This is to certify that the

thesis entitled

Apothecial development and ascospore maturation in Blumeriella jaapii

presented by

Stella M. Garcia

has been accepted towards fulfillment
of the requirements for

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Alan L. Jones
Major professor

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APOTHECIAL DEVELOPMENT AND ASCOSPORE MATURATION IN *Blumeriella jaapii*

By

Stella Maria Garcia

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ABSTRACT

APOTHECIAL DEVELOPMENT AND ASCOSPORE MATURATION IN *Blumeriella jaapii*

By

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Apothecial development of *Blumeriella jaapii* in cherry leaves was separated into three phases: before appearance of asci, from appearance of asci to mature asci, and from mature asci to apothecia disintegration. Field studies over a 2-yr period indicated that low temperatures during late winter or early spring delayed the appearance of the first asci in the spring. Rate of maturation of apothecia was affected by temperature and rain, and apothecial development was arrested during periods of dry weather even at favorable temperature. Disintegration of apothecia was accelerated by rain. In controlled experiments, temperature affected the number and maturation of asci and discharge of ascospores. Non-linear regression analyses were significant and quadratic for number of asci per apothecium and for percentage of mature plus empty asci after 7 days of incubation. The number of asci per apothecium was highest at 16 C, and ascus maturation was most rapid at 16 - 20 C. Apothecial development was poor at 4 and 24 C. The percentage of ascospores discharged from apothecia increased with increased temperature over a range of 8 - 30 C.

DEDICATION

To my parents, for their love, support and encouragement, not only during my education, but also during all my life.

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GENERAL INTRODUCTION

Cherry leaf spot, caused by Blumeriella jaapii (Rehm) Arx, is a major disease of sour and sweet cherry (Prunus cerasus L. and P. avium L.) throughout the cherry growing areas of United States and Canada. The fungus overwinters in infected leaves as a stroma-like structure that later develops into an apothecium (1,3). Since the ascocarps are formed in association with acervuli, their production depends on the quantity and conditions of the acervuli at the time of ascocarp initiation (1,3,7). Keitt (7), found that there was a relationship between the time of leaf fall and the number of apothecia formed. Fewer apothecia per leaf were formed in leaves that fell in August than in leaves that fell in September. Most of the leaves that fell early disintegrated before apothecia had an opportunity to develop. Normally, leaves that were infected late in the season had the largest number of apothecia per leaf.

Freezing temperatures before leaf fall had been reported to affect the presence of the ascocarps. Backus (1) mentioned that ascocarps failed to developed in infected cherry leaves that were frozen on the trees.

Environmental conditions during winter affects the development of stroma. Higgins (3), found that when

infected leaves were brought into the laboratory at intervals during fall and winter, fruiting bodies failed to developed if they were incubated before paraphyses had been differentiated. He speculated that excessive moisture or lack of freezing during incubation prevented the formation of the ascogenous hyphae. Under natural conditions during winter, ascocarps failed to develop when the leaves were packed closely together and therefore moist and poorly aerated. Ascoscarps also failed to develop on the side of the leaf that was in contact with the ground (4,8).

According to Kaszonyi (7), the further development of stromatic plectenchymae in spring depends on temperature and precipitation. If the overwintered leaves were soaked by rain and retained their moisture for 1 or 2 days and the temperature rose to 15 - 21 C, the stromata began to develop rapidly and produced within 24 - 48 h either apothecia or acervuli.

The time at which asci began to form in spring depended on weather conditions. Backus (1) found that under conditions prevailing in Madison, Wisconsin, the first appearance of asci could be expected in the early part of April; however, in winters unusually mild and snowless, asci began to form as early as the middle of March. In the laboratory asci could be induced to appear several weeks before their formation outside if the overwintering leaves were incubated in a moist chamber for a few days.

During the later part of April or the first of May the asci enlarged rapidly and lifted the covering of the apothecia until it finally broke in a more or less stellate manner. The breaks occurred before the ascospores were mature, but they matured very shortly thereafter (3). In most years the development of asci proceeded quite slowly, and it was not until the middle of May that mature ascospores were found in abundance. Asci in various stages of maturity were found in a single fruiting body (1,8). The asci open by a pore in the papillate apex and the ascospores are forcibly discharged (4).

Ascospores are responsible for initiating the disease in the spring. Ascospores are usually mature and available for discharge when the cherry trees start to bloom.

Ascospores discharge occurs when overwintered leaves are wet by rain. Apothecia continue to produce and discharge ascospores until about mid-summer (6).

Discharge of ascospores is affected by temperature and rain. Keitt (8) reported that when required moisture conditions were met, ascospore discharge occurred at temperatures ranging from 1 to 36 C, but discharge of ascospores was greatest at temperature > 16 C, less rapid at 12 C, and rare at 4 - 8 C.

Extensive spread of the disease in late spring and summer is caused by the conidial stage of B. jaapii. Control of cherry leaf spot is based in the use of fungicide sprays in a calendar schedule. Fungicide sprays are initiated when

the first susceptible leaf tissue is present. Cherry leaves are susceptible to the leaf spot fungus once they are unfolded, usually at the petal fall stage of blossom bud development, and remain susceptibles throughout the season (8), although susceptibility the cherry leaves to infection decreases with age (3).

Precise knowledge of ascospore maturity is important to management of the disease because sprays should be timed to coincide with ascospore discharge in order to prevent infection by primary inoculum, and to reduce later unnecessary sprays. Several approaches that are used to evaluate the progress of ascospore maturation in pseudothecia of Venturia inaequalis, such as microscopic examination of crushed pseudothecia, or the quantification of ascospores collected in spore traps (5,9), could be used for evaluating ascospore maturation and discharge of B. jaapii. However, these methods are time-consuming and labor-intensive. Therefore attempts should be made to develop a model for predicting ascospore maturity and discharge from temperature and rainfall measurements.

The data from the present study make it possible to quantify the influence of environmental factors on apothecial development and maturation and discharge of ascospore of B. jaapii in order to develop a model for predicting ascospore maturation and discharge that could be incorporated into a model developed by Eisensmith et al (2) for predicting infection periods. If fungicides sprays

could be applied only when infections occur, it should be possible to improve timing and effectiveness of sprays, reducing costs of chemical control and environmental problems.

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PART I

ENVIRONMENTAL FACTORS AFFECTING APOTHECIAL DEVELOPMENT AND ASCOSPORE MATURATION OF *Blumeriella jaapii*

ABSTRACT

Based on histological examination of apothecia in overwintering sweet and sour cherry (Prunus avium L. and P. cerasus L.) leaves, apothecial development of Blumeriella jaapii could be separated into three phases: (1) before appearance of asci, (2) from appearance of asci to presence of mature asci, (3) from mature asci to apothecial disintegration. Field studies made during a 2-yr period indicated that the appearance of asci in the spring is temperature-dependent. Low temperatures during late winter or early spring delayed the appearance of the first asci in the spring. Maturation of apothecia was affected by temperature and rain, and apothecial development was arrested during dry periods even at favorable temperatures. The disintegration of apothecia was accelerated by rain, especially May rains. Ascospore discharge usually started before blooming of the cherry trees and continued for about 5 - 7 wk. The major portion of the ascospores were discharged from bloom to about 2-wk after petal fall.

INTRODUCTION

Cherry leaf spot, caused by Blumeriella jaapii (Rehm) Arx, is the most important disease of sour and sweet cherry (Prunus cerasus L. and P. avium L.) in Michigan and throughout the eastern United States and Canada. The fungus overwinters in infected leaves as a stroma-like structure that later develops into an apothecium (1,4). Temperature and moisture govern the development of the apothecia and the maturation of the ascospores (1,4,8,10,11). Higgins (4) and Backus (2) indicated that mild temperatures during late winter accelerated the development of apothecia and asci were formed as early as the middle of March. Apothecia mature in the spring and ascospores are available for discharge by the time the cherry trees start to bloom.

Ascospore discharge occurs when the leaves are wet by rain. Apothecia continue to produce and discharge ascospores until mid-summer. Conidia formed on acervuli on newly infected leaves are responsible for the secondary spread of the disease throughout the growing season. Control is based in the use of fungicide sprays during the primary and secondary cycles based on a calendar schedule (8). Precise knowledge of ascospore maturity is critical to

disease management because fungicide sprays should be timed to coincide with ascospore maturity and discharge, in order to reduce unnecessary sprays.

Current methods used to evaluate the progress of ascospore maturation and discharge in pseudothecia of Venturia inaequalis (5,12) could be used in the cherry leaf spot disease, but these methods are time-consuming and labor-intensive. A better understanding of the factors affecting development of apothecia and ascospore discharge could enable us to develop a predictive model of ascospore maturation.

The objective of this research was to quantify the influence of environmental factors on the maturation of apothecia and discharge of ascospores of B. jaapii.

MATERIAL AND METHODS

Apothecial development. In 1990 studies were conducted in two orchards (orchards 1 and 2) near East Lansing, and in three orchards (orchards 3, 4, and 5) near Traverse City, MI. Orchards 1 and 2 were located at the Botany and Plant Pathology Research Farm, Michigan State University, and were sweet and sour cherry respectively. Orchards 3 and 4 were located at the Northwest Michigan Horticultural Experimental Station and were sweet and sour cherry respectively. Orchard 5 was located on the Old Mission Peninsula about 10 miles from the orchards 3 and 4,

and was sour cherry. In 1991 studies were conducted in East Lansing (orchard 1) and in Traverse City (orchards 3 and 4).

Leaves of sweet and sour cherries heavily infected with B. jaapii were collected beneath cherry trees starting on 11 April 1990 and 21 March 1991 at East Lansing and on 27 April 1990 at orchards 3, 4, and 5 and 26 April 1991 at orchards 3 and 4. Leaf sampling continued at 2-wk interval until the apothecia disintegrated. On each date 10 leaf disks, 1-cm in diameter, were cut from the leaves with a cork borer, fixed in a mixture of 2-propanol, water, propionic acid, and formaldehyde (45:45:5:5, v/v) (FPP), dehydrated in an alcohol series, embedded in Tissue Prep. (MP = 56 - 57 C, Fisher Scientific Co., Fair Lawn, NJ), sectioned with a microtome at thickness of 12 um, and stained according to a modified Conant's staining schedule schedule (7). In 1991 clove oil was replaced with wintergreen oil as a solvent for the dye Orange G. Apothecia were rated according to internal stage of development as follows: stage 1, lumen of the apothecium filled with paraphyses (Fig. 1); stage 2, asci formed but ascospores not differentiated (Fig. 2); stage 3, few asci with ascospores formed (Fig. 3); stage 4, most asci with ascospores and apothecia predominantly open (Fig. 4); stage 5, spores discharged from most asci (Fig. 5); and stage 6, apothecium disintegrated. Serial sections of each apothecium were observed, and at least 50 apothecia per

Fig. 1. Apothecial development of Blumeriella jaapii.

Stage 1 - only paraphyses present in lumen of apothecium.

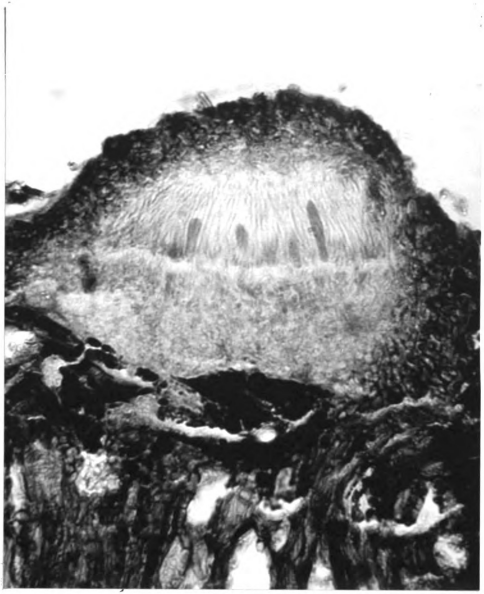


Fig. 2. Apothecial development of Blumeriella jaapii. Stage 2 - asci formed but no ascospores present.

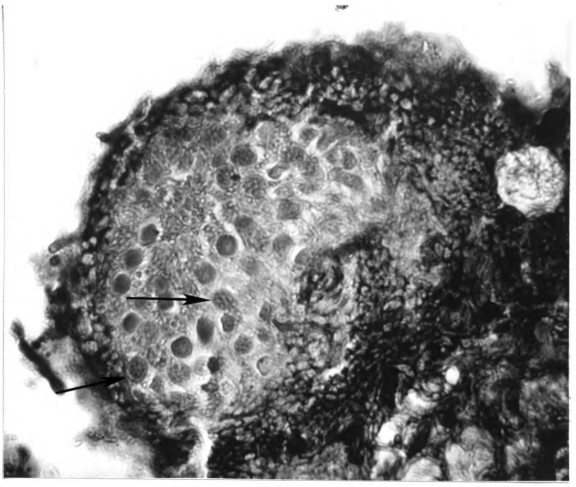


Fig. 3. Apothecial development of Blumeriella jaapii. Stage 3 - few asci with ascospore formed.

