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Identification of Three Fungi Associated with Stem and Twig Diseases of *Juglans sinensis* in Korea and Characterization of Factors Affecting Their Growth[1]

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호도나무 줄기와 가지의 病原菌 三種의 同定과 菌絲生長에 影響을 미치는 要因 究明

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ABSTRACT

This study was conducted to identify fungi causing canker dieback and melanconis disease of walnut trees (*Juglans sinensis* Dode) in Korea and clarify the pathogenicity and factors affecting the growth of these fungi. The causal fungi isolated from infected walnut stems and branches obtained from the commercial walnut orchards in Cheonwon, Goesan, Youngdong were identified as *Botryosphaeria dothidea* (Moug. ex Fries) Casati *et de* Notaris, *Phomopsis albobestita* Fairman, *Melanconis juglandis* (Ellis et Everhart) Graves and their pathogenicity was confirmed by inoculation test.

Temperature range for minimum growth of three fungi was 8 to 35℃ and the optimum temperature for mycelial growth of *B. dothidea* and *P. albobestita* ranged from 25 to 30℃, while the optimum temperature for *M. juglandis* ranged from 20 to 25℃. The optimum pH range for mycelial growth of *P. albobestita* was 4.0~5.0 and that for *B. dothidea* and *M. juglandis* 4.0~8.0. Glucose, sucrose, starch or maltose, as a carbon source, and histidine or potassium nitrate as a nitrogen source were more suitable compounds for growth of *B. dothidea*. *P. albobestita* grew very well on the medium containing alanine and potassium nitrate as a nitrogen source, and utilized well glucose and sucrose as a carbon source. *M. juglandis* grew well on the medium containing glucose, and sucrose as a carbon source and utilized well potassium nitrate as a nitrogen source.

The dieback and twig blight caused by *P. albobestita* were more severe than those by *B. dothidea* and *M. juglandis* at three locations investigated. Incidences of canker and dieback were more frequently observed in aged walnut trees than in young ones.

Key words : *Juglans sinensis*, *canker*, *dieback*, *melanconis disease*, *pathogenicity*, *Botryosphaeria dothidea*, *Phomopsis albobestita*, *Melanconis juglandis*

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要 約

本 實驗의 目的은 天原, 永東, 塊山에서 栽培하고 있는 호도나무(*Juglans sinensis* Dode)에 가지마름과 枯死를 일으키는 病原菌을 分離하여 同定하고 이들 病原菌의 病原性 및 培養的 特性, 發病率 等을 調査하는 것이었다.

分離된 病原菌의 菌學的 特性을 調査한 結果, 이들菌은 *Botryosphaeria dothidea*(Mou g. ex Fries) Casati *et de* Notaris(가지마름病), *Phomopsis albobestita* Fairman(Phomopsis 가지마름病), *Melanconis juglandis*(Ellis et Everhart) Graves(검은 돌기 가지마름病)로 同定 되었다. 溫室內에서 이들菌의 病原性을 檢定한 結果 2年生 호도나무 苗木에서 病原性이 立證 되었다. 菌絲生育에 미치는 溫度의 影響을 調査한 結果 *B. dothidea* 와 *P. albobestita*는 25~30℃ 사이에서 生育이 良好 하였으며, *M. juglandis*는 20~25℃ 에서 生育이 가장 좋았으며 세 가지 菌 모두 4℃와 40℃에서는 生長하지 않았다. 分離된 세 菌의 生育은 培地에 따라 差異를 보이지 않았으며 生育最適 pH는 *B. dothidea* 와 *M. juglandis*의 경우 4.0~8.0의 範圍이며 *P. albobestita*의 境遇 4.0~5.0으로 나타났다. 菌絲生育에 미치는 炭素源과 窒素源의 影響을 調査한 結果 *B. dothidea*의 境遇 炭素源으로는 glucose, sucrose, starch 및 maltose를, 窒素源으로는 histidine과 potassium nitrate를 添加한 培地에서 生育이 좋았다. *P. albobestita*의 境遇 炭素源으로 glucose 와 sucrose를 窒素源으로 alanine과 potassium nitrate를 含有한 培地에서 生育이 좋았으며, *M. juglandis*는 glucose, sucrose, potassium nitrate 含有 培地에서 生育이 좋았다.

