

Article



Characteristics of Meteorological Conditions during a Severe Outbreak of Onion Downy Mildew and Metalaxyl Sensitivity of *Peronospora destructor* in Saga, Japan, in 2016

Shin-ichirou Syobu * 🕩 and Sachiko Watanabe 🕩

Saga Prefectural Agriculture Technological Support Centre, Kawasoe, Saga, Saga 840-2205, Japan; watanabe-sachiko@pref.saga.lg.jp

* Correspondence: shobushin.1624@b2.bunbun.ne.jp

Abstract: In 2016, an onion downy mildew epidemic caused by *Peronospora destructor* severely damaged the commercial onion fields in Saga Prefecture, western Japan. To identify the factors underlying the outbreak, we investigated the symptoms of downy mildew caused by secondary infections and examined *P. destructor*'s sensitivity to metalaxyl, the most effective traditional fungicide used against this onion pathogen, in 2016–2018. Disease symptoms developed in late March 2016, which was earlier than symptom development in 2017 and 2018. Furthermore, there were synchronous repeated disease development and favourable meteorological conditions for infection in early and late April resulting in the development of polycyclic epidemics. In field trials from 2016 to 2018, the efficacy of chlorothalonil + metalaxyl-M application ranged 18–45%, as calculated by comparing disease severity at the final stage of each treatment to that in the untreated plots. On the basis of the metalaxyl sensitivity observed in 2016, the effective concentration, which reduced germ-tube elongation in *P. destructor* by 50%, exceeded 200 μ g ai/mL for certain strains. Our observations indicate that these characteristic meteorological conditions were major factors contributing to the severe disease outbreak in 2016. The emergence of less-metalaxyl-sensitive *P. destructor* strains may be an additional predisposing factor.

Keywords: onion downy mildew; *Peronospora destructor*; meteorological conditions; infection risk model; metalaxyl sensitivity

1. Introduction

Downy mildew of onions, caused by *Peronospora destructor* ((Berk.) Casp.), occurs in most onion-producing regions worldwide, causing yield and quality losses [1–4]. In western Japan, onion seeds are primarily sown in nursery beds in autumn; seedlings are then transplanted in main fields in autumn–winter, and onions are finally harvested in spring. In spring 2016, however, an epidemic caused by this disease in western Japan severely damaged commercial onion fields. The yield losses mainly resulted from severe infections, causing early defoliation, reduced bulb sizes, and an approximately USD 50 million loss in Saga Prefecture, the second-largest bulb-onion-producing area in Japan. Therefore, identifying the factors responsible for the outbreak is critical in the risk assessment of onion downy mildew.

Onion plants primary-infected by *P. destructor* show systemic symptoms, such as leaf chlorosis (i.e., pale green–yellowish leaves) and distortion (i.e., down-curled lower leaves), while the formation of sporangia plays a pivotal role as secondary inoculum for the spread of disease, thereby perpetuating the occurrence of downy mildew epidemics [5,6]. Reporting studies have also found that typical disease symptoms caused by secondary infections are pale green–yellowish areas of irregular size and shape (oval–cylindrical) on infected leaves, whereas sporulation on diseased leaves has been reported to occur above 90% [5] or 93% relative humidity (RH) [7]. The environmental requirements for



Citation: Syobu, S.-i.; Watanabe, S. Characteristics of Meteorological Conditions during a Severe Outbreak of Onion Downy Mildew and Metalaxyl Sensitivity of *Peronospora destructor* in Saga, Japan, in 2016. *Horticulturae* 2022, *8*, 578. https://doi.org/10.3390/ horticulturae8070578

Academic Editors: Vladimir K. Chebotar and Anastasiya M. Kamionskaya

Received: 22 May 2022 Accepted: 23 June 2022 Published: 26 June 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). secondary *P. destructor* sporangia infection and a step-like pattern in the epidemic have been reported [5,6,8]. Numerous infection risk models for onion downy mildew have been developed, such as DOWNCAST [9], modified DOWNCAST [10], and ZWIPERO [11]; however, to date, there have been no detailed reports assessing the underlying mechanisms of epidemic outbreaks via infection risk modelling.