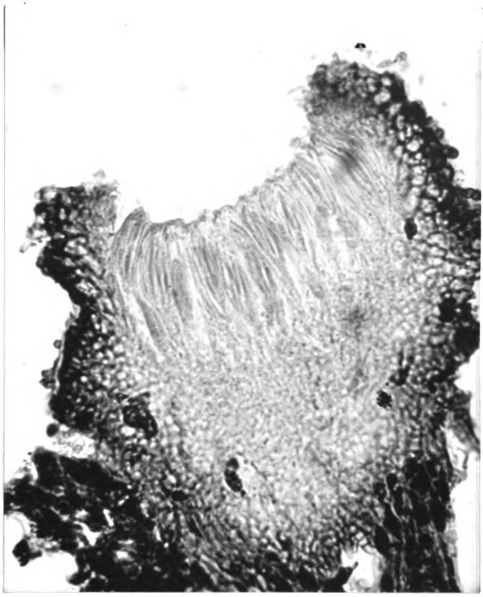


Fig. 4. Apothecial development of Blumeriella jaapii. Stage 4 - many asci with ascospores formed and apothecia open.

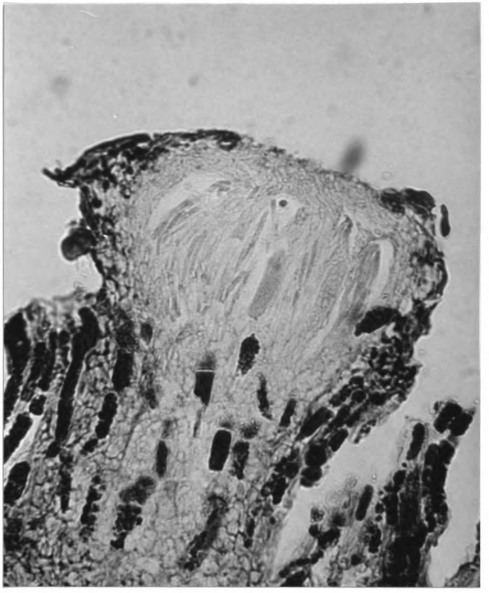


Fig. 5. Apothecial development of Blumeriella jaapii. Stage 5 - spores discharged from most asci.

Fig. 6. Apothecial development of Blumeriella jaapii. Stage 6 - apothecia disintegrated.

sampling date (if available) were rated according to their internal stage of development. Each apothecium was assigned a stage classification. The resulting classification number was averaged to give a mean value for each date (6).

Meteorological data were obtain from the Horticultural Research Center, Michigan State University for orchards 1 and 2; from the Northwest Michigan Horticultural Experimental Station for orchards 3 and 4; and from the Old Mission station (National Oceanic Atmospheric Administration) for orchard 5.

Ascospore discharge. During 1990, two to four rotorod samplers (Sampling Technologies, Inc., 26338 Esperanza Drive, Los Altos Hill, CA) were set between cherry trees in the row at orchards 3, 4, and 5. At East Lansing, the samplers were placed in an apple orchard and diseased cherry leaves were placed under the samplers the same day the samplers were set up. In 1991, three rotorod samplers were placed in the orchards 1, 3, and 4. Rotorod samplers with fixed sampling heads and plastic "I" rods were mounted on vertical pipes 20-mm in diameter and were operated at 2,400 rpm. In 1990 "I" rods 64 x 1.59 mm were used in orchards 1 - 4 and "I" rods 32 x 1.59 mm in a retractile head were used in orchard 5. In 1991 the last type of rods and head were used in all the orchards.

The height of the traps was such that the mid-point of their trapping surface was 0.50 m above the ground. A 25 x 25 cm

piece of sheet metal was positioned about 10 cm above each sampler as a rain shield. The plastic rods were thinly coated with silicone grease (General Electric G-697). After each rain the rods were removed and the total sampling surface of each rod was examined under the microscope (X200).

To reduce the accumulation of trash on the "I" rods during dry periods a transistorized control unit and a moisture sensor was used to activate the samplers from the beginning of the rain and for the duration of the wet period (12).

The traps were maintained from 24 April to 30 June 1990, and from 5 April to 30 June 1991 at East Lansing; from 27 April to 17 July 1990, and from 17 April to 30 June 1991 at Traverse City.

Infection periods. Meteorological data obtained from the Northwest Michigan Horticultural Experimental Station were used to determine infection periods for orchards 3, 4, and 5. The infection periods were determined according to the Enviromental Favorability Index (EFI) developed by Eisensmith et al (3).

Isolation of *Phloeosporrella padi* and *Blumeriella jaapii*. In the process of evaluating samples for apothecial development, acervuli with conidia resembling those of *P. padi* were observed in overwintered leaves (Fig. 7). To determine if these structures were acervuli of *P. padi*, single conidia from the acervuli were isolated and

Fig. 7. Cross-section through overwintered leaf showing acervuli with conidia of Phloeosporella padi

germinated on potato dextrose agar amended with 10 ug/ml streptomycin (PDAS).

Overwintering sweet cherry leaves infected with the leaf spot fungus were collected on 5 May 1991 from beneath trees in an orchard at East Lansing and brought to the laboratory, washed with tap water and held in a moist chamber. After 24 h, and with the aid of a dissecting microscope, small pieces of overwintered leaf containing acervuli were cut with a razor blade and rubbed across the surface of PDAS in petri dishes. After 24 h, pieces of agar containing uncontaminated germinated conidia, were cut with a sterile scarpel and rubbed across the surface of fresh PDAS. Monoconidial isolations were done 24 h later by transferring single germinating conidia to fresh PDA. The petri dishes were incubated at room temperature (21 - 23 C). For comparison, ascospores of B. jaapii and conidia of P. padi from acervuli formed on newly infected leaves, were isolated in the same way.

RESULTS

Apothecial development. Apothecia were observed on the lower surface of leaves where the lower surface was exposed.

During the 2 yrs of the study differences were observed in the date of maturation of the apothecia of B. jaapii (Table 1 & 2). In 1990 at East Lansing, apothecia in sweet cherry leaves started showing asci (Stage 1.2) by 11 April, and

asci with ascospores (stage 2.9) were observed 24 April. After apothecia reached stage 3, no changes were observed in the development of the ascocarps for 15 days. Apothecia disintegrated by 4 June, about 8 weeks after the first asci were found (Table 1). In sour cherry leaf samples, apothecia were in stage 1.5 on 24 April, 2-wk later than in sweet cherry; however apothecia on sour cherry leaves were in stage 3.2 at the same date as the apothecia in sweet cherry (Table 1). Above normal temperatures were recorded during March and rain was below normal during March and April, while above-normal rainfall was registered on May (Table 3).

At orchards 3 and 4, asci were found on 27 April, 2-wk later than at East Lansing. Apothecia matured to stage 3 on 11 and 18 May for sour and sweet cherry respectively. Apothecia disintegrated by 13 June, about 6-wk from appearance of asci (Table 1). Apothecia of B. jaapii did not overwinter well in the Traverse City area, especially in orchard 4, where very few normal apothecia were found. Above-normal temperatures and rainfall were registered during March at Traverse City, although the temperature was lower that at East Lansing. April temperatures were above normal while rainfall was below normal. During May above-normal rainfall was registered (Table 4).

Apothecia overwintering in orchard 5, showed asci (stage 1.3) on 4 May, a few days later than in orchards 3 and 4, but they quickly matured and apothecia were in stage 3.5 by

Table 1. Stage of apothecial development of the cherry leaf spot fungus (*Blumeriella jaapii*) in cherry leaves collected from two orchards near East Lansing and three orchards near Traverse City, Michigan, in 1990.

Date of sampling	Apothecia (no.)	Mean stage[a]	SD[b]	Apothecia in each stage (%)					
				St. 1	St. 2	St. 3	St. 4	St. 5	St. 6
East Lansing - sweet cherries									
April 11	41	1.3	0.3	68.3	31.7	0.0	0.0	0.0	0.0
April 24	55	2.9	0.1	0.0	14.5	83.7	1.8	0.0	0.0
May 7	12	3.3	0.4	0.0	0.0	75.0	25.0	0.0	0.0
May 21	33	4.3	1.3	0.0	0.0	0.0	81.8	15.2	3.0
June 4	21	6.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
East Lansing - tart cherries									
April 11	8	1.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
April 24	15	1.4	0.0	66.6	26.6	6.8	0.0	0.0	0.0
May 7	29	3.2	0.1	0.0	3.2	79.3	17.5	0.0	0.0
May 21	35	4.0	0.1	0.0	0.0	8.6	82.8	8.6	0.0
June 4	18	5.3	0.6	0.0	0.0	0.0	11.0	44.5	44.5
Orchard 3[c] - sweet cherries									
April 27	122	1.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
May 11	22	1.7	0.2	45.5	45.5	9.0	0.0	0.0	0.0
May 18	65	3.0	0.1	0.0	9.2	83.1	7.7	0.0	0.0
May 31	50	4.4	0.5	0.0	0.0	0.0	94.0	4.0	2.0
Orchard 4 - tart cherries									
April 27	13	1.4	0.1	61.5	38.5	0.0	0.0	0.0	0.0
May 11	15	2.5	0.1	0.0	53.3	46.7	0.0	0.0	0.0
May 18	3	4.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
June 13	2	6.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
Orchard 5 - tart cherries									
May 4	22	1.2	0.4	71.3	22.7	0.0	0.0	0.0	0.0
May 18	97	3.5	0.3	0.0	10.3	33.0	56.7	0.0	0.0
May 31	34	4.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
June 13	26	5.6	0.4	0.0	0.0	0.0	3.9	96.1	0.0
June 28	29	6.0	0.0	0.0	0.0	0.0	0.0	24.2	75.8

[a]Stages of development were: 1 = only paraphysis present in lumen of apothecium; 2 = asci formed but no ascospores present; 3 = few asci with ascospores formed; 4 = ascospores formed and apothecium open; 5 = spores discharged from most asci; and 6 = apothecium disintegrated. Each apothecium was assigned a stage classification. The resulting classification number was averaged to give a mean value for each date.

[b]Standard deviation of the mean.

[c]Orchards 3 and 4 were located near Northwest Michigan Horticultural Experimental Station. Orchard 5 was located on Old Mission Peninsula.

Table 2. Stage of apothecial development of the cherry leaf spot fungus (*Blumeriella jaapii*) in cherry leaves collected from orchards near East Lansing and Traverse City, Michigan, in 1991.

Date of sampling	Apothecia (no.)	Mean Stage[a]	SD[b]	Apothecia in each stage (%)					
				St. 1	St. 2	St. 3	St. 4	St. 5	St. 6
East Lansing - sweet cherries									
March 21	140	1.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
April 5	160	1.4	0.1	56.2	43.8	0.0	0.0	0.0	0.0
April 18	246	3.6	0.5	0.0	3.6	23.6	72.3	0.5	0.0
May 3	274	3.7	0.2	0.0	0.0	30.7	64.6	4.7	0.0
May 22	175	4.5	0.1	0.0	0.0	0.0	56.0	37.7	0.0
June 6	130	5.5	0.2	0.0	0.0	0.0	0.7	32.4	66.9
Orchard 3[c] - sweet cherries									
March 26	60	1.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
April 26	130	3.2	0.2	0.7	20.8	40.0	37.7	0.8	0.0
May 10	218	3.9	0.2	0.0	0.0	17.4	80.3	0.0	2.3
May 25	133	4.6	0.0	0.0	0.0	0.0	45.8	47.4	6.8
June 7	207	5.2	0.2	0.0	0.0	0.0	4.3	67.6	28.1
Orchard 4 - sour cherries									
March 26	30	1.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
April 26	77	1.5	0.8	71.5	14.3	6.4	7.8	0.0	0.0
May 10	144	3.8	0.1	0.0	0.7	19.4	78.5	1.4	0.0
May 25	133	4.8	0.5	0.0	0.0	1.5	33.1	54.1	11.3
June 7	40	4.9	0.1	0.0	0.0	0.0	30.0	57.5	12.5

[a]Stages of development were: 1 = only paraphysis present in lumen of apothecium; 2 = asci formed but no ascospores present; 3 = few asci with ascospores formed; 4 = ascospores formed and apothecium open; 5 = spores discharged from most asci; 6 = apothecium disintegrated. Each apothecium was assigned a stage classification. The resulting clasification number was averaged to give a mean value for each date.

[b]Standard deviation of the mean.

[c]Orchards 3 and 4 were located near Northwest Michigan Horticultural Experimental Station.

Table 3. Average temperature, total rainfall, departure from normal, and accumulated degree days for the period March through June of 1990 and 1991 at East Lansing, Michigan.

