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# Outbreak of Alfalfa *Verticillium* Wilt in Hokkaido

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**Abstract**
A wilt disease of alfalfa caused by *Verticillium* albo-atrum Reinke et Berthold was detected in most of the alfalfa meadows in Hokkaido. This was the first report of the occurrence of diseases caused by V. *albo-atrum* in Japan along with the report by Saito (1981) on potato. The fungus which was first introduced from infected seeds from abroad survived in plant residues contaminated during harvesting. Alfalfa cultivars showed different reactions to the pathogen: all the cultivars grown in Hokkaido were susceptible, whereas those selected for resistance were resistant. The disease caused a yield loss of 33% in susceptible cultivars, while the resistant cultivars were not affected. Strains of V. *albo-atrum* from alfalfa and potato differed in the host range. Preinoculation of alfalfa plants with the potato strain decreased the disease severity.

**Discipline:** Plant diseases
**Additional key words:** alfalfa strain, *Medicago sativa, Verticillium albo-atrum*

## Introduction

In June 1981, an unknown disease of alfalfa, which resembled *Fusarium* wilt[20], was detected in two meadows in Sorachi, Hokkaido. After extensive surveys throughout Hokkaido, the disease was found to occur all over the island, and severely damaged meadows had to be renovated.

The causal fungus was identified as *Verticillium albo-atrum* Reinke et Berthold. The disease was first reported in Sweden in 1918, and has been a problem in northern Europe[7], New Zealand[27], Canada[1], and U.S.A.[5]. Since the disease was new to Japan, I studied the ecological characteristics and control of the disease, and the results are presented here.

## Characteristics

**(1) Symptoms:** Leaflets wilt, producing a triangular, yellow discoloration along the vein. Leaves show reddish lesions and finally turn white.

With the progression of the disease, plants become stunted and are covered by neighboring, healthy plants. The growth of plant tops resumes after harvest but they show the same symptoms, and plants eventually die. There is no discernible symptom on stems or roots, but vascular bundles turn brown.

**(2) The pathogen:** The causal fungus was isolated and identified.

Stems and roots of diseased alfalfa plants were excised (ca. 5 mm in length), surface-

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sterilized in sodium hypochlorite solution (0.125% active chlorine) for 1–2 min, and incubated on potato-dextrose agar (PDA) at 22°C. *Verticillium* sp. and *Fusarium* spp. emerged from both types of segments, and *Verticillium* sp. was frequently recovered from stems.

Alfalfa plants (cv. Thor) were inoculated with the fungi isolated by dipping seedlings in a spore suspension ($10^6$ spores/m*l*) for 24 hr. *Fusarium* spp. did not produce the symptoms, but *Verticillium* sp. caused the same symptoms. *Verticillium* sp. was reisolated from inoculated plants showing symptoms, and this fungus was found to be the pathogen, with the following characteristics: Mycelium on PDA white, later becoming dark. Aerial mycelium short. Conidia produced in heads on verticillate phialides, ovoid to cylindrical, hyaline, and mostly aseptate. Dimensions 5.6 × 3.0 $\mu$m. Dark mycelia and torulose hyphae present, microsclerotia absent. Basal cells of conidiophores dark. Optimal growth at 20–25°C, no growth at 30°C. Based on these characteristics, the fungus was identified as *V. albo-atrum* Reinke et Berthold.

**(3) Specialization in pathogenicity:** *Verticillium albo-atrum* is known to cause wilt of potatoes[19] and alfalfa[15]. Both strains were compared for their pathogenicity. Tunika potatoes (*Solanum tuberosum* L.) and Thor alfalfa were inoculated with both pathogens separately as described above. Both strains were pathogenic to their respective hosts but not to the other. Inoculation experiments were repeated to determine whether the pathogenicity changed after consecutive inoculations, i.e. potato plants were inoculated with the alfalfa strain, and the inoculated fungus was isolated 3–4 weeks later and used for the next inoculation. The pathogenicity of the alfalfa strain did not change after 7 generations (Table 1). The same results were obtained for the potato strain. The above results indicate that both strains of *V. albo-atrum* from alfalfa and potatoes are different pathogenically[21].

## Dissemination

This disease occurs exclusively in cool regions and is difficult to control due to the various ways of dissemination[3,4,6,8–11,13,16,22]. Here, major ways of disease dissemination are described.