Fungicide control is an effective means for controlling downy mildew of onion and avoiding crop loss. Among commercial fungicides, metalaxyl and formulations containing metalaxyl-M, the active R-isomer, which has exhibited strong preventive and curative activities [12], have been the most effective ones against *P. destructor* since the late 1980s in Japan. However, phenylamide fungicides, including metalaxyl, generally pose a high risk of resistance development in pathogens [13,14]. There have been numerous reports on the resistance of downy mildew to metalaxyl. For example, since metalaxyl resistance was found in the 1980s in grapevine downy mildew caused by *Plasmopara viticola*, fungicide resistance has been detected in many vineyards around the globe [12,13]. To date, a few studies have reported metalaxyl resistance in onion downy mildew in Australia [15,16] and in some strains of *P. destructor* with a certain degree of metalaxyl resistance in New Zealand [17]. Although no metalaxyl-resistant strains have been reported other than those from these two countries, it is vital to monitor *P. destructor* sensitivity to this fungicide in Japan.

The objectives of this study were essentially two-fold: (i) to identify and validate the characteristics of the 2016 epidemic of onion downy mildew caused by secondary infections based on the monitoring of disease symptoms in spring in the years 2016–2018 and on the estimation of the dates of infection using the infection risk model developed in this study using meteorological data; and (ii) to determine the sensitivity of *P. destructor* to metalaxyl, the most effective traditional fungicide used against this onion pathogen, based on field and laboratory experiments.

2. Materials and Methods

2.1. Disease Development

Field surveys were performed in the onion fields of the Saga City and Shiroishi Town branches of the Prefectural Research Centre of Saga, except for one commercial onion field (field D), in 2016 (Figure 1; Tables 1 and S1). All areas investigated in this study lay at elevations of <10 m above sea level. Onion seeds were sown in nursery beds during September and October and raised for ~60 days. Seedlings were then transplanted in main fields (experimental areas) in autumn–winter (Table 1). In Japan, artificial irrigation is rarely performed in main fields after transplanting onion seedlings. Therefore, the experimental onion fields were not irrigated after transplanting the seedlings and depended on natural rainfall for irrigation. Fertiliser application and insect control procedures (mainly for onion thrips) included standard district practices to maintain normal crop growth. Weeds were controlled using herbicides and manual clearing and did not influence disease development.

	D: 0			Onion Cultivation		Assess		
Year ^b	Disease S (Loo	cation)	Variety (Maturity)	Planting Date	Harvest Date	Disease Survey Field (Unsprayed Plots)	Fungicide Experiment Plots	Placement of Infected Plants (No. ^c)
2016	А	Saga	Takanishiki (very early)	12 November 2015	15 April 2016	1 March–7 April	1 March–7 April (expts. 1-1, 1-2)	2–29 March (4 ^d)
	В	Shiroishi	Shippo-wase 7 (early)	12 November 2015	25 April 2016	3 March-1 April	3 March–1 April (expt. 2)	-
	С	Saga	Spart (early)	24 November 2015	2 May 2016	29 March–29 April	29 March–29 April (expt. 3)	30 March–23 April (2 ^d)
	D	Shiroishi	Advance (early)	20 December 2015	18 May 2016	30 March-25 April	_	-
	Е	Saga	Tarzan (medium–late)	8 December 2015	27 May 2016	11 April–15 May	_	-
2017	F	Saga	Spart (early)	2 December 2016	1 May 2017	22 March–28 April	19–25 April (expt. 4)	23 March–19 April (4)
	В	Shiroishi	Shippo-wase 7 (early)	12 December 2016	2 May 2017	4 April–28 April	_	_
	E	Saga	Tarzan (medium–late)	12 December 2016	31 May 2017	10 April–15 May	_	-
	G	Shiroishi	Tarzan (medium–late)	21 November 2016	25 May 2017	4 April–17 May	-	-
2018	Н	Saga	Spart (early)	21 November 2017	1 May 2018	15 March–26 April	18–26 April (expt. 5)	15 March–22 April (3)
	Ι	Saga	Shippo-wase 7 (early)	21 November 2017	11 May 2018	15 March–27 April	_	_
	В	Shiroishi	Shippo-wase 7 (early)	27 November 2017	2 May 2018	14 March–27 April	_	-
	G	Shiroishi	Tarzan (medium–late)	6 December 2017	15 May 2018	14 March–14 May	_	-

Table 1. Onion cultivation and disease assessment of onion downy mildew in the disease survey fields ^a.