	1990		1991		Departure from normal 1990		Departure from normal 1991		Degree Days[c]	
Month	Temp[a]	Rain[b]	Temp	Rain	Temp	Rain	Temp	Rain	1990	1991
March	2.7	36.5	3.8	70.0	4.0	-0.9	4.6	1.5	80.0	70.0
April	8.4	41.6	10.1	106.3	1.5	-0.5	3.9	1.6	173.0	195.0
May	12.4	81.5	17.7	42.3	-3.1	0.9	6.5	-0.8	263.6	432.0
June[d]	16.0	3.5	19.7	18.3	-0.3	-1.0	2.5	-0.9	62.0	95.0

[a] Temperature C.
[b] Millimeters.
[c] Base temperature 3.9 C.
[d] First 6 days.

Table 4. Average temperature, total rainfall, departure from normal, and accumulated degree days for the period March through June of 1990 and 1991 at orchards near Traverse City, Michigan.

	1990		1991		Departure from normal 1990		Departure from normal 1991		Degree Days[c]	
Month	Temp[a]	Rain[b]	Temp	Rain	Temp	Rain	Temp	Rain	1990	1991
March	0.0	63.3	1.0	85.0	5.3	0.3	3.9	1.1	53.1	21.0
April	7.6	42.3	8.5	84.8	3.0	-0.8	2.7	0.9	158.0	153.0
May	11.0	86.5	15.6	72.0	-1.8	1.6	4.7	1.7	220.8	357.8
June[d]	17.5	78.0	18.7	13.3	0.6	-0.5	2.8	-1.4	230.3	148.1

[a]Temperature C.
[b]Millimeters.
[c]Base temperature 3.9 C.
[d]First 10 days.

18 May. Apothecia disintegrated by 28 June (Table 1).

During spring 1991 at East Lansing apothecia were in stage 1.4 on 5 April, and apothecia matured to stage 3.6 by 18 April. Apothecia disintegrated by 6 June (Table 2). Above-normal temperatures and rainfall was registered during March and April. Below-normal rainfall was registered during middle of May and early June (Table 3).

Apothecial development at the Traverse City area occurred approximately 15 days later than at East Lansing, and apothecia in sweet cherry leaves were mature (stage 3.2) by 26 April (Table 2). Apothecia did not overwinter well at Traverse City area, especially in orchard 4. Above normal temperatures were registered during March - June and rainfall was above-normal except for June (Table 4).

In each sampling date, the stage of maturity between apothecia was similar, as shown by the standard deviation of the samples (Tables 1,2). However, not all the asci in the same apothecium matured at the same time.

Ascospore discharge. There was little difference in the initiation date of ascospore discharge from apothecia on leaves of sweet and sour cherry between different locations (Figs. 8,12). During 1990 at East Lansing, the first ascospores were trapped on 5 and 11 May in sweet and sour cherry, respectively. In Traverse City area, the first discharge was register on 11 May in orchard 3 and on 10 May in orchard 4. In orchard 5, the first spores were caught on 18 May. The peak of ascospore discharge was similar for

East Lansing and Traverce City orchards (Figs. 8 - 10). During 1991, the first ascospore discharge was on 20 April for East Lansing and on 29 April for Traverse City. However, the peak of discharge of ascospores was a few days earlier at Traverse City than at East Lansing (Figs. 11,12).

Ascopores were not caught in the spore traps before apothecia reached stage 3 of development (Figs. 8 - 12). The discharge of ascospores started before bloom and continued for about 4 - 7 weeks for both 1990 and 1991. The major portion of the ascospores were discharged after petal fall until fruit set (Figs. 8 - 12).

Infection periods. During 1990, according to the EFI, there were four infection periods at Traverse City: on 8, 15, 16 May; and 2 June. The first lesion were seen on 8 June (Figs. 9,10). During 1991, leaf spot infection periods were identified as 6, 17, 25, 28 May; and 2 June. The first lesions were discovered on 25 May and were confined to bracht leaves around the flower cluster, indicating that infection had occurred earlier than normal (Fig. 12).

Isolation of *P. padi* and *R. jaapii*. Conidia of *C. padi* germinated slowly in agar. After 24 h germs tubes could only be detected under the dissecting microscope at high magnification (X 50). The mycelium also grew very slowly and it was not apparent to the unaided eye until after 20 days. At 30 days, colonies appeared as small whitish specks. When colonies were examined microscopically, they consisted of stromata covered with conidia. The conidia

Fig. 8. Mean stage of apothecial development and ascospore discharge of Blumeriella jaapii from March to 5 June 1990 at East Lansing, Michigan, compared with daily degree days and rain.

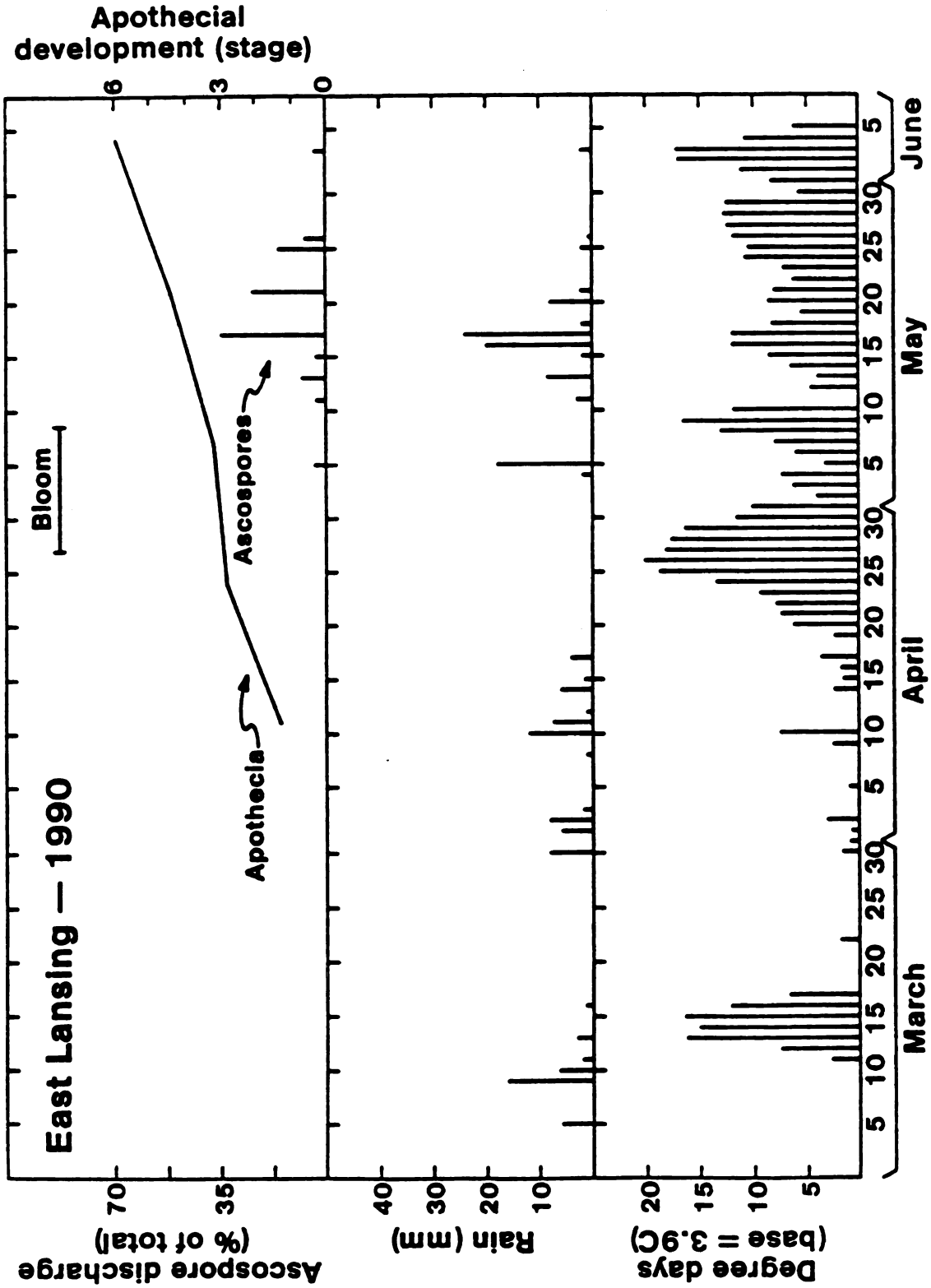

Apothecial development (stage)
6
3
0
East Lansing — 1990
Bloom
Ascospores
Apothecia
70
35
Ascospore discharge (% of total)
40
30
20
10
Rain (mm)
20
15
10
5
Degree days (base = 3.9C)
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5
March
April
May
June

Fig. 9. Mean stage of apothecial development and ascospore discharge of *Blumeriella jaapii* from March to 8 June 1990 at Traverse City, Michigan, compared with daily degree days and rain.

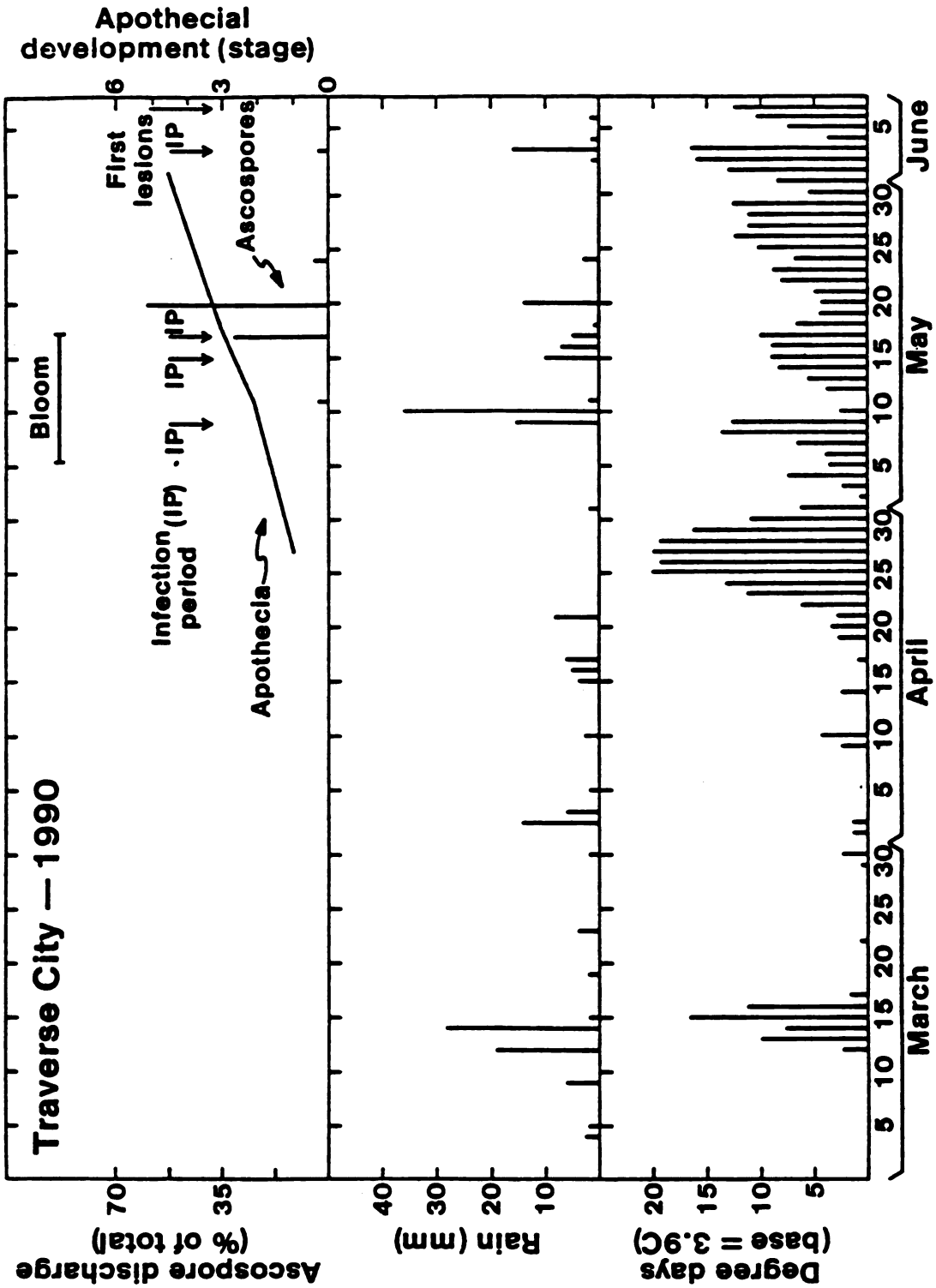

Apothecial development (stage)
6
3
0
First lesions
IP
Ascospores
Bloom
Infection period (IP)
IP
IP
IP
Apothecia
Traverse City — 1990
70
35
Ascospore discharge (% of total)
40
30
20
10
Rain (mm)
20
15
10
5
Degree days (base = 3.9C)
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5
March
April
May
June

Fig. 10. Mean stage of apothecial development and ascospore discharge of Blumeriella jaapii from April to 8 July 1990 at Old Mission Peninsula, Michigan, compared with daily degree days and rain.