**(1) Seed dissemination:** The disease had never been observed in Hokkaido previously and was presently introduced from seeds from abroad. The possibility of seed dissemination was examined. Diseased plants after inoculation (susceptible cv. Thor) received a conidial suspension during pollination, and resultant seeds were collected. They were then placed on a selective medium[2] and incubated at 22°C to analyze the emergence of the fungus:

**Table 1. Stability of pathogenicity of *V. albo-atrum* strains from alfalfa and potato to alfalfa and potato plants after repeated inoculation**

| Isolate | Plants inoculated | | Isolate | Plants inoculated | |
|---|---|---|---|---|---|
| | Alfalfa | Potato | | Alfalfa | Potato |
| A1 | 47/60 (78.3) | 0/48 | P1 | 0/87 | 32/32 (100) |
| A2 | 40/43 (93.0) | 0/28 | P2 | 0/42 | 22/22 (100) |
| A3 | 28/32 (87.5) | 0/40 | P3 | 0/40 | 16/16 (100) |
| A4 | 30/30 (100) | 0/27 | P4 | 0/53 | 16/16 (100) |
| A5 | 16/18 (88.9) | 0/25 | P5 | 0/10 | 10/10 (100) |
| A6 | 12/15 (80.0) | 0/9 | P6 | 0/30 | 7/10 (70.0) |
| A7 | 9/10 (90.0) | 0/9 | P7 | 0/28 | 2/6 (33.8) |

**Table 2. Detection of *V. albo-atrum* from seeds produced on greenhouse-inoculated alfalfa plants**

| No. of seeds tested | No. of seeds with *V. albo-atrum* recovered | Detection (%) | Treatment |
|---|---|---|---|
| 1,428 | 0 | 0.0 | Surface-sterilized |
| 1,362 | 3 | 0.2 | Nonsterilized |
| 2,182 | 609 | 27.9 | Immature, nonsterilized |

**Table 3. Infection of *V. albo-atrum* by various treatments**

| Treatment | No. of plants used | No. of diseased plants | Diseased plants (%) |
|---|---|---|---|
| Clipping healthy stems with infected scissors[a)] | 52 | 4 | 7.7 |
| Covering trimmed plants with diseased plants tops | 46 | 13 | 28.3 |
| Clipping with disinfected scissors | 37 | 0 | 0.0 |

a): Scissors were infected by cutting diseased stems.

*Verticillium albo-atrum* was not detected from surface-sterilized seeds but was isolated from 0.2% of nonsterilized seeds, and moreover, immature seeds very frequently (27.9%) produced the fungus (Table 2). The pathogen on the surface of the seeds disappeared after 3 months of incubation at room temperature but persisted for more than one year when the seeds were stored at 5°C (Fig. 1). These results indicate that seeds produced on diseased plants are infected and can be the source of disease occurrence. Most seeds used in Japan are imported from U.S.A. and Canada. The disease was reported in those countries in the late 1970s[5,25], which coincided with the first occurrence in Hokkaido in 1981. Seed dissemination of this disease has been reported by many authors. Kreitlow[16] and Christen[3] suggested that the disease was introduced from infected seeds from Europe to U.S.A., and Shepard et al.[25] found that 3.2 and 8.7% of seeds from Canada and U.S.A. were contaminated, respectively. The pathogen was present inside the seed with a frequency of 0.4%[3], and ca. 18,000 plants were considered to become infected when such seeds were sown at 11 kg/ha.

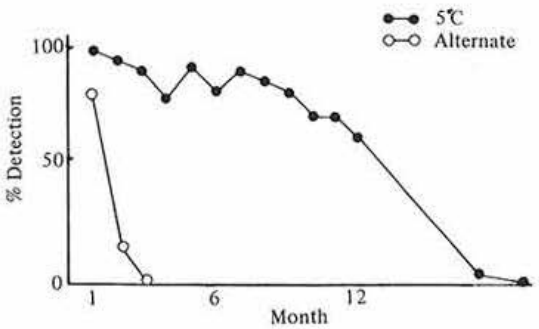


Fig. 1. Longevity of *V. albo-atrum* on artificially infected alfalfa seeds

**(2) Dissemination by mowing:** Alfalfa is usually harvested three times a year. Dissemination during harvest, when diseased and healthy plants were likely to be in close contact, was examined. Plant debris were collected from a mower conditioner used in an infected field and kept under humid conditions. Growth of *V. albo-atrum* was observed from the cut surface of stems and leaflets after 24–48 hr.

Dissemination by contact between diseases and healthy plants was examined in the cv. Thor at 25°C in a glasshouse. When healthy plants were trimmed with scissors, with which diseased plants had been excised, few plants were infected, and the percentage of infection increased when trimmed plants were covered with diseased plant tops (Table 3). Conidia were produced on the cut surface of diseased plants. Conidia were trapped at the base of diseased plants but seldom detected 1–5 m apart from the source under field conditions (no wind, 80% relative humidity).