주요 호도나무 栽培地域에서 發病 狀況을 調査한 結果 調査地域 모두에서 *P. albobestita*에 의한 被害가 가장 심하였으며, 15年生 以上된 樹木이 어린나무보다 더 被害가 큰 것으로 나타났다.

INTRODUCTION

Walnut trees(*Juglans sinensis* Dode) are native to China and Korea and widly cultivated in south of Kyonggi province in Korea. Domestically cultivated area of walnut trees is estimated to be 11,246ha. Cheonan, and Youngdong are famous for major walnut producing areas.

Dead twigs and branches are common in walnut trees and it is most serious limiting factor in production of walnuts in Korea. Symptoms of canker and dieback were frequently encountered in gardens of walnut trees during the investigation of walnut tree diseases in 1989. Eight species of fungi causing canker and dieback of walnut trees have been reported in Japan(Ito, 1971) and 27 species in USA(Crops Research Division, Agricultural Research Service United States Department of Agriculture). However, there have been no studies on the canker and dieback of walnut trees in Korea.

The aim of this work was to identify causal fungi, and to determine the pathogenicity and factors affecting growth of fungi causing canker and dieback in walnut trees. Surveys were conducted throughout the country in 1989 and 1990 for diseased branches and twigs of walnut trees, and three causal fungi were isolated. Morphological characteristics were also given to determine the identity of the fungi.

MATERIALS AND METHODS

1. Fungal collection and identification

In 1989 and 1990 the diseased walnut(*Juglans sinensis* Dode) stem and branch samples with necrosis or lesions were collected from commercial walnut tree farms in Cheonwon, Goesan, Youngdong, Jungwon, and Suwon. At least 50 canker samples were collected from each region.

Diseased tissues from the margins of the lesions were sectioned in 5mm size, surface sterilized with 1% sodium hypochlorite solution(Commenwealth Mycological Institute, 1983), plated onto 2% water agar, incubated at 25℃, and subcultured on potato dextrose agar(PDA) plates. To enhance the produc-

tion of sporulating pycnidia, agar plates were incubated under fluorescent light for 15 days at room temperature (Dhingra and Sinclair, 1985).

Isolates were investigated with light microscope, scanning electron microscope and stereoscopic microscope for morphological characteristics. Diseased samples were cut into 1~2mm square, prefixed for 90 min in 2.5% glutaraldehyde and postfixed in graded series of ethanol and acetone. And specimens were dried using a Hitachi Hcp-2 critical point dryer, with CO_2 as the intermediate fluid. The specimens were then mounted on stubs and coated with gold in a Eiko IB-3 ion coater. Observations were made using a Hitachi S-570 scanning electron microscope operated at 20Kv.

2. Pathogenicity test in greenhouse

Pathogenicity of isolated fungi was tested on 1-year-old walnut trees in greenhouse. Five-day-old cultures of the fungi were used as inocula, and agar plugs were inserted into the open wounds to make contact with the freshly cut phloem. Unwounded inoculations were also made. Inoculation points were selected on twigs in 1.0~1.5cm diameter. Thirty days after inoculation, the presence of the tested fungi were verified by plating the tissues on PDA. Inoculations with each fungus were replicated three times, and plants were kept in a greenhouse at ambient temperature (20~25℃).

3. Investigation on factors affecting fungal growth

Mycelial growth of three fungi identified was examined for the selection of media and the optimum cultural conditions of temperature, pH, carbon sources and nitrogen sources.

To measure the effects of temperature on radial growth, cultures of 3 fungi were incubated on PDA plates in the dark at 4, 8, 15, 20, 25, 30, 35, 40℃ for seven days.

Effect of pH on mycelial growth was examined on PDA adjusted from pH 4 to 10 by 1 interval at 25± 1℃ after 7 days in culture. pH was adjusted by adding with 1N HCl and 1N NaOH solution.

The influence of different media on mycelial growth of the fungi were examined on potato dextrose agar, cornmeal agar, Czapeck solution agar, malt extract agar, yeast extract agar and V-8 juice agar.

The influences of various carbon sources on mycelial growth at 25±1℃ were examined. Tested carbon sources were glucose, mannose, galactose, maltose, sucrose, cellulose, starch and glycerol. Each carbon source was added to 1000ml of the basal medium. Basal medium was composed of 5g yeast extract and 15g agar powder. Colony diameters were measured seven days after inoculation.

The influences of various nitrogen sources on mycelial growth were examined using alanine, proline, glycine, asparagine, arginine, histidine, ammonium sulfate, potassium nitrate and sodium nitrate.