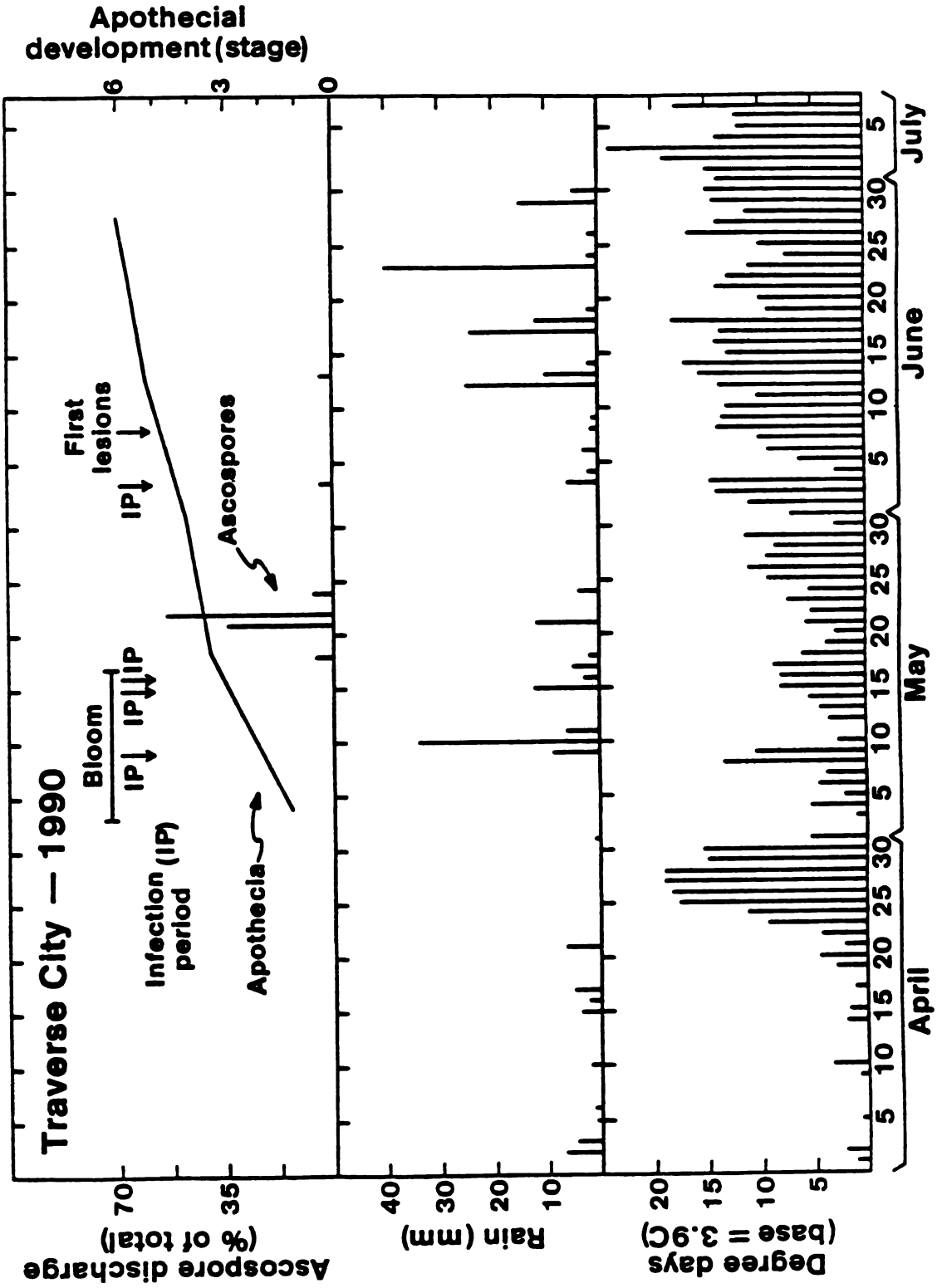

Apothecial development (stage)
6
3
0
Traverse City — 1990
Bloom
IP
IP
IP
Infection period (IP)
First lesions
IP
Ascospores
Apothecia
70
35
Ascospore discharge (% of total)
40
30
20
10
Rain (mm)
20
15
10
5
Degree days (base = 3.9C)
April
May
June
July
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5

Fig. 11. Mean stage of apothecial development and ascopore discharge of Blumeriella jaapii from March to 8 June 1991 at East Lansing, Michigan, compared with daily degree days and rain.

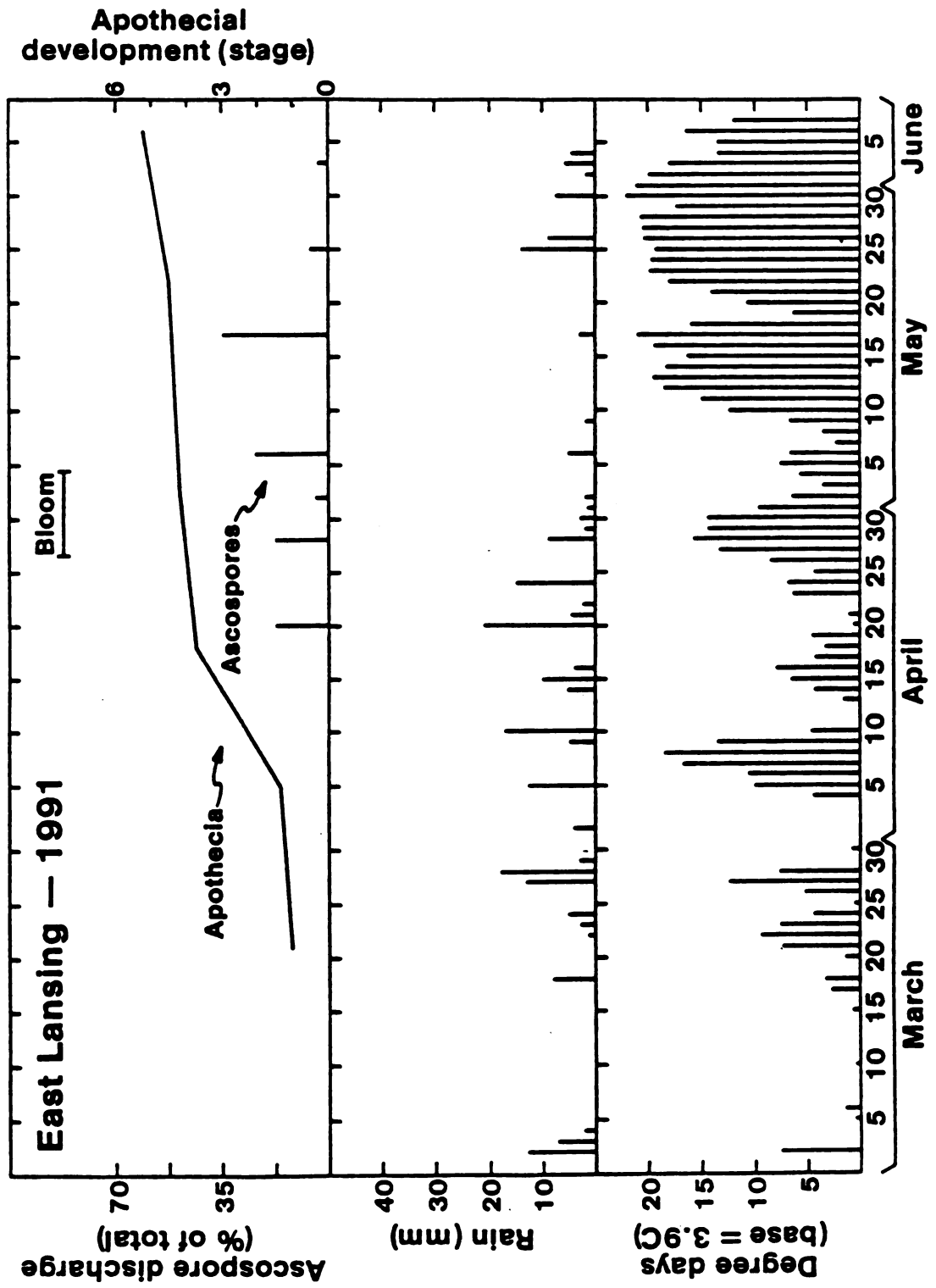
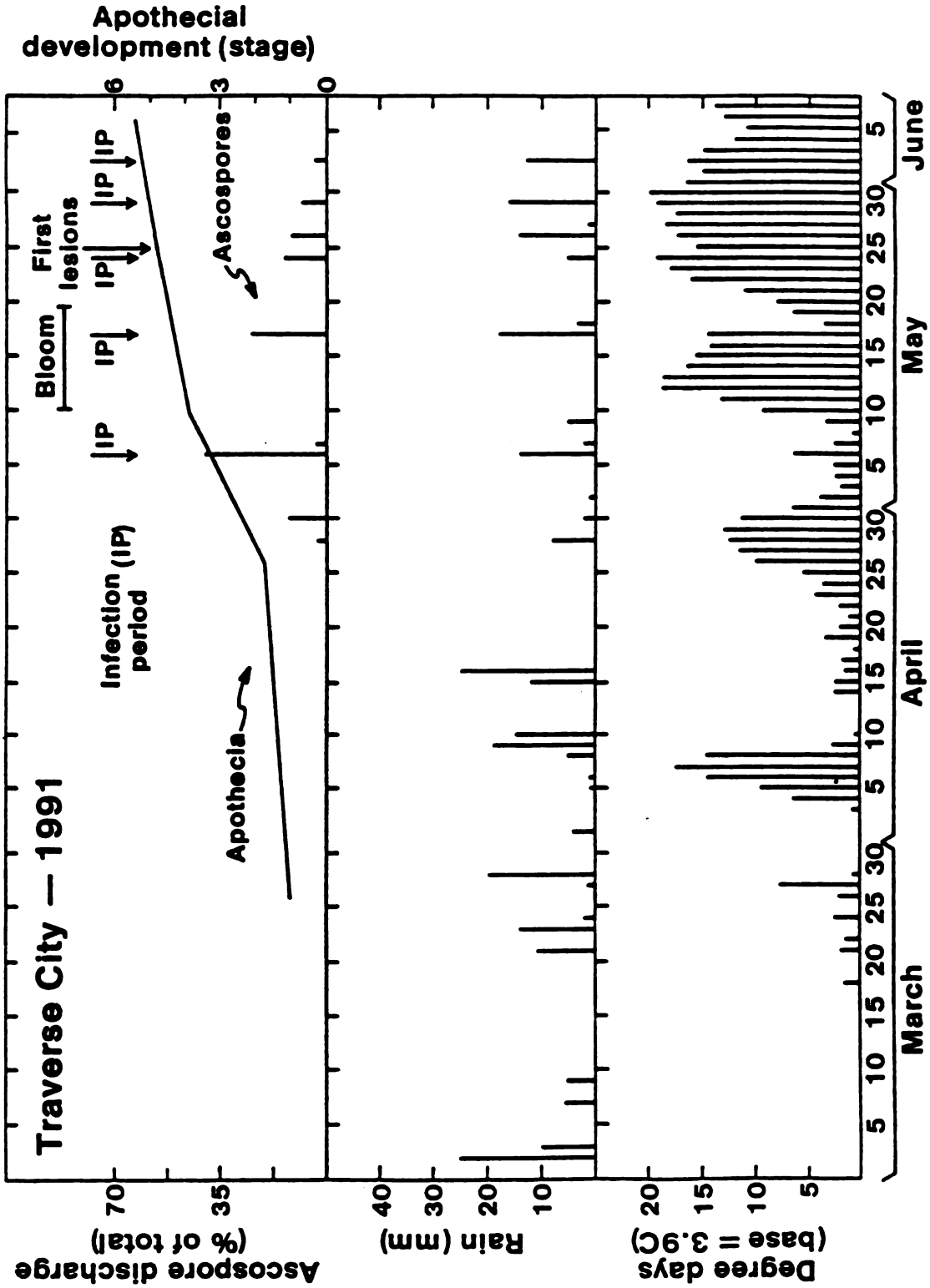

Apothecial development (stage)
6
3
0
East Lansing — 1991
Bloom
Apothecia
Ascospores
70
35
Ascospore discharge (% of total)
40
30
20
10
Rain (mm)
20
15
10
5
Degree days (base = 3.9C)
March
April
May
June
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5

Fig. 12. Mean stage of apothecial development and ascospore discharge of Blumeriella jaapii from March to 8 June 1991 at Traverse City, Michigan, compared with daily degree days and rain.