These findings suggest that machines are involved in secondary infection during harvest by transferring diseased plant debris. Heale et al.[7] consider that this mode of dissemination is important, emphasizing that the infection rate was enhanced by vigorous growth of the pathogen when harvested plants were left in the field due to rainfall. Conidia are thus effective as inoculum but do not seem to play an important role in long-distance dispersal[12] since they can survive for only 24 hr under field conditions.

**(3) Soil-borne dissemination:** Soil from an infected field was placed in plastic pots, and in each pot single plant species was planted. The pot was placed outdoors to observe the changes in the amount of pathogen in soil. Soil was retrieved periodically, and 1.0 m*l* of soil solution (×100) was spread over a plate containing the selective medium. Colonies of *V. albo-atrum* were counted up to 36 months: soil planted with alfalfa always contained a larger number of colonies, while timothy soil showed the smallest number but still contained the fungus (Fig. 2). Soils planted with eggplant, red clover or *Plantago asiatica* L. showed intermediate values.

*Verticillium albo-atrum* is reported to display a weak saprophytic ability, and the fungus disappeared from diseased alfalfa stems from 9[6] to 11 months[14] after burial in soil. However, when the soil was planted with host or non-host plants, the fungus was found to survive

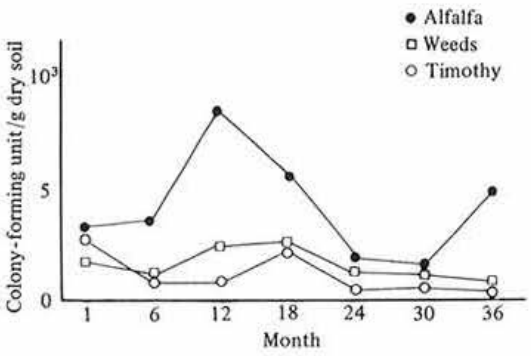


Fig. 2. Plate counts of *V. albo-atrum* in soil planted with alfalfa, timothy or weeds

for at least 3 years, suggesting that the inoculum level increased when susceptible alfalfa was again planted.

**(4) Latent infection of weeds:** Many weed species growing in infected fields were investigated to determine whether they were a potential inoculum source. The fungal isolates were obtained from crown or roots of 24 species belonging to 13 families as described above and identified, and *V. albo-atrum* was detected in 14 species belonging to 9 families (Table 4). Of these, isolates from *Rumex obtusifolius* L. and *Plantago asiatica* L. were selected for inoculation experiments with alfalfa: they were found to be pathogenic to alfalfa with typical symptoms. These results indicate that many weeds can carry the pathogen although they do not show symptoms. Weeding is known to be one of the control measures[26].

## Varietal resistance

Diseases of forage crops should be controlled by using resistant cultivars. Screening methods were developed to select resistant individual plants, and control experiments with resistant cultivars were conducted under field conditions.

**(1) Screening for resistance:** Seeds were sown in rows in plastic pots (57 × 32 × 12 cm); plant tops were removed when the seedlings grew to a length of 10 cm; and then, 40 m*l*

**Table 4. Detection of *V. albo-atrum* from various plants growing in infected fields**

| Plants | No. of plants examined | No. of plants with *V. albo-atrum* | Detection (%) |
|---|---|---|---|
| *Taraxacum officinale* Weber | 160 | 40 | 2.5 |
| *Erigeron annuus* Pers. | 195 | 2 | 1.0 |
| *Sonchus brachyotis* DC. | 74 | 4 | 5.4 |
| *Artemisia princeps* Pampan. | 51 | 0 | 0 |
| *Solidago* sp. | 23 | 0 | 0 |
| *Senecio vulgaris* L. | 26 | 4 | 15.4 |
| *Plantago asiatica* L. | 77 | 7 | 9.1 |
| *Plantago lanceolata* L. | 48 | 0 | 0 |
| *Rumex obtusifolius* L. | 120 | 10 | 8.3 |
| *Rumex acetosella* L. | 32 | 0 | 0 |
| *Polygonum longisetum* De Bruyn | 74 | 1 | 1.4 |
| *Polygonum aviculare* L. | 9 | 1 | 11.1 |
| *Commelina communis* L. | 60 | 1 | 1.6 |
| *Amaranthus lividus* L. | 20 | 2 | 10.0 |
| *Rorippa sylvestris* (L.) Besser | 32 | 10 | 31.3 |
| *Brassica campestris* L. | 19 | 0 | 0 |
| *Oxalis corniculata* L. | 59 | 0 | 0 |
| *Geranium thunbergii* Sieb. et Zucc. | 52 | 0 | 0 |
| *Solanum nigrum* L. | 20 | 3 | 15.0 |
| *Portulaca oleracea* L. | 24 | 2 | 8.3 |
| *Elsholtzia ciliata* Hylader | 29 | 0 | 0 |
| *Chenopodium album* L. | 87 | 2 | 2.3 |
| *Salix integra* Thumb. | 35 | 0 | 0 |
| *Trifolium repens* L. | 39 | 0 | 0 |

