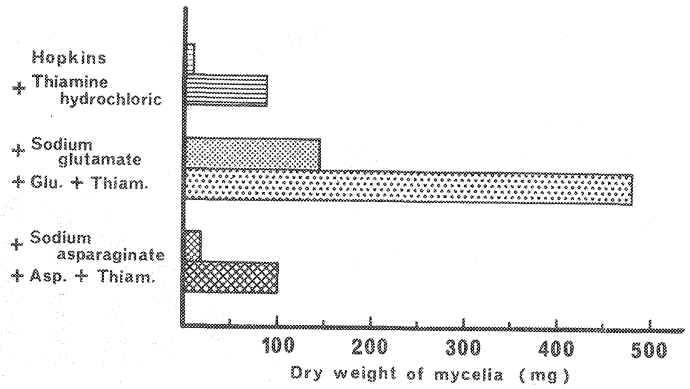

Fig. 12 Effect of adding thiamine on mycelial growth of *Monochaetia unicornis* (after 14 days at 25°C)

pH range from 3.6 to 8.0 after a week of incubation. At pH 8.8 the conidia were produced after 3 weeks.

According to CICCARONE (1949), mycelial growth of *Monochaetia unicornis* in CZAPEK's solution modified by MACINNES was best at the pH range from 4.6 to 5.9, and was inhibited at pH 2.8 and 8.5. JONES (1953) obtained an accordant result on PDA + 0.5% yeast extract plates, their H-ion concentrations regulated without buffering. The optimal pH for the mycelial growth of Japanese isolate was more on the acidic side than that of Kenyan isolates.

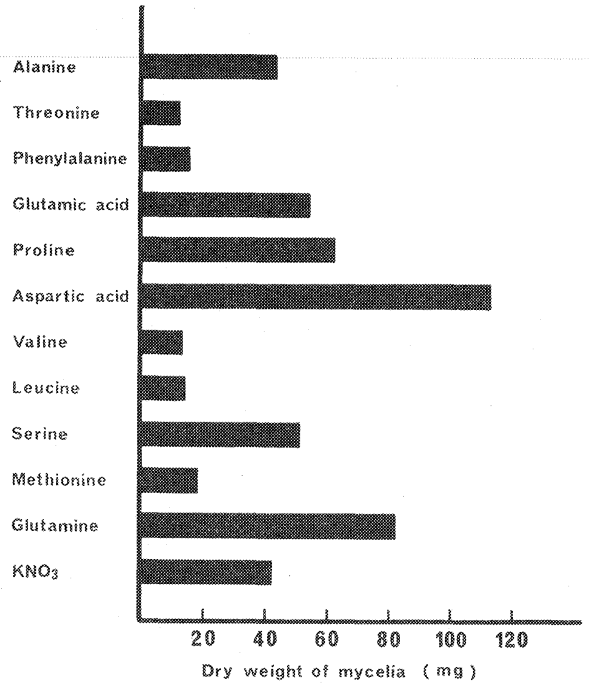

Fig. 13 Effect of different kinds of amino acids as a nitrogen source on mycelial growth of *Monochaetia unicornis* (after 10 days at 25°C)

8. **Effect of kinds of sugars to mycelial growth in solution media**

Glucose-free WAKSMAN's solution was employed as a basal medium. The sugar was added to the basal solution medium at an equal amount to carbon included 10 g of glucose per liter. As shown in Figure 11, glucose, mannose, galactose, fructose, maltose and sucrose seemed to be better than xylose and lactose as the carbon source. Best mycelial growth was recorded in the solution medium containing soluble starch.