Apothecial development (stage)
Traverse City — 1991
Bloom
First lesions
IP
Infection period (IP)
Apothecia
Ascospores
Ascospore discharge (% of total)
Rain (mm)
Degree days (base = 3.9C)
March
April
May
June

conidia were elongated, curved or flexuous, continuous or 1 - 2 septate. These characteristics agree with the description of the conidia of P. padi described by Higgins (4). The stroma grew slowly, enlarging until a hemispherical mass 0.5-1 cm in diameter was formed, which occured in about 2 month. Before this time the stroma turned darker in color, some being dark brown while other isolates were creamy to brown. The appearance of the stromata was floccose. Isolates from ascospores of P. jaapii and summer conidia of P. padi showed the same characteristics described above.

DISCUSSION

The results obtained in these studies confirm early findings reported by Backus (1), Higgins (4), and Keitt (11) with respect to the epidemiology of the cherry leaf spot fungus. In Michigan this is the first time that development of the apothecia and its relationship with environmental factors has been documented. Temperature and rainfall have an important effect in the three phases of apothecial ontogeny. During the first phase, apothecial development is retarded by low temperatures. Mild temperatures during March 1990-91 at East Lansing, accelerated development of apothecia and asci were formed during the the first days of April. Low temperatures occurring during the same period at Traverse City delayed

the appearance of the first asci by 2-wk, even though the amount of rainfall for that period was higher at Traverse City. These results confirm observations of Higgins (4) and Backus (1) that mild temperatures during late winter, and early spring accelerate the appearance of the first asci. When temperature is not limiting, rainfall could accelerate the development of the apothecia. During March 1991 at East Lansing above-normal rainfall was registered and the first asci were found earlier than the previous year.

During the second phase of apothecial development rain appeared to be the limiting factor. During April 1991 above-normal rainfall was registered at East Lansing and apothecia matured to stage 4 almost 1-mo earlier than in 1990. A similar pattern was observed for Traverse City. During April- May 1991 rainfall was higher than in 1990 and apothecia matured to stage 4 about 20 days earlier than in 1990. Keitt (11) indicated that moisture and temperature were cardinal factors in relation to the time of maturity of ascocarps, and their development was sharply arrested when the leaves were air dried, even at a comparatively high humidity and favorable temperature. Spring rains are therefore of great importance in relation to maturation of the ascospores.

After apothecia reached maturity (stage 3) they were capable of rapid development under periods of favorable temperature and moisture and apothecia disintegrated in about 4 - 5 wk. As temperature is usually not limiting

during this time of the year, rainfall has a major influence, especially May rains. During May 1990 above-normal rainfall occured at East Lansing and apothecia disintegrated in 4-wks. Meanwhile, in 1991, apothecia disintegrated about 7-wk after stage 3, even though the total amount of rainfall after that stage was higher than in 1990. Rains were concentrated at the end of April and at the beginning of June.

During the winters of 1990 and 1991, apothecia in orchards at Traverse City, especially those at orchard 4, did not overwinter well, and few normal apothecia were formed. As the leaf samples were taken close to orchards that had been treated during the previous season with fungicides with postinfection activity, the problem of poor overwintering could be due to inhibitory effects of the fungicides on the formation of asci.

During this 2-yr study, ascospores were mature before cherry leaves had reached their susceptible stage. The first discharge started before bloom and continued for about 6-wk and the highest numbers of ascospores discharged were between petal fall and fruit set.

Apothecia in the same leaf did not differ greatly in their stage of maturity; however, not all the asci in the same apothecia matured at the same time, but furnished the material for a number of successive discharge periods. These observation agree with the findings done by Backus (2) and Keitt (11).

Although apothecial development was initiated earlier in East Lansing than in Traverse City, the discharge of ascospores occurred on similar dates for both areas and cultivars. Discharge of ascospore was always associated with rains. During 1990 the lack of rain after the ascospores were mature delayed the first discharge of ascospores at East Lansing and Traverse City.

None of the infection periods occurred early in the season during 1990, caused infection because the amount of susceptible tissue at this time was very low or absent. Infection periods predicted to occur during the peak of discharge of ascospores did not resulted in actual infections. This might be due to low temperatures occuring during this period. According to Keitt (11), low temperatures are an important factor in the suppression of primary infection. They lengthen the moist period requisite for infection, retard the ascospore discharge or even preclude infection notwithstanding favorable condition of moisture.

Conidia in acervuli found in overwintering leaves were identified as P. Padi. This results confirm early finding of Higgins (4) and Kaszonyi (10). We do not know the role that spring conidia play in primary infection. In Hungary, Veghelyi (13) reported that during very cold and dry winters few apothecia were formed and primary infection was mainly induced by spring conidia. Further studies to clarify the role of spring conidia in the epidemiology of

the cherry leaf spot fungus in Michigan should be conducted.

Currently, control of the cherry leaf spot is based in fungicide applications on a calendar schedule, starting when the first susceptible tissue is present. This implies that in some years unnecessary sprays may be applied because no ascospores are available for discharge. Timing fungicide applications to the actual threat of infection reduces the number of sprays per season without reducing efficacy of control measure (2,9).

Data obtained in this study would be analyzed in order to develop a predictive model for ascospore maturation that could be incorporated into the model developed by Eisensmith et al (3) that predict infection period of the cherry leaf spot fungus.

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PART II

INFLUENCE OF TEMPERATURE ON APOTHECIAL DEVELOPMENT AND ASCOSPORE DISCHARGE OF *Blumeriella jaapii*

ABSTRACT

Cherry leaves bearing apothecia of Blumeriella jaapii with either immature or mature asci were collected from a sweet cherry (Prunus avium) orchard near East Lansing, MI, for experiments on apothecial development or ascospore discharge, respectively. Disks cut from the leaves were incubated at six temperatures in experiments on apothecial development (4 - 24 C), and on ascospore discharge (8 to 30 C). Non-linear regression analyses were significant ($P > 0.05$) and quadratic for number of asci per apothecium and for the percentage of mature plus discharged asci after 7 days of incubation. Temperature affected the number and maturation of asci and discharge of ascospores. The number of asci per apothecium was highest at 16 C, and ascus maturation was highest at 16 - 20 C. Temperatures of 4 and 24 C were unfavorable for apothecial development. The percentage of ascospores discharged from apothecia increased with increasing temperature over a range of 8 - 30 C.

INTRODUCTION

Cherry leaf spot, caused by Blumeriella jaapii (Rehm) Arx, is a major disease of sour and sweet cherry (Prunus cerasus L. and P. avium L.) throughout Michigan's cherry-growing region. The fungus overwinters in infected leaves as stroma-like structures that develop in spring into apothecia (1,4). The apothecia then mature and ascospores are available for discharge during the blossoming period of the cherry trees. Ascospore discharge occurs when the leaves are wet by rain. The danger of primary infection continues until the supply of ascospores is exhausted, usually by mid-summer.

Extensive spread of the disease in late spring and summer is caused by the conidial stage of B. jaapii. Control is based on the use of fungicide sprays during the primary and secondary disease cycle based on a calendar schedule. Fungicide spray programs are initiated when the first susceptible leaf tissue is present at about the petal fall stage of blossom bud development (7).

Conditions of temperature and moisture in spring govern the rate of development of apothecia (1,4,8,9). If temperatures are 15 - 21 C and the overwintering leaves are wet for 1 to 2 days, the stroma develops rapidly and

apothecia are produced in 24 to 48 h (8). Moisture and temperature are also cardinal factors in maturity of apothecia and discharge of ascospores. Periods of ascospore discharge begin when ascocarps are thoroughly wet. Discharge may occur at temperatures ranging from 1 to 36 C but discharge is greatest at temperatures above 16 C, less rapid at 12 C, and rare at 4 to 8 C (9).

Precise knowledge of ascospore maturity is important in disease management because fungicide sprays should be timed to coincide with ascospore discharge. Several approaches that are used to evaluate the progress of ascospore maturation in pseudothecia of Venturia inaequalis, such as microscopic examination of crushed pseudothecia or the quantification of ascospores collected in spore traps (5,10) should be useful for evaluating ascospore maturation of B. jaapii. However, these methods for determining ascospore maturation and discharge are time-consuming and labor-intensive. A better understanding of the effects of temperature on apothecia development and ascospore discharge is needed to be able to develop a model for predicting ascospore maturity and discharge.

The objective of this study was to determine the effects of temperature on apothecial development and ascospore discharge.

MATERIALS AND METHODS

Effect of temperature on apothecial development. Sweet cherry leaves with immature apothecia of B. jaapii were collected on 4 April 1991 from beneath trees of the cultivar Hedelfingen in an orchard at the Botany and Plant Pathology Research Farm, Michigan State University, East Lansing. Disks, 1-cm in diameter, were cut from the leaves with a cork borer. Moist cellulose sponges were placed in the bottom of 12 plastic boxes (12.5 x 8 x 5.5 cm) and 10 leaf disks were placed on the surface of the sponge in each box. Moisture from the sponges was adequate to maintain the leaf disks in a pliable condition without watersoaking throughout the experiment. The boxes were enclosed in plastic bags and then placed in incubators in the dark at 4, 8, 12, 16, 20, or 24 C.

At weekly intervals, a piece of leaf tissue bearing apothecia was removed from each of the 10 disks at each temperature and fixed in a mixture of 2-propanol, water, propionic acid, and formaldehyde (45:45:5:5, v/v) (FPP). The leaf tissue was examined with the aid of a dissecting microscope (X25). Two apothecia near the center of the microscope field were removed with a dissecting needle, transferred to a drop of cotton blue-lactophenol on a glass slide, crushed, and examined with a compound microscope (X200). A total of 20 apothecia were examined for each

incubation temperature.

Asci in each apothecium were rated individually based on their stage of maturity as follows: immature = no ascospores present; mature = some to all ascospores delimited; and discharged = ascospores not present and the apex of the ascus open (Fig. 1&2). The number of asci in each maturity class was determined weekly and the results presented as a percentage of the total number of asci per apothecium. Weekly observations were continued until the apothecia disintegrated.

Effect of temperature on ascospore discharge. Overwintered leaves with mature apothecia were collected on 26 April and 5 May from the orchard used in the first experiment. Leaf disks were cut with a cork borer and 20 disks were placed in each box as described above. The boxes enclosed in plastic bags were held at room temperature for 2 h. The disks were transferred to 250 ml beakers coated with dichlorodimethysilane to prevent ascospores from sticking to the inside surface of the beakers. The beakers contained 75 ml of distilled water adjusted to 8, 12, 16, 20, 24, and 30 C. Temperature controlled air from within each incubator was bubbled through the water in the corresponding beaker for 2 h with a Dinatomic Hi-Tech P-310 pump (Ethical Product, Inc., 216 First Avenue, Newark, NJ), the disks were removed from the beakers, and the water along with the water used for rinsing the beakers was filtered through a 47-mm cellulose acetate grid filter of 0.8-um pore size (Millipore

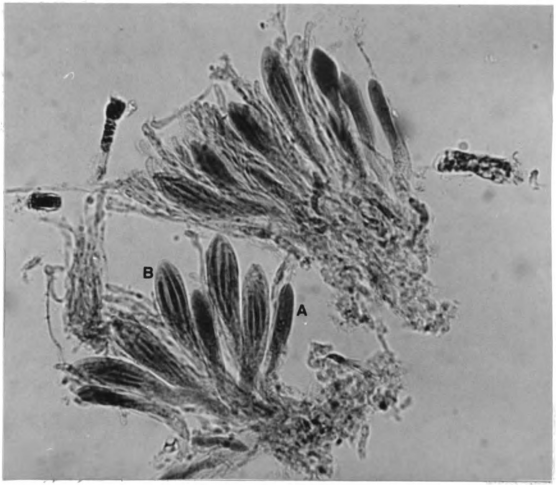


Fig. 1. Maturity classes of asci of Blumeriella jaapii. **(A)** immature asci = no ascospores present and **(B)** mature = some to all ascospores delimited.

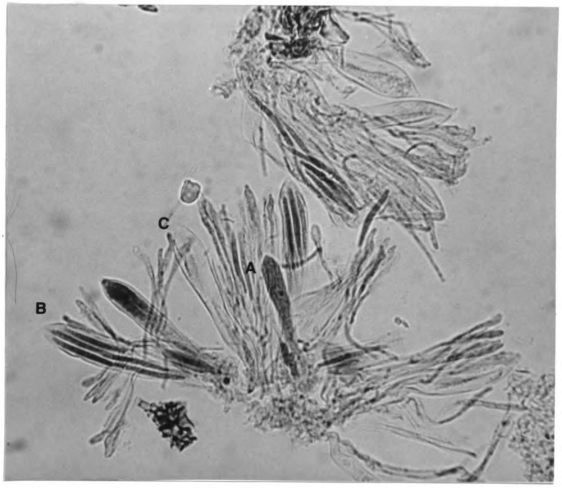


Fig. 2. Maturity classes of asci of Blumeriella jaapii. (A) immature asci = no ascospores present (B) mature = some to all ascospores delimited and (C) discharged = ascospores not present and the apex of the ascus open.