4. Disease survey

Symptoms of canker and dieback of walnut trees were surveyed at 3 locations in 1989 . In May 1990, 1600 trees on 8 farms located in Cheonwon, Goesan, Naju were investigated. Aadditional 1600 trees on 3 farms were investigated in Sep. 1990. Disease severity at different ages and incidence of the diseases on local varieties of walnut trees were also investigated.

RESULTS AND DISCUSSION

1. Isolation and identification of canker and dieback causing fungi.

Fungi associated with canker and dieback in walnut trees were identified by their morphology and cultural characteristics. Three species of fungi were identified according to the mycological characteristics described by previous workers.

1) *Botryosphaeria dothidea* (가지마름病)

This disease was characterized by the development of extensive cankers elliptical in shape along the hardened wood. As the canker developed the trunk above the canker eventually died. The surface of the cankered area was sunken and flattened. The outer bark showed orange to purplish appearance and exposed pycnidia. Immature pycnidia and mature perithecia were observed in canker lesions of walnut

stems.

Black pycnidial and ascogenous stromata of *B. dothidea* found in lenticels on the bark of diseased walnut trees were also produced in culture. Under some conditions white cirrhi exudated from pycnidia immersed in the outer layer of bark. In contrast to other host (Britton and Hendrix, 1982, Weaver 1974) gum exudation was not observed on affected walnut trees. The pycnidia contained hyaline, non septate, fusiform conidia. Perithecia were black, globose to conical and 160~320×190~360μm in size (Fig.1). Ascospores, pycnidia, pycndiospores were similar to those described by CMI. Stromata formed blackish erumpent circular crusts up to 1.5cm long. Asci were cylindric, clavate, thick walled above 8-spored ascospores, elliptical 24.5~16×12~7μm in size, hyaline to faintly yellowish, non-septated (Fig.2). Colonies of the B. dothidea grown on PDA produced mycelia which were initially white and cottony but turned gray and then black as they matured.

2) *Phomopsis albobestita* (Phomopsis 가지마름病)

This fungus was frequently isolated from lesions of stems. The infected area become rugged, dark brown to black in colour. On the host pycnidial conidiomata occur immersed within the dead walnut tissue. Pycnidia were usually solitary, immersed, stromatic, ostiolate. Colonies on PDA were variably floccose, whitish, grey brown or white with greyish black ; reverse of colonies colourless to pale brown with black spots and blotches. α-conidia were hyaline, fusiform, unicellular, 5.5~9.5×1.5~3.0 um. β-conidia are not observed on the host plant, but those produced on PDA were measured 25.0~32.0×1.0~1.4um, curved at the apex (Fig.3). These mophological characters were similar to those reported by Ito (1971).

3) *Melanconis juglandis* (검은 돌기 가지마름病)

The conidial state of this fungus is almost invariably seen on dead walnut branches, at first developing small, rounded, pimply, blackened elevations, scattered irregularly and later bursting through the epidermis and young cork layers with rounded, irregular openings revealing the pustules of apparently black conidia. Pustules on the bark of cankered or dead stems and twigs, were first immersed under epidermal layer, and then erumpent and broken through the bark. If the weather is damp or rainy, they resemble tiny drops of thick ink, or if the twigs are placed in moist chambers, with the humidity of correct percentage, the conidia are extended. Acervuli are flat or elevated at the center part of the layer, composed of conidiophores and their basal layers. Conidiophores are hyaline to pale brown, simple 15~38×3~5um in size, and produce conidia acrogenously . Conidia are elliptic, dark brown to olive brown, granular, unicellular, 17~34×10~14.5um in size (Fig.4). Conidia often ooze out from acervuli as black surface under wet condition. Colony on PDA developed flat and became yellowish brown to dusty yellow in colour. Compared with the Ito's record, the size of conidia is almost similar. Pycnidia were produced in culture, black, spherical irregular, partly sunk in the medium, 150~400um in diameter, with a rounded apical pore and surrounded by a dense felt of mycelia.

Cultural studies indicated that the mophological characteristics of *B. dothidea*, *P. albobestita* and *M. juglandis* were similiar to those described for isolates from other host (Anagnostakis and Aylor, 1984 ; Fenner, 1925 ; Filler, 1967 ; Matuo and Sakurai, 1954 ; Mcpartland and Schoeneweiss, 1984).