9. **Effect of kinds of amino acids on mycelial growth in solution media**

Amino acids which are contained in potato extract (Eiken brand) and glutamine were used in this test. As a preliminary test two amino acids were added to HOPKINS' solution medium. Mycelial growth in the solution media, in which sodium glutamate or sodium asparaginate was solely added to the basic medium, was relatively poor. Addition of 1 mg/*l* of thiamine hydrochloric to these media, however, stimulated mycelial growth of the fungus (Fig. 12). Therefore, multiplied effect of thiamine plus another amino acid to the mycelial growth of the fungus was examined. Basal medium was KNO_3-free HOPKINS' solution containing 1 mg of thiamine hydrochloric per liter. Each amino acid calculated as equivalent nitrogen content as 2 g of KNO_3 was added to this solution medium. Mycelial growth after 10 days at 25°C is shown in Figure 13. Good mycelial growth was recorded in HOPKINS' solution containing aspartic acid or glutamine instead of KNO_3. Threonine, phenylalanine, valine, leucine and methionine were quite unsuitable for growth of the fungus.

Table 2. Production of conidia on steam-sterilized twigs of several coniferous trees

Tree species	Experiment-1	Experiment-2	Experiment-3	Index
Chamaecyparis obtusa	++—+++[1]	+—++	++—+++	2.1[2]
Abies firma	++—+++	++	+—+++	2.4
Pinus densiflora	+—++	+—++	+—+++	1.6
Cryptomeria japonica	+	+	——	1.0
Larix leptolepis	+—+++	+—++	——	1.6

1) + : Conidial masses were scatteredly produced.
++ : many +++ : abundant — : none

2) $\text{Index}=\frac{(+_n\times1)+(++_n\times2)+(+++_n\times3)}{(+_n)+(++_n)+(+++_n)+(-_n)}$

10. Formation of fruiting bodies on steam-sterilized twigs

The fungus usually forms a little amount of conidia on various agar media. Jones (1953) reported that one isolate of *Monochaetia unicornis*, which was originated from a conidium, produced perithecia on a sterilized *Cupressus lusitanica* twig after 8 months. Therefore, twig culture was also attempted in the present fungus. Experiment was repeated three times, and results obtained were given in Table 2. All twigs were overgrown by the fungus within a half to one month after inoculation at 20°C, and many conidia appeared in a little while as small black masses on the surface of the twigs. Amount of the conidial production differed not only by the kinds of tree species but also by the difference of twigs in the same tree species. The fungus formed conidia more regularly and abundantly on the twigs of *Chamaecyparis obtusa* and *Abies firma* than on others. Perfect stage did not develop on these twigs during this experiment.

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Explanation of plates

Plate 1

A. Growth of mycelial colony of *Monochaetia unicornis* on several kinds of agar media, after 3 weeks at 25°C.
   a. V-8 juice　b. Potato　c. Malt　d. WAKSMAN　e. HOPKINS
   f. Corn meal

B. Growth of mycelial colony of *Monochaetia unicornis* on potato dextrose agar controlled at various temperatures, after 2 weeks.
   a. 10°C　b. 15°C　c. 20°C　d. 25°C　e. 30°C　f. 35°C

C. Growth of mycelial colony of *Monochaetia unicornis* on potato dextrose agar regulated at various H-ion concentrations with HCl and NaOH solutions, after 3 weeks at 25°C.
   a. pH 2.0　b. pH 2.8　c. pH 3.6　d. pH 4.5　e. pH 5.2
   f. pH 6.1　g. pH 8.0　h. pH 10.0

D. Formation of conidia of *Monochaetia unicornis* on several steam-sterilized twigs.
   a. *Chamaecyparis obtusa*　b. *Cryptomeria japonica*　c. *Larix leptolepis*
   d. *Pinus densiflora*　e. *Abies firma*

Monochaetia unicornis (Cke. & Ell.) Sacc. による ヒノキ・ピャクシン類の樹脂胴枯病 (II)

病原菌の生理的性質

佐々木克彦[1]・小林享夫[2]

摘　要

本報は，ヒノキ・ピャクシン類の樹脂胴枯病に関する一連の試験研究の一部で，病原菌 *Monochaetia unicornis* (Cke. & Ell.) Sacc. の生理的性質について第II報としてとりまとめたものである。