Corporation, Belford, MA). Each filter was cut in half and the halves were mounted in cotton-blue lactophenol on microscope slides. Ascospores were counted in five grid-squares chosen at random on each half filter. In order to compare data between experiments, the replication with the highest number of ascospore in each experiment was set at 100 ascospores. Then the data for the remaining replications in the experiment were adjusted proportionally, and the results expressed as a percentage.

Statistical analyses. The experiments were conducted in a completely randomized design with two replications for each temperature treatment. The experiment on apothecial development was repeated two times, the one on ascospore discharge three times. Data were analyzed using non-linear regression in Systat, Wilkinson, Leland. SYSTAT: The System for Statitics. Evanston, IL. SYSTAT, Inc., 1990.

RESULTS AND DISCUSSION

Effect of temperature on apothecial development. Development of apothecia varied with temperature. When the experiment was initiated, there was a mean of 13.7 asci per apothecium and 99.8% of the asci were immature. After 7 days of incubation, there were 24.1 - 105.8 asci per apothecium and the highest numbers of asci were observed in apothecia maintained at 16 C (Table 1). The optimum temperature range for maturation of the asci was 16 - 20 C.

At these temperatures, 75.1 - 86.7% of the asci either contained mature ascospores or had discharged their ascospores. Apothecia at 16 C had the highest rate of increase in the number of asci and those at 20 C had the highest rate of change in maturation of the asci (Table 1).

The number of asci per apothecium and changes in stages of maturity of the asci after 7 days of incubation were significantly ($P > 0.05$) affected by temperature. The relationship between temperature (X) and apothecial development (Y) was non-linear and best described by quadratic regression equations (Fig. 3). The regressions accounted for 93.4% of the variation in the number of asci per apothecium and for 73.7% of the variation in the percent of mature (including discharged) asci per apothecium.

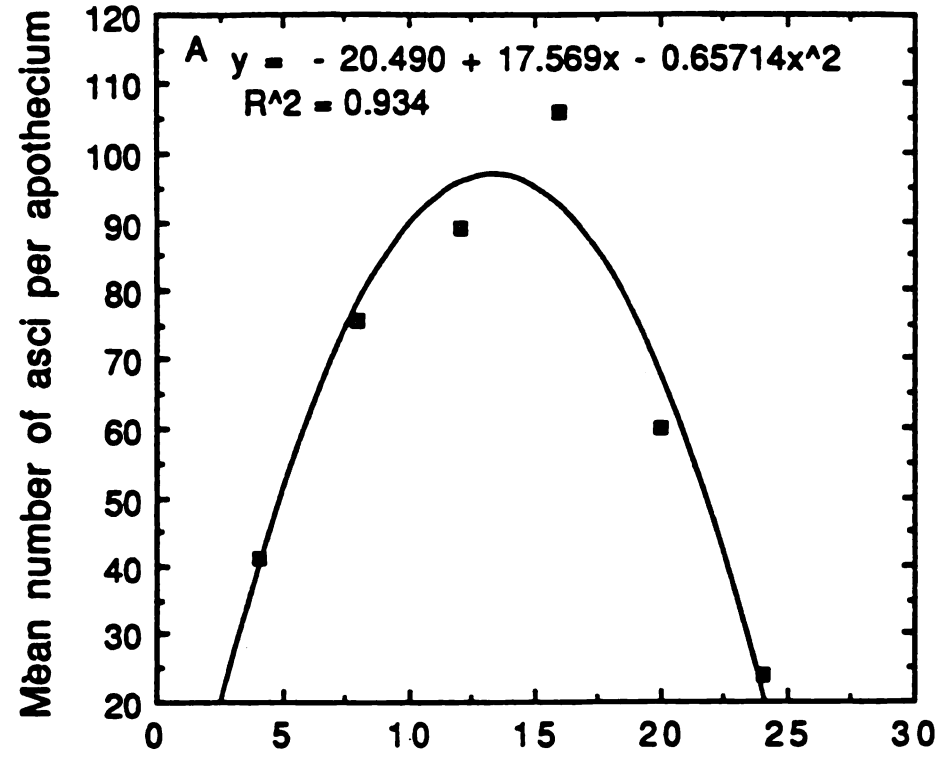
When leaf disks were held at the respective temperatures for an additional 35 days, moisture from the sponges was adequate for ascospore release and eventual exhaustion of the apothecia. Apothecia incubated at 16 or 20 C developed quickly; most of the ascospores were discharged by day 14 and many of the apothecia had started to disintegrate (Fig. 4A). Discharged asci were difficult to count because they failed to stain or were lost in the process of preparing the samples for microscopic examination. High temperature and high humidity accelerated the disintegration not only of the apothecia but also of the leaf itself. Keitt et al (8) found that when apothecia kept were under optimum conditions of moisture and temperature the ascospores were discharged

Table 1. Effect of temperature on apothecial development of Blumeriella jaapii after 7 days of incubation.

Temperature (C)	Asci per apothecium (no.)[a]	SD[b]	Rate of change (asci/day)[c]	Mature + discharged asci (%)[d]	SD	Rate change (asci/day)
4	41.4	6.6	4.0	11.5	7.6	1.6
8	75.5	5.0	8.8	30.7	3.6	4.4
12	89.1	5.6	10.8	49.7	9.3	7.1
16	105.8	3.6	13.2	75.1	1.3	10.7
20	60.0	15.6	6.6	86.7	5.1	12.4
24	24.1	10.5	1.5	24.9	4.0	3.5

[a]Each value is the mean of 20 apothecia per repetition. Data are the result of one experiment. The other experiment gave similar result.
[b]Standard deviation of the mean.
[c]The number of asci per apothecium after 7 days of incubation minus 13.7 (number of asci per apothecium at the beginning of the experiment) divided by 7.
[d]Asci in each of the 20 apothecia were rated individually based on their stage of maturity as follows: immature (no ascospore present); mature (some or all of the ascospores were delimited); discharged (no ascospore present and the ascus apex broken). Data are expressed as a percentage of the number of asci per apothecium.
[e]The percentage of mature + discharged asci after 7 days of incubation minus 0.2 (percentage of mature + discharged asci at the beginning of the experiment) divided by 7.

Fig. 3. Non-linear relationships of the effect of temperature 7 days after incubation on (A) the number of asci per apothecium and (B) the percentage of mature asci (including discharged) asci per apothecium of Blumeriella jaapii. Data are the result of one experiment. The other experiment gave similar result.


A y = - 20.490 + 17.569x - 0.65714x^2
R^2 = 0.934
Mean number of asci per apothecium
120
110
100
90
80
70
60
50
40
30
20
0
5
10
15
20
25
30

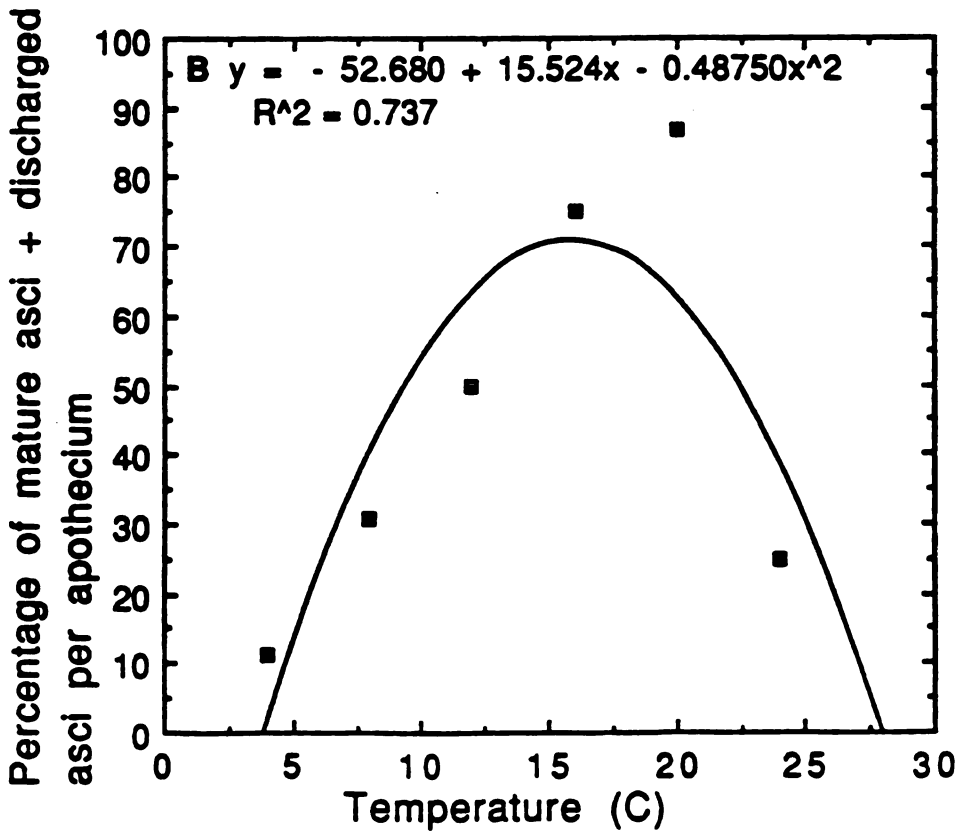
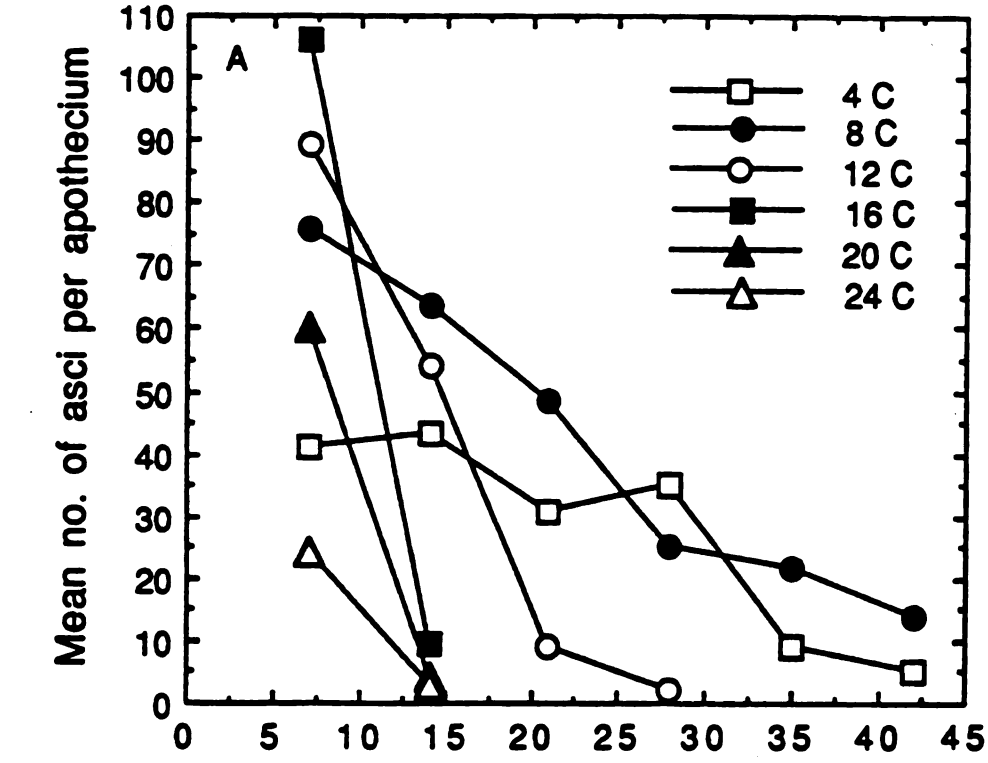
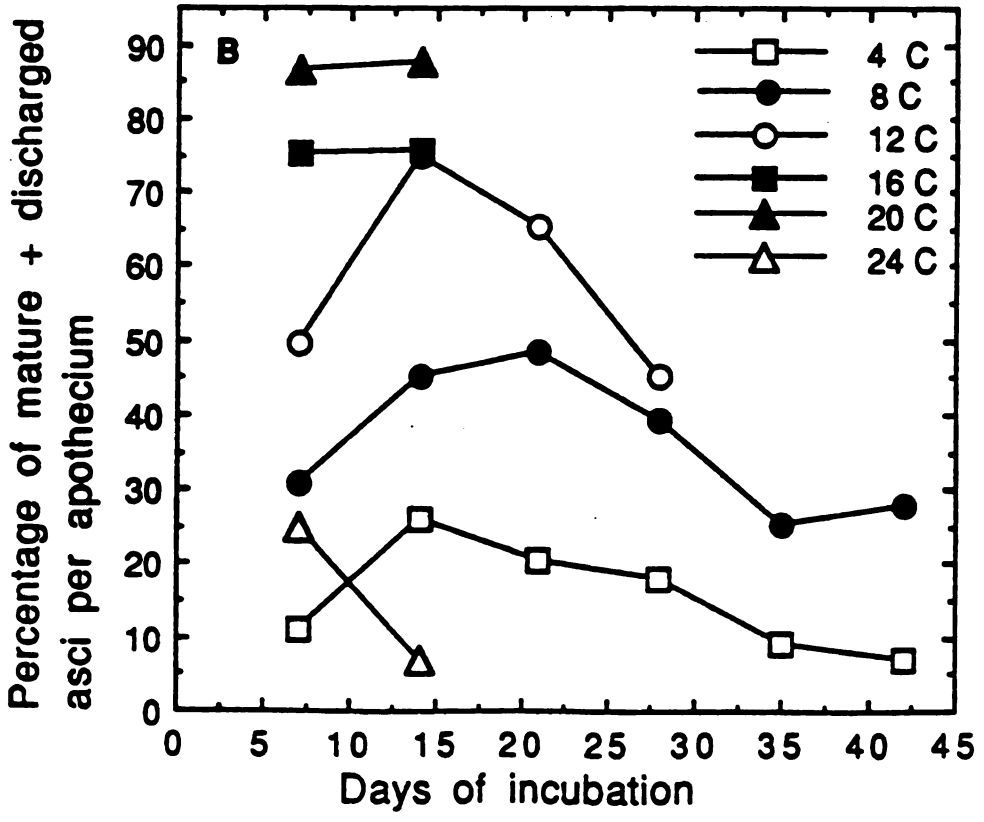