2. Pathogenicity test

All three fungi were pathogenic and were reisolated from lesions produced on inoculated stems of walnut trees. Symptoms were first observed on all the inoculated trees 10 days after inoculation, and they appeared as circular, brown lesions and then enlarged later, coalesced and turned dark brown to black. Within 3 month after inoculation, canker developed in the wounded portion of each stem inoculated with *B. dothidea*, *P. albobestita*, and *M. juglandis*, while those inoculated with PDA alone were healed over.

Inoculation on the other host plant have indicated that B. dothidea is usually a wound parasite (Smith 1934, Brown and Hendrix, 1981). However, the fungus invades unwounded stem of blueberry (Milholland 1970 ; Milholland and Galleta, 1969) and almond (English and De Vay, 1975). Smith (1934)

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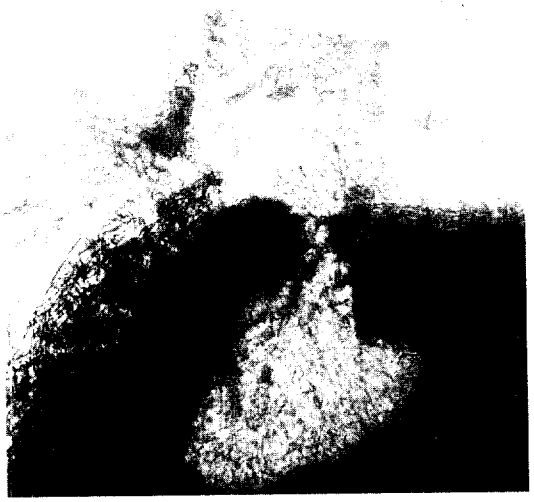

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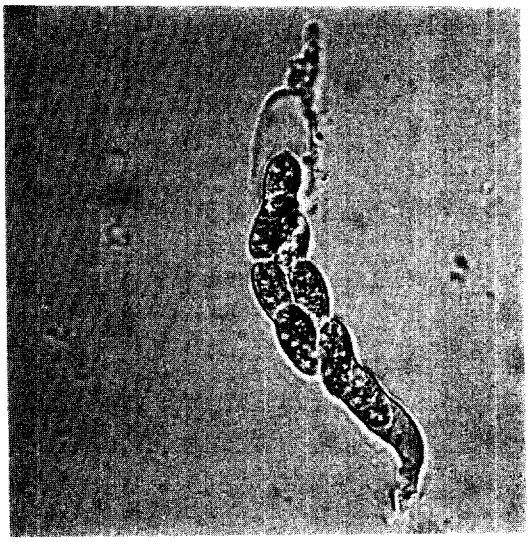

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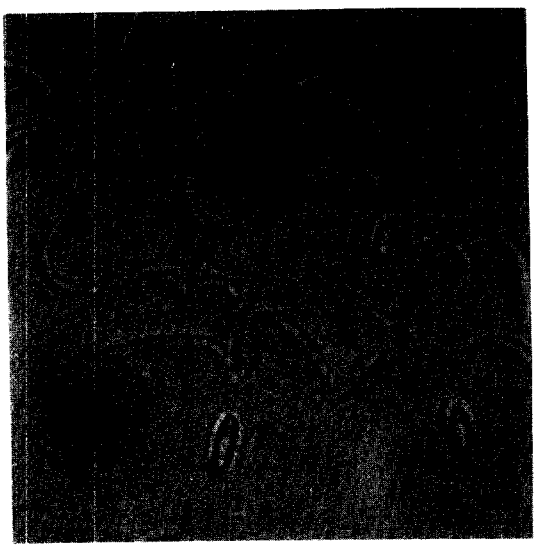

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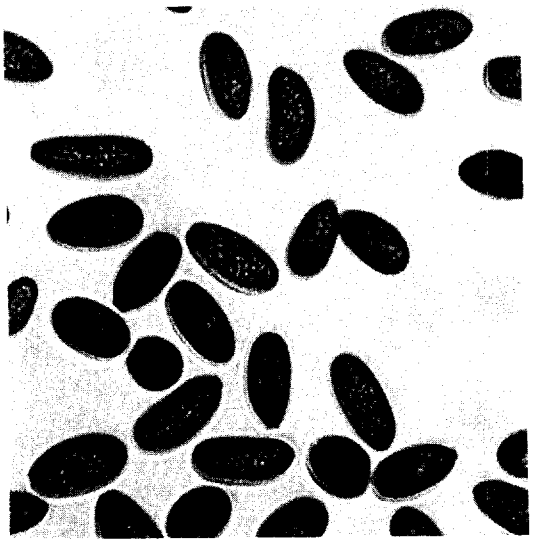