^a See Table S1 for further explanation of location, onion cultivation, and disease assessment. ^b The year when the disease development was assessed. ^c Number of plants placed in each plot. ^d Containing one plant collected from Shiroishi 1 described below.



Figure 1. Locations of meteorological stations (I–IV), disease survey fields (1–3), and onion plant collection areas (a–h) for the examination of *Peronospora destructor's* sensitivity to metalaxyl. Meteorological stations: I, Saga City; II, Sasebo City; III, Fukuoka City; and IV, Kumamoto City. Disease survey: 1—Fields A, C, E, F, H, and I in Saga City; 2—Fields B and G in Shiroishi Town; and 3—Field D in Shiroishi Town. Plant collection: a, Saga City Honjou; b, Saga City Kawasoe; c, Saga City Kubota; d, Kashima City 1–3; e, Kanzaki City; f, Miyaki Town 1, 2; g, Shiroishi Town 1; and h, Shiroishi Town 2. Map data were derived from Geospatial Information Authority of Japan.

Primary-infected plants with systemic symptoms (Figure 2a–d) collected from onion fields in Shiroishi were transferred to vinyl pots containing artificial culture soil and then evenly distributed throughout the field plots as an inoculum source for selected experimental treatments (Table 1). In this study, all additional primary-infected plants under natural conditions were removed from the field as soon as they were identified from late winter to early spring under all experimental conditions. The assessment of onion downy mildew disease was conducted at ~5–7-day intervals in onion trial plots that were untreated with fungicides (disease survey fields in Tables 1 and S1). Disease incidence was quantified as the percentage of plants with downy mildew symptoms caused by secondary infections (Figure 2e–h), and disease severity was estimated as the percentage of infected leaf area per plant. Only the typical symptoms on infected leaves were used to assess disease incidence and severity. A disease index per plant of 0–5 was set (0, no downy mildew; 1, 1–20% leaf area per infected plant; 2, 21–40%; 3, 41–60%; 4, 61–80%; 5, >81%), and disease severity was calculated using the following formula:

Disease severity = $100 \times (B + 2C + 3D + 4E + 5F)/5 (A + B + C + D + E + F)$

where A, B, C, D, E, and F represent the numbers of plants rated with disease indices of 0, 1, 2, 3, 4, and 5, respectively.



Figure 2. Symptoms of onion downy mildew induced by (**a**–**d**) primary infection and (**e**–**h**) secondary infection. (**b**) Solid arrow shows infected onion plants; (**f**) initial symptoms; (**d**,**g**,**h**) dashed arrows show sporangia formed in onion plants. Photographs were taken in (**g**) April 2016, (**a**–**d**) March 2017, (**e**,**h**) April 2018, and (**f**) May 2018.

2.2. Estimating Infection Date Using Risk Model

With reference to the literature, a simple infection risk model of onion downy mildew (Figure 3) was here developed using hourly meteorological data of air temperature, RH, and wind speed (based on the average of the 10 min period preceding hourly observations). The dates of downy mildew infection were estimated via an infection risk model (Figure 3) using data from four meteorological stations (Figure 1; Table S2).

In the present study, the daily mean temperatures on the days preceding sporulation, the sporulation periods, and the temperatures during the night of sporulation were set according to previously reported values [5,8,18] (Figure 3). As mentioned above, sporulation is generally found to occur above 90% RH. However, the measurements of humidity were significantly affected by the location of the sensors. The humidity within crops was generally higher and was also affected by the density and size of crops and the amount and size of foliage [19]. Relative humidity can be measured at a normal meteorological station 2 m above the ground, but it is considered that 5% should be added to the RH values to adapt them to the conditions present on the soil surface [20]. In the present study, we used RH measured at 1.5 m above the ground at all meteorological stations (Table S2); therefore, we set the boundary value for sporulation to 80–90% RH and the favourable condition for sporulation to 90% RH or more. *Peronospora destructor* sporangia least survived at 33% RH, and the differences in survival rates between 53% and 76% RH were generally not significant [21]. Moreover, *P. destructor* sporangia can remain viable for several days after formation [21,22]. In this study, it was assumed that the sporangia survived for 2 days when the average hourly RH from 09:00 to 18:00 h was \geq 55% (Figure 3). The duration of leaf wetness required for *P. destructor* infection varies from 6 h [6], 7 h [5], or 8 to 12 h (temperature dependent) [23]. Thus, in this study, the boundary value for infection was set from 6 to 10 h, and the favourable condition for infection was set to more than 10 h. Leafwetness duration was not measured in this study, as analyses were conducted solely using