B y = - 52.680 + 15.524x - 0.48750x^2
R^2 = 0.737
Percentage of mature asci + discharged asci per apothecium
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Temperature (C)

Fig. 4. Influence of temperature over 42 days on the maturation of apothecia of Blumeriella jaapii. The apothecia were evaluated by assessing (A) the percentage of mature (including discharged) asci per apothecium. Values are means of 20 apothecia. Data are the result of one experiment. The other experiment gave similar results.


A
Mean no. of asci per apothecium
110
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45
4 C
8 C
12 C
16 C
20 C
24 C


B
Percentage of mature + discharged asci per apothecium
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45
Days of incubation
4 C
8 C
12 C
16 C
20 C
24 C

until ascocarps were exhausted. During springs with wet and warm weather most of the spores might be discharged in 2 or 3 weeks period, and therefore the length of the primary infection period would be short.

Apothecia incubated at 4, 8, and 12 C contained mature and/or discharged asci for up to 42, 42, and 28 days, respectively, before they disintegrated. Productivity of the apothecia increased with temperature from 4 - 12 C (Fig. 4). At 4 C most of the asci failed to mature. There was little or no increase in the size of the asci nor were ascospores delimited in the asci. By day 21, some asci were distorted and others were observed to contain large vacuoles (Fig. 5). Distorted asci were also observed at 12 and 8 C, but they were observed earlier in the incubation period. Many small atypical apothecia were observed among the apothecia that formed at 24 C. Some failed to form paraphyses while others formed paraphyses that were shorter than normal and lacked the enlarged, hooked apex typical of paraphyses observed in apothecia that developed at lower temperatures. Apothecia maintained at 24 C produced very few ascospores and disintegrated in 2 wk.

Additional studies are needed to establish if apothecia containing asci with vacuoles and distorted paraphyses have lost the ability to develop normally when they are switched to optimum conditions for apothecial development. If they do not resume normal development, the amount of primary inoculum may be reduced in years with abnormally cold, wet

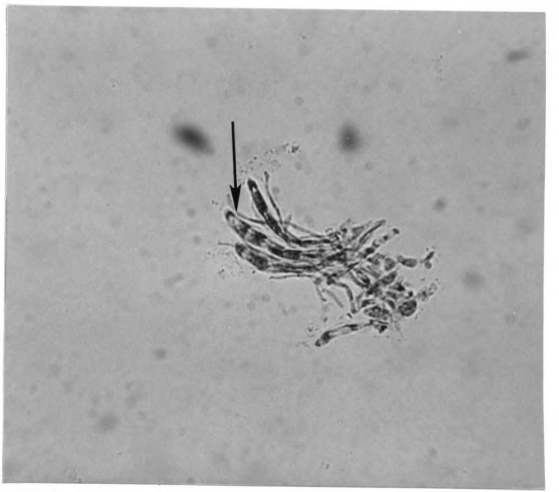


Fig. 5. Asci of Blumeriella jaapii showing large vacuoles after 21 days incubation of apothecia at 4 C.

weather in spring.

Effect of temperature on ascospore discharge. Ascospore discharge occurred at all temperatures tested, but the percentage of ascospores discharged increased as the temperature of the water that held the disks was increased (Fig. 6). The relationship between percentage of ascospores discharged and temperature during the discharge period was best described by an exponential equation. The equation was: $Y = 17.586 \times 10^{(2.22570\ e-2x)}$, in which Y is an estimate of the percentage of ascospores discharged and X is temperature from 8 to 30 C. The regression accounted for 90% of the variation in the percentage of ascospores discharged. These results confirm the early work of Keitt et al (9) that temperature influences the release of ascospores and that temperatures of 16 C and above favor abundant discharge.

There was considerable variation between replications in the number of ascospores discharged. This variation may have been due to differences in maturation between apothecia on the same leaf, in maturation of asci between apothecia of similar maturity, and in the number of apothecia per disk. Even though efforts were made to select leaf disks with a similar number of apothecia for all the temperature treatments, the exact number of apothecia per disk was not determined.

There were other factors beside temperature and maturity of the apothecia such as relative wetness which could have

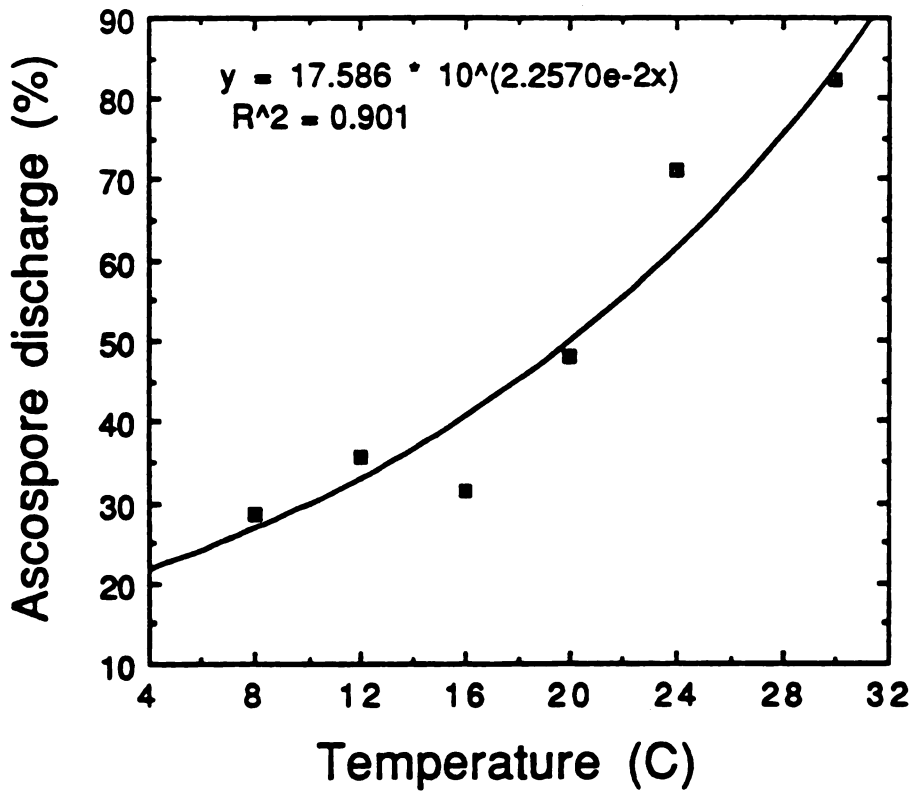


Fig. 6. Relationship of the effect of temperature on discharge of ascospores of Blumeriella jaapii. The replication with the highest number of ascospores discharged in each experiment was set at 100 ascospores, then the data for the remaining replications in the experiment were adjusted proportionally. The results are expressed as a percentage and each value is the mean of three experiment

affected release of the ascospores. In these experiments the apothecia were kept in water throughout the 2 h period. Although Keitt et al (9) reported that ascospore release started once physiologically mature apothecia were thoroughly wet, they observed that the release was highest when apothecia were drying at the end of the wet period. Further experiments are needed to define the effect of drying of apothecia in ascospore release. If ascospore release is accelerated by drying, then interrupted wet periods may be more favorable for ascospore discharge than a continuous wet period.

The data obtained in this study could be the basis for developing a model to predict maturity of ascospores and potential inoculum dosage. Eisensmith et al (3) developed a model that predicted infection periods of B. jaapii, using the assumption that inoculum is always present, which may not be true. As we reported in this paper, low temperature delays the maturation of the ascospores and reduces the number of ascospores discharged in physiologically mature apothecia. Therefore a model is needed that combines ascospore maturation and discharge with infection period predictions. This will allow management of the disease based on the use of fungicides with post-infection activity (2,6,11).

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APPENDICES

APPENDIX A

HISTOLOGY PROCEDURES

DEHYDRATION PROCEDURE

Leaf disks were immerse in the following solutions:

1. 50% Ethol for 2 h.
2. Solution 1 for 2 h.
3. Solution 3 for 2 h.
4. Solution 4 for 1 h.
5. Solution 5 for 1 h.
6. 100% TBA for 6 h (or overnight).
7. 100% TBA for 6 h.
8. 100% TBA overnight.

The following steps should be done in the oven.

9. Add 20% Paraffin + 80% TBA for 12 h.
10. Add 20% Paraffin to previous solution let in oven for 12 h.
11. Add 20% Paraffin to previous solution and let in oven for 12 h.
12. Add 20% Paraffin. Leave the tubes overnight with the cup off.
13. 100% Paraffin for 12 h.
14. 100% Paraffin for 12 h.

SOLUTIONS USED

Solution 1. 50% distill water
40% Ethanol (95%)
10% TBA (tert-Butyl Alcohol)

Solution 2. 30% distill water
50% Ethanol (95%)
20% TBA

Solution 3. 15% distill water
50% Ethanol (95%)
35% TBA

Solution 4. 45% Ethanol
55% TBA

Solution 5. 75% TBA
25% Ethanol (100%)

TRIARCH QUADRUPLE STAIN

1.	XYLENE	5 min.
2.	XYLENE	5 min.
3.	XYLENE: abs. ETOH (1:1)	5 min.
4.	95% ETOH	5 min.
5.	70% ETOH	5 min.
6.	1% Safranin in 50% ETOH	5 min.
7.	Water wash	rinse (6 - 7 times)
8.	1% Aqueous Crystal Violet	1 - 2 min.
9.	Water wash	rinse
10.	100% ETOH	30 sec.
11.	100% ETOH	30 sec.
12.	Orange G - Fast Green (135ml - 15ml)	3 mon.
13.	Orange G - Fast Green (145ml - 5ml)	3 min.
14.	Orange G - Fast Green (148ml - 2ml)	2 min.
15.	Orange G - Fast Green (150ml - 0)	2 min.
16.	100% ETOH	1 min.
17.	XYLENE	5 min.
18.	XYLENE	5 min.

Preparation of the dyes

1% Safranin in 50% ETOH. 1 gram Safranin in 100 ml 50% ETOH. Filter.

0.4% Orange G in Clove Oil. 0.4 grams of Orange G in 100 ml of clove oil. Filter.

1% Fast Green in Absolute ETOH. 1 gram of Fast Green in 100ml of absolute ETOH. Filter.

Modifications. Steps 8 and 9 were omitted, and clove oil was substituted by Wintergreen oil. This is not recommended because the quality of the stain is reduced.

APPENDIX B

EXPERIMENTAL DATA

EFFECT OF TEMPERATURE ON APOTHECIAL DEVELOPMENT

Experiment 1.