of a spore suspension ($10^6$ spores/m*l*) was sprayed with an atomizer for each pot. Inoculated plants were incubated in a moist box at 22–25°C for 24 hr. Disease severity was estimated for each plant according to the rating shown in Table 5, 30 days after inoculation. Plants with indices 1–2 were considered to be resistant and those with indices 3–5 susceptible. The extent of the resistance in the cultivars and lines was expressed as a mean index of the total number of plants examined and as a percentage of resistant plants. A part of the results with 241 cultivars and lines is presented in Table 6[24]. In summary, all the cultivars except for a few resistant ones were found to be susceptible, including recommended cultivars in Hokkaido such as Kitawakaba,

**Table 5. Disease rating**

| Index | Symptoms |
|---|---|
| 1 | No symptoms |
| 2 | 1 or 2 leaflets show chlorosis. |
| 3 | Leaflets on more than one shoot are chlorotic. |
| 4 | Most leaflets are chlorotic. |
| 5 | Plants dead |

**Table 6. Reactions of alfalfa cultivars to *V. albo-atrum***

| Varieties | No. of plants tested | Disease index | | | | | Severity | Resistant plants (%) |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | | |
| Alfa | 106 | 33 | 1 | 68 | 3 | 1 | 2.4 | 32.1 |
| Arrow | 282 | 218 | 15 | 56 | 0 | 0 | 1.5 | 82.6 |
| Citation | 89 | 33 | 17 | 35 | 4 | 0 | 2.1 | 56.2 |
| Du Puits | 152 | 40 | 21 | 56 | 30 | 7 | 2.7 | 40.1 |
| Elevation | 100 | 40 | 1 | 52 | 6 | 1 | 2.3 | 41.0 |
| Europe | 146 | 38 | 34 | 51 | 19 | 4 | 2.4 | 49.3 |
| Euver | 238 | 143 | 15 | 86 | 4 | 0 | 1.9 | 66.4 |
| Everest | 137 | 64 | 17 | 51 | 5 | 0 | 2.0 | 59.1 |
| Excalibur | 255 | 121 | 10 | 115 | 8 | 1 | 2.1 | 51.4 |
| Florida 77 | 177 | 72 | 20 | 61 | 19 | 5 | 2.2 | 52.0 |
| Gemini | 180 | 63 | 25 | 78 | 11 | 2 | 2.2 | 48.9 |
| Lutéce | 397 | 274 | 23 | 97 | 1 | 0 | 1.5 | 74.8 |
| Maris Kabul | 253 | 174 | 44 | 35 | 0 | 0 | 1.5 | 86.1 |
| Maya | 238 | 188 | 0 | 50 | 0 | 0 | 1.4 | 79.0 |
| Resis | 243 | 155 | 11 | 73 | 3 | 1 | 1.7 | 68.3 |
| Rhizoma | 101 | 25 | 2 | 69 | 5 | 0 | 2.5 | 26.7 |
| Sabilt | 107 | 73 | 19 | 15 | 0 | 0 | 1.5 | 86.0 |
| Saranac AR | 124 | 31 | 17 | 58 | 14 | 4 | 2.5 | 38.7 |
| Thor | 537 | 179 | 33 | 264 | 53 | 14 | 2.5 | 39.5 |
| Vertus | 487 | 375 | 32 | 83 | 0 | 0 | 1.4 | 83.6 |
| 5444 | 267 | 210 | 14 | 43 | 0 | 0 | 1.4 | 83.9 |
| WWB-13 | 277 | 183 | 17 | 77 | 0 | 0 | 1.6 | 72.2 |
| Kitawakaba | 221 | 102 | 12 | 94 | 13 | 0 | 2.1 | 51.6 |