Fig. 1 .A perithecium and asci of *Botryosphaeria dothidea*.
Fig. 2 .An ascus of *Botryosphaeria dothidea* containing eight ascospores.
Fig. 3 .α conidia(fusiform) and conidia(hooked) of *Phomopsis albobestita*.
Fig. 4 .Conidia of *Melanconis juglandis* with granular content.

demonstrate the susceptibility of walnut by stem wounds with three isolates of *B. dothidea*, one from walnut(*Juglans regina*) one from avocado(*Persea americana*) and one from palm(*Arecastrum romanzoffianum americana*). In the present study, wounding was a prerequisite for infection of all three fungi. This is in contrast to results obtained by previous workers. This study confirmed the susceptibility of wounded walnut stem tissue.

3. Cultural characteristics

1) Mycelial growth under different temperature

As shown in Table 1, *Botryosphaeria dothidea*, and *Phomopsis albobestita* grew well at temperature range of 25~30℃ and temperature range for their mycelial growth was between 8~35℃. Maximum mycelial growth of *Melanconis juglandis* occurred at 20~25℃. No growth occurred at 40℃ and sparse or no growth at 4℃.

These results were similar to those reported by Witcher and Clayton(1962). *B. dothidea* can grow over a wide range of temperature (Milholland 1970). A broad temperature range for growth suggest that this fungi may be active in walnut trees during all but the coldest month in Korea. Brown and Hendrix (1981) reported that the optimum growth temperature for the *B. dothidea* was 25 to 30℃. Their results are confirmed in the present study.

2) Mycelial growth under different pH

The effect of pH on mycelial growth of three fungi are shown in Table 2. There were no significant differences in mycelial growth of *Botryosphaeria dothidea* and *Melanconis juglandis*. This is contrast to the result obtained by Drake and Moore(1966). They reported that optimum pH for mycelial growth of *B. dothidea* was 6~7. The optimum pH range for mycelial growth of *Phomopsis albobestita* was 4.0~5.0.

3) Mycelial growth on different media

Mycelial growth of 3 fungi on PDA, cornmeal agar, Czapeck solution agar, malt & yeast extract agar, V-8 juice agar are shown in Table 3. There

Table 1. Effect of different temperature on the mycelial growth of three pathogenic fungi isolated from *Juglans sinensis* after five days in culture.

unit : colony diameter(cm)

Temperature	*B. dothidea*	*P. albobestita*	*M. juglandis*
4	0 D	0 D	0 D
8	1.1 CD	1.3 C	2.9 C
15	2.3 C	3.2 C	4.5 B
20	5.5 B	6.7 B	6.9 A
25	8.2 A	8.5 A	7.5 A
30	8.8 A	8.5 A	4.7 B
35	1.6 C	2.2 C	0.2 D
40	0 D	0 D	0 D

Letters within a column indicates Ducan's Multiple Range Test at 5% level.

Table 2. Effect of different pH on the mycelial growth of three pathogenic fungi isolated from *Juglans sinensis* after five days in culture.

unit : colony diameter(cm)

pH	*B. dothidea*	*P. albobestita*	*M. juglandis*
4.0	8.3 A	6.2 AB	5.7 B
5.0	8.0 A	6.8 A	7.1 A
6.0	8.0 A	5.6 B	7.6 A
7.0	8.4 A	5.6 B	7.8 A
8.0	8.6 A	5.4 B	7.7 A
9.0	6.3 B	4.0 C	7.1 A
10.0	7.4 AB	2.4 D	2.4 C

Table 3. Effect of different media on the mycelial growth of three pathogenic fungi isolated from *Juglans sinensis* after five days in culture.

unit : colony diameter(cm)

Agar medium	*B. dothidea*	*P. albobestita*	*M. juglandis*
Cornmeal	7.1 B	6.7 B	7.2 A
Czapeck	8.8 A	5.9 C	6.5 B
Malt and Yeast extract	8.0 AB	8.2 A	7.0 B
Oat meal	8.7 A	8.8 A	6.9 B
Potato dextrose	8.2 AB	6.4 B	7.5 A
V-8 juice	8.8 A	8.8 A	6.7 B

were no differences in mycelial growth among the 3 fungi. But in cultures of *Melanconis*, various color changes develop under the influence of different culture media. It is the same result that as Tisdale (1916) observed. He noted that the fungus showed marked differences in behavior in the different media. In contrast to this study, culture studies of *Phompsis* indicated that this species grew well on cornmeal agar(Hahn, 1928). Wehmeyer(1927) reported that mycelial growth and pycnidial development was obtained on oatmeal agar and Leonian's agar.