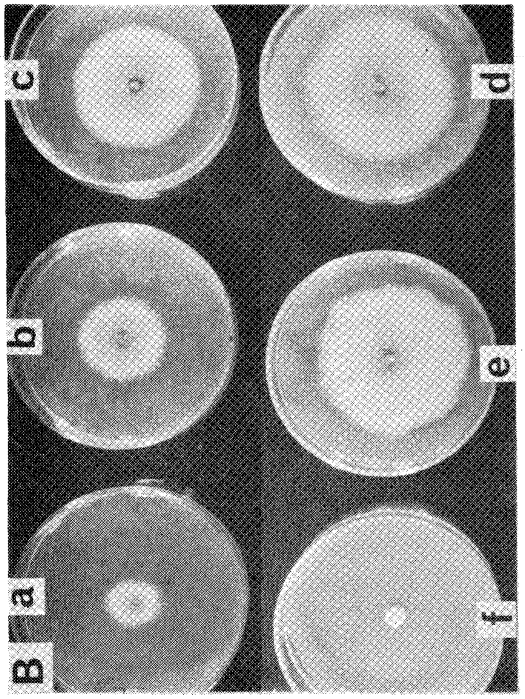
本菌の新鮮な分生胞子は，素寒天培養基上できわめて容易に発芽でき，25°C, 6時間内外で90%以上の高い発芽率を示す。発芽は4個の有色細胞のいずれからも行なわれるが，両端の有色細胞から発芽する場合が最も多く，中央有色細胞からの発芽は，高温あるいはアルカリ域でわずかながら増加する傾向が認められた。発芽管の生育は30°C で最も速く，0° および 40°C ではまったく認められない。水素イオン濃度は，pH 3.9～10.6 の間で発芽率にほとんど影響を与えないが，発芽管の伸びからみて pH 5.8～7.1 が好適と思われる。

本菌は potato dextrose, malt extract, V-8 juice および Waksman 氏の培養基で良好な生育を示した。これに対し，corn meal, Czapek 氏，Hopkins 氏および Richards 氏の培養基では著しく不良である。分生胞子は，potato dextrose および malt extract 上では生育した菌そう上に生じたが，他の培養基上では接種原の周囲のみに形成された。PDA 培地上における菌糸生育と温度との関係は，発芽の場合と大体において一致し，25°～30°C を適温とし，10°～30°C で分生胞子の形成が認められた。水素イオン濃度は菌糸生育に強く影響し，pH 2.0～10.0 の実験範囲で生育が可能なものの，好適 pH は 3.6～4.5 の間で狭い。しかしながら，pH 3.6～8.8 の比較的広い範囲で分生胞子の形成がみられた。

Waksman 氏の培養液は本菌の増殖のためにおおむね良好であったが，この培養液から glucose を取り除くと，菌糸の生育は著しく減少した。一方，他の糖類と置換したところ，soluble starch が最も良好で，mannose, galactose, fructose, maltose および sucrose は，glucose と同程度の増殖を示した。生育に対する thiamine hydrochloric およびアミノ酸類の影響を Hopkins 氏の培養液を用いて調べた結果，thiamine hydrochloric と sodium glutamate の加用が本菌の生育を顕著に高めた。また，供試したアミノ酸類では，特に aspartic acid が良く，次いで glutamine が良好であった。

本菌は，供試したどの培養基上でも分生胞子を形成した。しかし，その形成は不安定で形成量もわずかであった。しかしながら，熱殺菌した針葉樹の枝，とりわけヒノキとモミの枝が本菌の分生胞子を多量に形成させる基質として，最適であることを認めた。