meteorological station observation data. Therefore, high-humidity (\geq 80% RH) duration data were used rather than leaf-wetness duration.

Figure 3. Criteria for estimating the date of occurrence of favourable meteorological conditions for secondary infection of onion downy mildew using the infection risk model. Air temperature (Temp), relative humidity (RH), and wind speed (Wind) are hourly data.

In some downy mildew pathogens, wind negatively affects sporangia production. For instance, in onion downy mildew [24] and lettuce downy mildew caused by *Bremia lactucae* [25], sporulation was prevented at wind speeds of 0.5–1.0 m/s. Wind can also reduce leaf wetness and infection by *B. lactucae* [26,27]. At meteorological stations in Japan, anemometers are mostly installed on rooftops and towers to secure a stable environment, free from the interference of surrounding objects. Accordingly, the wind speed data employed in the present study were obtained from measurements at heights of 15.3 m, 34.6 m, 35.0 m, and 56.1 m above ground level (Table S2). However, given the occurrence of frictional effects, the wind speed near the surface tends to be lower than that at greater heights above ground [28]. Consequently, in this study, instead of the aforementioned value of 0.5–1.0 m/s, a wind speed of 3 m/s or less was set as a favourable condition for sporulation and infection by *P. destructor* (Figure 3).

2.3. Infection Risk Model Ability to Predict Disease Development

The variables (number of days in which the prespecified meteorological conditions were met during the putative infection period) were evaluated for correlation with subsequent disease development (N = 78 cases; Table S3) using binary logistic regression (i.e., logit model). Independent data (i.e., data not used in building the risk model; Figure 3) collected during the three growing seasons from 2016 to 2018 according to the above-described disease survey fields (Tables 1 and S1) were used to assess disease development (Figure 4). Field data investigated every 3–7 days were used to assess the timing of disease development occurrence. DEFRA [23] evaluated the ability of the onion downy mildew predictive model based on whether disease incidence or severity increased by ~10% when the model predicted disease development. In this study, the data obtained on a given investigation date (Date A) were compared with those from the following date (Date B), and when the disease incidence increased by \geq 10% over 5 days (\geq ~2% points per day), disease development was considered to have occurred (Figure 4). If the incidence was \geq 90%, a severity increase of \geq 10% over 5 days was also used to determine the presence of disease development.



Figure 4. Flow chart for evaluating the relationship between meteorological conditions during the 3-day putative infection period and the probability of onion downy mildew disease development.

The date in the middle of Dates A and B (Date C) was considered the putative date of disease development (Figure 4). In the case of investigations every 3, 5, and 7 days, the first, second, and third days from Date A were set as the middle days, respectively. As latent periods of *P. destructor* are affected by temperature [23], here, the calculations were

performed from each Date C (Figure 4; Table S3), inferred using the latent days under each temperature condition (Table S4) and the observed daily mean temperature in Saga City (Table S2) by summing the inverse of the latent period (in day⁻¹) corresponding to the daily mean temperature (for example, 1/25 at 10 °C, 1/15 at 13 °C, 1/12 at 15 °C, and 1/10 at 20 °C). The day when the total value reached '1' was defined as the putative last day of infection (Date D). In the assessment of onion downy mildew infection, potted onion plants were placed as trap plants in artificially inoculated fields for 1–7 days [10]. In a previous assessment of grapevine downy mildew infection, leaf samples were collected from the vineyard at 2–3 day intervals and incubated under optimal conditions for infection [29,30]. Accordingly, the previous 3 days including Date D were set as the putative infection period in the present analysis (Figure 4). Notably, onion plants become more susceptible to downy mildew when bulb enlargement commences [6]. In western Japan, these periods for early maturing and medium–late-maturing onion varieties correspond to early and late March, respectively; thus, they were here analysed.