4) Effect of different carbon sources on mycelial growth

The effects of carbon sources on mycelial growth of these fungi are shown in Table 4. Maximum growth of 3 fungi varied with different carbon sources. Mycelia of *Botryosphaeria dothidea* grew well on glucose, sucrose, starch and maltose. Mycelial growth of *Phomopsis albobestita* was good on medium containing glucose and sucrose. The mycelia of *Melanconis juglandis* grew faster on the media containing glucose, sucrose than other sources.

5) Effect of different nitrogen sources on mycelial growth

Mycelial growth on different nitrogen sources are shown in Table 5.

As a nitrogen source, histidine, and potassium nitrate were more suitable compounds for the growth of *B. dothidea*. *P. albobestita* grew well on the medium containing alanine and potassium nitrate as a nitrogen source. *M juglandis* grew well on the medium containing potassium nitrate.

4. Disease Incidences in the Field

Field survey showed that canker and dieback caused by *P. albobestita* occurred more severely than those by *B. dothidea* and *M. juglandis* at 3 locations investigated(Table 6). However in Youngdong, the disease caused by *M. juglandis* occurred more severely than that by the other two fungi.

Incidence of canker and dieback at different grow-

Table 4. Effect of different carbon sources on the mycelial growth of three pathogenic fungi isolated from *Juglans sinensis* after seven days in culture.

unit : colony diameter(cm)

Carbon source	*B. dothidea*	*P. albobestita*	*M. juglandis*
Glucose	9.0 A	9.0 A	5.1 A
Mannose	7.8 B	8.1 C	4.3 C
Galactose	8.3 AB	7.1 D	4.7 B
Maltose	8.8 AB	7.7 C	4.5 C
Sucrose	9.0 A	8.5 B	5.4 A
Cellulose	6.8 B	6.2 E	3.6 D
Starch	9.0 A	6.1 E	4.7 B
Glycerol	8.5 AB	6.2 E	4.6 B
Control	5.3 C	5.6 F	3.2 D

Basal medium was composed of 5g yeast extract and five grams of each carbon source was added to 1,000 ml of basal medium.

Table 5. Effect of different nitrogen sources on the mycelial growth of three pathogenic fungi isolated from *Juglans sinensis* after seven days in culture.

unit : colony diameter (cm)

Nitrogen source	*B. dothidea*	*P. albobestita*	*M. juglandis*
Alanine	4.4 C	6.5 A	4.4 CD
Proline	6.2 B	5.0 E	4.8 BC
Glycine	2.5 E	4.4 G	4.5 CD
Asparagine	5.8 B	5.2 DE	3.8 EF
Arginine	3.6 D	2.1 H	3.2 G
Histidine	7.0 A	4.7 F	3.5 FG
Ammonium sulfate	3.3 D	4.2 G	2.2 H
Potassium nitrate	6.8 A	6.2 B	5.4 A
Sodium nitrate	6.1 B	5.4 CD	4.2 DE
Control	3.1 DE	5.5 C	5.1 AB

Basal medium was same as in Table 4.

Table 6. Incidences of canker and dieback on walnut trees in 1989 at three locations in Korea

Location investigated	Percentage of walnut trees infected with		
	B. dothidea	*P. albobestita*	*M. juglandis*
Cheonwon	10.5	65.5	35.5
Goesan	15.0	20.4	10.5
Youngdong	16.5	21.2	26.5
Average	14.0	38.4	24.0

ing stages of walnut trees was checked. Old walnut trees were more severely infected with the three fungi than young trees. It was also found that more than 50% of 20-year-old trees were infected with the three canker causing fungi.

Disease incidences among the different varieties of walnut trees seemed to show no difference. However, *P. albobestita* was more frequently observed on Pochon variety (16%) than in other four varieties (3~9%) investigated.

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