1975年8月30日受理
(1)(2)　保護部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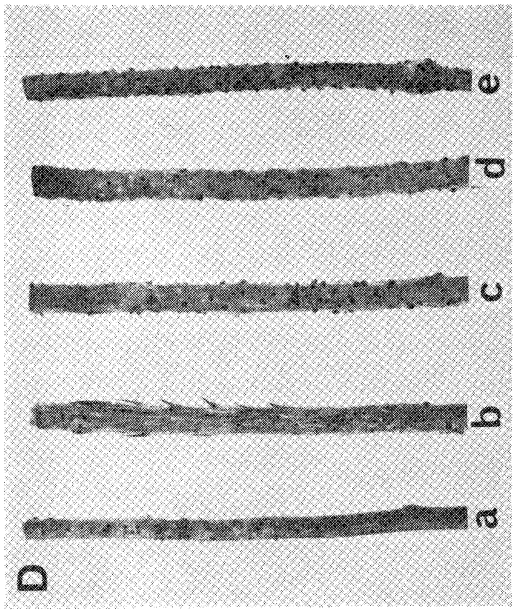
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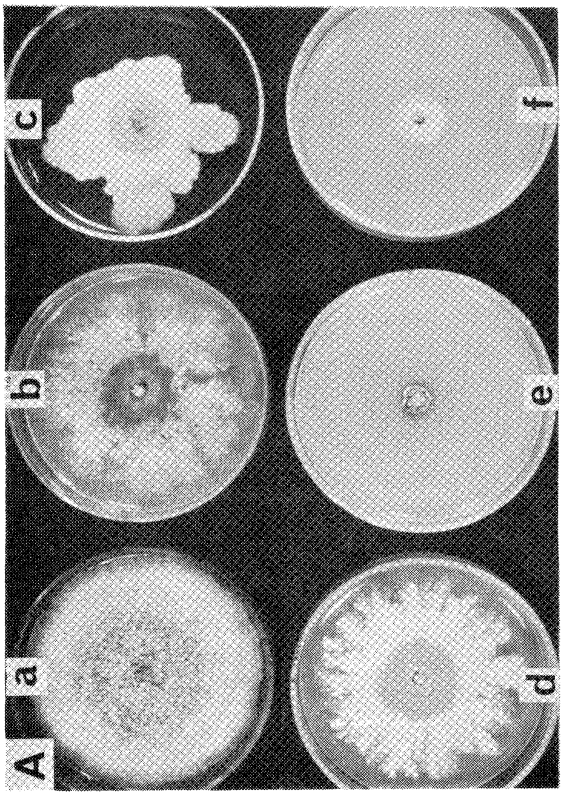
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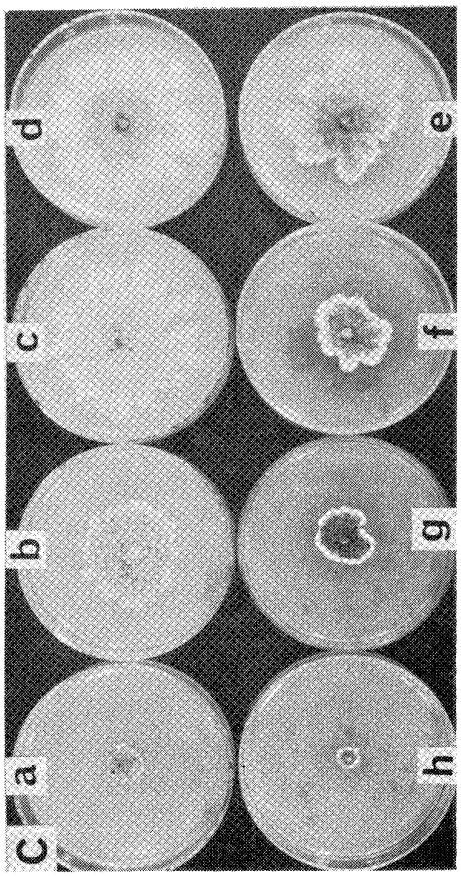
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