**Table 7. Incidence of *Verticilium* wilt disease and effect on alfalfa hay yield**

| Alfalfa cultivar | Inoculation | Disease incidence (%) | | Yield (kg) | | | t/ha |
|---|---|---|---|---|---|---|---|
| | | 1986 | 1987 | 1st | 2nd | 3rd | |
| Thor | − | 0.0 | 1.3 | 27.5 | 25.5 | 16.4 | 5.8 |
| | + | 58.0 | 89.5 | 16.9 | 15.9 | 13.9 | 3.9 |
| Vertus | − | 0.0 | 0.0 | 21.6 | 23.2 | 15.2 | 5.0 |
| | + | 4.2 | 2.1 | 24.2 | 22.0 | 16.5 | 5.2 |

Europe, Saranac, and Thor. On the contrary, cultivars and lines, which had been selected for resistance in foreign countries, were resistant in terms of disease severity and percentage of resistant plants[25]. Although these cultivars or lines included unsuitable ones for practical cultivation in Hokkaido due to their low productivity and low winter hardiness level, Arrow, Euver, Maya, Resis, Vertus, and 5444 were registered as recommended cultivars in 1990.

The methods described here are suitable for resistance screening due to the following advantages: the methods are highly reproducible and enable to save space, hence a large number of plants can be screened compared with the soil-inoculation method[23].

**(2) Control with resistant cultivars:** Field experiments were conducted using 12 $m^2$ plots with duplication. Thor and Vertus were selected as susceptible and resistant cultivars, respectively.

They were sown in rows in May 1986 and inoculated with a spore suspension ($10^6$ spores/m*l*) in mid-September 1986 just after harvest. Number of plants and yield were determined the following year. Percentage of diseased plants increased from 58 to 89.5% in Thor, whereas Vertus showed few changes (Table 7). The decrease in the number of plants which survived in the inoculated plots was ca. 15% higher than that in the uninoculated plots. Thor yielded 5.8 t/ha in uninoculated plots, but the yield decreased to 3.9 t/ha (33% decrease) in the inoculated plots. There was no difference in the yield of Vertus between inoculated and uninoculated plots. The use of resistant cultivars was thus found to be effective, since the damage was reduced to a negligible level. However, the resistant cultivars carry the pathogen without symptoms and are a potential source of inoculum[18]. Although data supporting this assumption were not obtained, the inoculum level in the soil was found to decrease by the use of resistant cultivars.

## Disease alleviation by preinoculation with the nonpathogenic strain

The nonpathogenic strain from potato was assessed as a biocontrol agent by cross-protection. Preliminary experiments with *Rhizobium meliloti*, *Gliocladium roseum* and the potato strain of *V. albo-atrum* revealed that *V. albo-atrum* from potato was most effective in reducing the disease severity when used before the inoculation of the alfalfa strain. Roots of 1-month-old alfalfa seedlings (cv. Thor) were removed and dipped in a spore suspension of the potato strain ($10^6$ spores/m*l*) for 24 hr (preinoculation) and then in a spore suspension of the pathogenic strain from alfalfa ($10^6$ spores/m*l*) for 24 hr (postinoculation). Plants inoculated with the potato or alfalfa strain and uninoculated plants were used as controls. All the plants inoculated with the alfalfa strain were affected by the disease with the highest severity, whereas those preinoculated with the potato strain showed a lower percentage of disease and a lower disease severity. Plants inoculated with the potato strain and uninoculated plants did not show any symptoms (Table 8). The interval between pre- and postinoculation to suppress the disease was less than 144 hr. The effect of cross-protection has been demonstrated in wilt diseases caused by *Fusarium*, *Verticillium*, and bacteria, and the effect was pronounced when the strains used for preinoculation were taxonomically close to the pathogens[17]. Strains of *V. albo-atrum* from alfalfa and potato belong to the same species with a different pathogenicity. The appearance of the symptoms was delayed when alfalfa plants were protected by preinoculation with the potato strain, and the disease severity decreased. This method of control is, however, considered to be unpractical because the effect did not last long and because the critical period for infection, i.e, harvesting time, occurs three times a year.

**Table 8. Control of Alfalfa *Verticillium* wilt by a potato strain of *V. albo-atrum***

| Pre-inoculation | Post-inoculation | No. of plants used | No. of diseased plant (%) | Diseased plants (%) | Disease index |
|---|---|---|---|---|---|
| A[a)] | - | 4 | 4 | 100.0 | 3.5 |
| P[b)] | - | 4 | 0 | 0.0 | 1.0 |
| P | A | 18 | 8 | 44.4 | 1.8 |
| - | - | 2 | 0 | 0.0 | 1.0 |

a): Alfalfa strain, B): Potato strain.

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