In wet or extremely humid conditions, onion downy mildew develops more rapidly, with rain having a diversity of effects on disease growth [22]. Therefore, in the binary logistic regression analysis, the number of days experiencing the following conditions during the 3-day putative infection period (Figure 4) was used as variable: (i) favourable and marginal conditions according to the infection risk model (Figure 3); (ii) daily mean RH was \geq 90%, \geq 85%, \geq 80%, and \geq 75%; and (iii) daily precipitation (Rain) was 0.5 mm \leq Rain < 5.0 mm, 0.5 mm \leq Rain < 20.0 mm, 0.5 mm \leq Rain < 40.0 mm, and 0.5 mm \leq Rain. The number of days of occurrence was calculated based on data from the four meteorological stations (Figure 1; Table S2), and the average number of days at the four points was used in the analysis. These variables were subsequently evaluated for their correlation with subsequent onion downy mildew disease development, and odds ratios with 95% confidence intervals (CIs) were calculated. Based on the results of the above analyses, a receiver operating characteristic (ROC) curve was created for the logit model and was considered to be the most effective for predicting the probability of disease development, as it provides a more comprehensive method to evaluate and compare risk algorithms or forecasters and has been previously applied to plant diseases [31]. The area under the ROC curve (AUC) is used to summarise overall model accuracy, where an AUC = 0.5 generally suggests no discrimination; 0.7–0.8 is characterised as acceptable; 0.8–0.9 is considered excellent; and >0.9 is deemed outstanding [32]. Sensitivity (the proportion of disease development correctly classified as such) and specificity (the proportion of no disease development correctly classified as such) were also calculated, both of which were derived after estimating the cut-off value that maximised the sum of sensitivity and specificity (Youden's index) [33].

2.4. Effectiveness of Metalaxyl in the Field

We evaluated the field efficacy of metalaxyl fungicide to manage downy mildew of onion in springs 2016–2018 (fungicide experiments 1–5). In 2016, the experiment was conducted in three different fields, with different onion varieties (fungicide experiments 1-1, 1-2, 2, and 3); whereas in both 2017 (fungicide experiment 4) and 2018 (fungicide experiment 5), only a single experiment was conducted. Fungicide experiments 1-1, 1-2, 2, and 3 were randomised complete block designs with two replicates, while experiments 4 and 5 were randomised complete block designs with three replicates (Tables 1 and S1). The significance of the differences between the objects in fungicide experiments 4 and 5 were determined using Dunnett's test. In all fungicide experiments, chlorothalonil + metalaxyl-M was used as the metalaxyl fungicide (Tables 2 and S5). Chlorothalonil and chlorothalonil + benthiavalicarb-isopropyl were used as the control fungicides in experiments 1-1, 1-2, 2, and 3, whereas mancozeb was used as the control fungicide in experiments 4 and 5. The fungicide solution in all the experiments was supplemented with a 0.033% agrochemical spreader (Kumiten; Kumiai Chemical Industry, Tokyo, Japan).

		Date of the Sprays											
Fungicide Active Ingredients (% ai)	Dosage (%)	2016 Field A (Fungicide Expt. 1-1)	2016 Field A (Fungicide Expt. 1-2)	2016 Field B (Fungicide Expt. 2)	2016 Field C (Fungicide Expt. 3)	2017 Field F (Fungicide Expt. 4)	2018 Field H (Fungicide Expt. 5)						
Chlorothalonil 50% +Benthiavalicarb- isopropyl 5%	0.1	1, 8, and 15 March	15, 22, and 29 March	3, 14, and 25 March	29 March; 5 and 12 April	not tested	not tested						
Chlorothalonil 32% +Metalaxyl-M 3.3%	0.125	1, 8, and 15 March	15, 22, and 29 March	3, 14, and 25 March	29 March; 5 and 12 April	23 and 30 March; 5 and 12 April	15, 23, and 30 March; 5 and 12 April						
Chlorothalonil 40%	0.1	1, 8, and 15 March	15, 22, and 29 March	3, 14, and 25 March	29 March; 5 and 12 April	not tested	not tested						
Mancozeb 80%	0.25	not tested	not tested	not tested	not tested	23 and 30 March; 5 and 12 April	15, 23, and 30 March; 5 and 12 April						

Table 2. List of the fungicides included in fungicide experiments 1–5, doses, and dates of spray ^a.