Rep.	Temp.	Time	Asci No.	% mature + discharge asci
1.00	1.00	1.00	11.500	2.610
2.00	1.00	1.00	20.900	24.400
1.00	2.00	1.00	106.200	49.600
2.00	2.00	1.00	94.400	39.300
1.00	3.00	1.00	40.400	45.000
2.00	3.00	1.00	91.200	48.800
1.00	4.00	1.00	52.200	72.800
2.00	4.00	1.00	79.000	65.780
1.00	5.00	1.00	59.000	84.320
2.00	5.00	1.00	47.500	80.080
1.00	6.00	1.00	19.400	46.820
2.00	6.00	1.00	16.000	52.400
1.00	1.00	2.00	98.300	22.310
2.00	1.00	2.00	52.500	28.590
1.00	2.00	2.00	133.000	43.450
2.00	2.00	2.00	138.100	45.620
1.00	3.00	2.00	114.700	64.760
2.00	3.00	2.00	75.500	49.700
1.00	4.00	2.00	10.800	84.300
2.00	4.00	2.00	12.700	69.330
1.00	5.00	2.00	4.000	47.200
2.00	5.00	2.00	0.010	80.080
1.00	6.00	2.00	7.000	57.700
2.00	6.00	2.00	20.220	55.200
1.00	1.00	3.00	68.400	24.580
2.00	1.00	3.00	156.300	35.830
1.00	2.00	3.00	121.300	27.140
2.00	2.00	3.00	67.600	49.890
1.00	3.00	3.00	39.500	53.700
2.00	3.00	3.00	113.400	53.960
1.00	4.00	3.00	8.351	42.000
2.00	4.00	3.00	0.010	69.330
1.00	5.00	3.00	0.010	47.200
2.00	5.00	3.00	0.010	80.080
1.00	6.00	3.00	0.010	57.700
2.00	6.00	3.00	2.000	87.400
1.00	1.00	4.00	80.100	25.600
2.00	1.00	4.00	63.900	14.900
1.00	2.00	4.00	52.300	27.000

Rep.	Temp.	Time	Asci No.	% mature + discharge asci
2.00	2.00	4.00	35.300	34.840
1.00	3.00	4.00	30.700	70.500
2.00	3.00	4.00	57.200	43.700
1.00	4.00	4.00	0.010	100.000
2.00	4.00	4.00	0.010	100.000
1.00	5.00	4.00	0.010	100.000
2.00	5.00	4.00	0.010	100.000
1.00	6.00	4.00	0.010	100.000
2.00	6.00	4.00	0.010	100.000
1.00	1.00	5.00	75.300	34.930
2.00	1.00	5.00	54.700	13.350
1.00	2.00	5.00	21.900	27.400
2.00	2.00	5.00	11.900	15.130
1.00	3.00	5.00	25.000	59.000
2.00	3.00	5.00	0.330	70.000
1.00	4.00	5.00	0.010	100.000
2.00	4.00	5.00	0.010	100.000
1.00	5.00	5.00	0.010	100.000
2.00	5.00	5.00	0.010	100.000
1.00	6.00	5.00	0.010	100.000
2.00	6.00	5.00	0.010	100.000
1.00	1.00	6.00	80.700	21.310
2.00	1.00	6.00	96.600	17.290
1.00	2.00	6.00	3.600	6.000
2.00	2.00	6.00	0.010	15.130
1.00	3.00	6.00	11.000	75.400
2.00	3.00	6.00	6.300	72.000

Variables description.

Temperature 1 = 4 C; Temperature 2 = 8 C; Temperature 3 = 12 C; Temperature 4 = 16 C; Temperature 5 = 20 C; Temperature 6 = 24 C.

Time = Evaluation date. Time 1 = 7 days after incubation; Time 2 = 14 days after incubation; Time 3 = 21 days after incubation; Time 4 = 28 days after incubation; Time 5 = 35 days after incubation; Time 6 = 42 days after incubation.

Experiment 2.

Rep.	Temp.	Time	Asci No.	% mature + discharge asci
1.00	1.00	1.00	36.700	16.900
2.00	1.00	1.00	46.000	6.200
1.00	2.00	1.00	79.100	33.200
2.00	2.00	1.00	72.100	28.100
1.00	3.00	1.00	93.000	56.300
2.00	3.00	1.00	85.100	43.100
1.00	4.00	1.00	103.300	74.200
2.00	4.00	1.00	108.300	76.000
1.00	5.00	1.00	49.000	90.300
2.00	5.00	1.00	71.000	83.100
1.00	6.00	1.00	31.500	27.300
2.00	6.00	1.00	16.600	22.400
1.00	1.00	2.00	50.100	31.700
2.00	1.00	2.00	36.600	20.500
1.00	2.00	2.00	73.400	46.300
2.00	2.00	2.00	54.200	43.800
1.00	3.00	2.00	63.100	75.000
2.00	3.00	2.00	45.000	74.300
1.00	4.00	2.00	10.000	61.000
2.00	4.00	2.00	9.400	90.000
1.00	5.00	2.00	7.400	83.000
2.00	5.00	2.00	1.600	91.000
1.00	6.00	2.00	1.500	5.000
2.00	6.00	2.00	4.500	8.500
1.00	1.00	3.00	31.500	15.000
2.00	1.00	3.00	30.300	25.700
1.00	2.00	3.00	64.200	45.100
2.00	2.00	3.00	33.200	52.000
1.00	3.00	3.00	13.000	72.500
2.00	3.00	3.00	5.000	58.000
1.00	1.00	4.00	26.600	20.200
2.00	1.00	4.00	43.700	15.500
1.00	2.00	4.00	27.400	40.800
2.00	2.00	4.00	22.500	38.000
1.00	3.00	4.00	2.000	60.000
2.00	3.00	4.00	2.000	30.000
1.00	1.00	5.00	11.100	9.900
2.00	1.00	5.00	7.100	8.600
1.00	2.00	5.00	28.600	29.600

Rep.	Temp.	Time	Asci No.	% mature + discharge asci
2.00	2.00	5.00	14.300	21.000
1.00	3.00	5.00	0.010	60.000
2.00	3.00	5.00	0.010	30.000
1.00	1.00	6.00	8.300	6.600
2.00	1.00	6.00	2.100	7.000
1.00	2.00	6.00	15.000	36.000
2.00	2.00	6.00	12.400	21.000
1.00	3.00	6.00	0.010	60.100
2.00	3.00	6.00	0.010	30.000

Variables description.

Temperature 1 = 4 C; Temperature 2 = 8 C; Temperature 3 = 12; Temperature 4 = 16 C; Temperature 5 = 20 C; Temperature 6 = 24 C.

Time = Evaluation date. Time 1 = 7 days after incubation; Time 2 = 14 days after incubation; Time 3 = 21 days after incubation; Time 4 = 28 days after incubation; Time 5 = 35 days after incubation; Time 6 = 42 days after incubation.

EFFECT OF TEMPERATURE ON ASCOSPORE DISCHARGE

Experiments: 1 - 2 - 3.

Rep.	Temp.	Ascospore discharged (%)
1.00	1.00	16.90
1.00	1.00	6.74
2.00	1.00	52.50
2.00	1.00	32.50
3.00	1.00	26.30
3.00	1.00	36.00
1.00	2.00	19.10
1.00	2.00	9.00
2.00	2.00	57.50
2.00	2.00	42.50
3.00	2.00	42.00
3.00	2.00	43.00
1.00	3.00	7.87
1.00	3.00	9.00
2.00	3.00	45.00
2.00	3.00	45.00
3.00	3.00	38.00
3.00	3.00	44.00
1.00	4.00	20.22
1.00	4.00	12.40
2.00	4.00	92.50
2.00	4.00	100.00
3.00	4.00	32.00
3.00	4.00	32.10
1.00	5.00	100.00
1.00	5.00	18.50
2.00	5.00	78.00
2.00	5.00	78.00
3.00	5.00	86.00
3.00	5.00	66.30
1.00	6.00	86.50
1.00	6.00	47.00
2.00	6.00	95.00
2.00	6.00	78.00
3.00	6.00	100.00
3.00	6.00	87.40

Temperature 1 = 8 C; Temperature 2 = 12 C; Temperature 3 = 16 C; Temperature 4 = 20 C; Temperature 5 = 24 C; Temperature 6 = 30 C.

APPENDIX C

TABLES

Comparison of mean stage and rate of change of apothecium development of *Blumeriella jaapii* with average temperature, accumulated degree days, and total rain for each sampling date at orchards near East Lansing and Traverse City, Michigan, in 1990 and 1991.

Comparison of mean stage and rate of change of apothecium development of *Blumeriella jaapii* with average temperature, accumulated degree days, and total rain for each sampling date at East Lansing and Traverse City, Michigan, in 1990.

Mean stage[a]	Rate of change	Rate of change/day	Temp avg.[b]	Accum. dd[c]	Total rain[d]
Orchard[e] 1 - Sweet cherries					
1.3	-	-	-	-	-
2.9	1.6	0.12 (13)[f]	6.8	55.5	16.3
3.3	0.4	0.03 (13)	15.4	161.3	19.8
4.3	1.0	0.07 (14)	14.5	128.9	61.0
6.0	1.7	0.12 (14)	14.6	160.7	6.5
Orchard 2 - Sour cherries					
1.0	-	-	-	-	-
1.4	0.4	0.03 (13)	6.8	55.5	16.3
3.2	1.8	0.14 (13)	15.4	161.5	19.8
4.0	0.8	0.06 (14)	14.5	128.9	61.0
5.3	1.3	0.09 (14)	14.6	160.7	6.5
Orchard 3 - Sweet cherries					
1.0	-	-	-	-	-
1.7	0.7	0.05 (14)	12.2	125.8	51.3
3.0	1.3	0.19 (7)	10.3	51.7	24.8
4.0	1.4	0.10 (13)	12.2	115.6	14.6
Orchard 4 - Sour cherries					
1.4	-	-	-	-	-
2.5	1.1	0.08 (14)	12.2	125.8	51.3
4.0	1.5	0.20 (7)	10.3	51.7	24.8
6.0	2.0	0.08 (25)	14.2	278.5	67.4
Orchard 5 - Sour cherries					
1.2	-	-	-	-	-
3.5	2.3	0.16 (14)	9.5	86.7	102.2
4.0	0.5	0.04 (13)	10.8	97.1	16.8
5.6	1.6	0.12 (13)	15.2	157.9	45.5
6.0	0.4	0.03 (15)	17.2	209.9	89.1

[a]Stages of development were: 1= only paraphysis present in lumen of apothecium, 2 = asci formed but no ascospores present, 3 = few asci with ascospores formed, 4 = ascospores formed and apothecium open, 5 = spores discharged from most asci, and 6 = asci empty and apothecium disintegrated. Each apothecium was assigned a stage classification. The resulting classification number was averaged to give a mean value for each date.

[b]Temperature C.

[c]Degree days. Base temperature 3.9 C.

[d]Millimeters.

[e]Orchards 1 - 2 were located near East Lansing, orchards 3 - 4 were located near Northwest Michigan Experimental Station, and orchard 5 was located on Old Mission Peninsula.

[f]Numbers in parenthesis are intervals between samplings.

Comparison of mean stage and rate of change of apothecium development of *Blumeriella jaapii* with average temperature, accumulated degree days, and total rain for each sampling date at East Lansing and Traverse City, Michigan, in 1991.

Mean stage[a]	Rate of change	Rate of change/day	Temp avg.[b]	Accum. dd[c]	Total rain[d]
Orchard[e] 1 - Sweet cherries					
1.0	-	-	-	-	-
1.4	0.4	0.03 (15)[f]	7.0	68.4	57.8
3.6	2.2	0.17 (13)	11.0	101.4	53.4
3.7	0.1	0.00 (15)	10.9	112.4	50.9
4.5	0.8	0.04 (19)	16.0	240.4	89.6
5.5	1.0	0.07 (15)	22.0	292.4	43.8
Orchard 3 - Sweet cherries					
1.0	-	-	-	-	-
3.2	2.2	0.07 (30)	7.0	115.3	95.5
3.9	0.7	0.05 (14)	10.3	96.5	26.9
4.6	0.7	0.05 (15)	18.1	219.8	26.3
5.2	0.6	0.05 (13)	19.7	221.5	20.3
Orchard 4 - Sour cherries					
1.0	-	-	-	-	-
1.5	0.5	0.02 (30)	7.0	115.3	95.5
3.8	2.3	0.16 (14)	10.3	96.5	26.9
4.8	1.0	0.07 (15)	18.1	219.8	26.3
4.9	0.1	0.00 (13)	19.7	221.5	20.3

[a]Stages of development were: 1 = only paraphysis present in lumen of apothecium, 2 = asci formed but no ascospores present, 3 = few asci with ascospores formed, 4 = ascospores formed and apothecium open, 5 = spores discharged from most asci, and 6 = asci empty and apothecium disintegrated. Each apothecium was assigned a stage classification. The resulting classification number was averaged to give a mean value for each date.
[b]Temperature C.
[c]Degree days. Base temperature 3.9 C.
[d]Millimeters.
[e]Orchards 1 - 2 were located near East Lansing, orchards 3 - 4 were located near Northwest Michigan Experimental Station, and orchard 5 was located on Old Mission Peninsula.
[f]Numbers in parenthesis are intervals between samplings.