^a See Table S5 for further explanation regarding the list of fungicides included in experiments 1–5.

In fungicide experiments 1-1, 1-2, 3, 4, and 5, the primary-infected potted plants with systemic symptoms were evenly placed to serve as inoculum sources, not only in the unsprayed plots described above (Table 1) but also in all fungicide-sprayed plots. Infected plants were placed in plots the day after the first fungicide application in fungicide experiments 1-1 and 3 and ~5 h later in fungicide experiments 4 and 5, whereas the first application of fungicide came 13 days following the placement of infected plants in fungicide experiment 1-2. In fungicide experiment 2 without infected plants (inoculum sources), the first application came prior to the appearance of onion downy mildew.

All fungicides were applied using manual pressure sprayers 3–5 times at 7–10-day intervals (Table 2). In fungicide experiments 1–5, downy mildew incidences and severities were calculated using the same method as in the disease survey fields. However, in fungicide experiments 1–3 conducted in 2016, when a severe outbreak was observed, the effect of the fungicides was evaluated only using disease severity.

2.5. Metalaxyl Sensitivity Assay

Primary-*P. destructor*-infected onion plants with systemic symptoms were collected from 11 fields in 2016 (Figure 1): Kanzaki and Miyaki (the eastern parts of Saga Prefecture); Saga Honjou, Saga Kawasoe, and Saga Kubota (the central parts of Saga Prefecture); and Shiroishi and Kashima (the western parts of Saga Prefecture), in 2016. Onion plants were collected from 1 field on 26 February (Shiroishi 1), 1 field on 9 March (Shiroishi 2), 3 fields on 14 March (Kashima 1–3), 5 fields on 16 March (Saga Honjou, Saga Kubota, Kanzaki, and Miyaki 1–2), and from 1 field on 14 April (Saga Kawasoe). One field (Shiroishi 2) was located at Saga Prefectural Research Centre (Shiroishi Town branch), whereas the others were commercial onion fields, among which Saga Kawasoe was an organic farm. Some of the inoculum plants used in fungicide experiments 1-1, 1-2, and 3 were collected from Shiroishi 1 (Table 1). Shiroishi 2 was adjacent to the field where fungicide experiment 2 was conducted.

In this study, the metalaxyl sensitivity of *P. destructor* collected from the 11 fields was examined, with the sensitivity to metalaxyl-M being examined in 4 of these fields (Saga Kawasoe, Saga Kubota, and Kashima 2–3). The collected onion plants were planted in pots and grown in a glass greenhouse for approximately 10–20 days; then; they were transferred to high-humidity conditions to promote sporangia formation. In each experiment, the leaves of the 2–3 pot-grown plants were gently wiped with a soft, wet piece of paper the evening prior to spore collection. Then, the plants were sealed in plastic boxes containing moistened paper towels to maintain high humidity, and sporangia formation was induced overnight in a growth chamber (Biotron NC-350H; Nippon Medical & Chemical Instruments, Osaka, Japan) at 15 °C in the dark. The following day, the individual plant

with the most abundant spore formation was selected, and the sporangia were collected from the surfaces of these leaves with a soft brush and suspended in distilled water. The concentration of sporangia was adjusted to ~1 × 10⁴ mL⁻¹ via a haemocytometer to be used for subsequent experiments within 1 h.

Phenylamide fungicides, including metalaxyl, inhibit various life stages of oomycetes, such as hyphal growth, haustoria, and sporangia formation, but do not inhibit the early stages in the disease cycle, such as the germination of sporangia [34]. Therefore, we investigated the inhibitory effect of metalaxyl on germ-tube elongation in sporangia [6,35], not sporangial germination. Technical-grade metalaxyl (99% ai; Kanto Chemical, Tokyo, Japan) and metalaxyl-M (95.7% ai; Hayashi Pure Chemical, Osaka, Japan) were used in this study. Dilutions of metalaxyl and metalaxyl-M were prepared in distilled water immediately before mixing with the above-described sporangial suspension. Then, the fungicide solution (50 µL) and sporangial suspension (50 µL) were mixed in cavity slides, using two slides for every concentration examined in each experiment. The final metalaxyl concentrations used in the experiments were 0.32, 1.6, 8, 40, and 200 µg ai/mL. However, metalaxyl-M, the R-enantiomer of metalaxyl, provides the same activity level at half of metalaxyl's rate [36]. Therefore, the following final metalaxyl-M concentrations were used: 0.064, 0.32, 1.6, 8, 40, and $200 \ \mu g \ ai/mL$. The cavity slides were then sealed in plastic boxes containing moistened paper towels to maintain high humidity and incubated in the growth chamber at 15 °C with a 10 h light: 14 h dark cycle. After 24 h of incubation, sporangia were examined under a light microscope (BX51; Olympus, Tokyo, Japan), and separate photographs of all cavity-filled areas with spore suspension were taken using a digital camera (COOLPIX 4500; Nikon, Tokyo, Japan) attached to the microscope (~10 photos were necessary to cover each cavity). The germ-tube length was measured using Photo measure software (KENIS, Osaka, Japan), which calculated the distance between multiple points in the captured digital image. Relative germ-tube length (RGTL) was thereafter calculated for each fungicide concentration as follows:

$$RGTL = 100Lx/Ly;$$

where *Lx* is the mean length of the germ tube in the fungicide-amended water and *Ly* is the mean length of the germ tube in the control. Half maximal effective concentrations (EC_{50}) were determined by calculating the inhibition according to:

$$1 - \left(\frac{\text{mean germ tube length in the fungicide} - \text{amended water}}{\text{mean germ tube length in the control}}\right)$$

then, the data were subjected to probit analysis [37].

All statistical analyses in this study were conducted using EZR v.1.53 [38].

3. Results

3.1. Disease Development in the Field

During the 3 years of the study (Table 3; Figures 5–7), although the dynamics of downy mildew epidemics varied in the disease survey fields, the maximum disease incidences and the highest disease severities were observed in 2016 (Figure 5). For all three years, the symptoms of downy mildew appeared earlier in early maturing onion varieties than in the medium–late-maturing onion varieties. In 2016, favourable meteorological conditions for *P. destructor* infection occurred from early to mid-March (Table 3), increasing the disease incidences and severities in Fields A and B from late March to early April (Figure 5). After favourable meteorological conditions occurred in early April, disease incidences and severities in Fields C and D, and disease incidence in Field E increased from mid- to late April. Then, favourable meteorological conditions in late April increased disease incidence and severity in Field E, from early to mid-May.

Date	2016				2017				2018			
	Saga	Sasebo	Fukuoka	Kumamoto	Saga	Sasebo	Fukuoka	Kumamo	to Saga	Sasebo	Fukuoka	Kumamoto
1 March												+
2 March					+							
3 March									+	+		
4 March	+ +	+							+ +	+	+	+ +
5 March					+	+		+			+	+
7 March	+ +	+ +	+ +	+ +								
8 March			+	+					+	+	+	+
9 March			+	+								
10 March			+									
11 March									+	+		
12 March			+							+		
13 March	+	+	+	+					+	+		+
14 March									+	+		
15 March									+	+		+
17 March												
18 March												
19 March				+ +					+ +	+	+	+
20 March									+	+	+	+
21 March								+ +	+	+	+	+
22 March									+	+	+ +	
23 March										+		
24 March									+			
25 March									+	+		
26 March 27 March								+ +		++		
27 March					+			+		+		
20 March										+		
30 March								+				
31 March	+ +	+ +	+	+ +	+		+	+				
1 April	+	+ +	+ +	+ +			+					
2 April	+	+ +	+ +	+ +			+			+		
3 April	+	+	+	+					+	+		
4 April	+	+ +	+ +	+ +					+	+ +		+
5 April		+							+	+	+	+ +
6 April 7 April			+								+	+
8 April	+ + +	++	+++	+ +	++	++	++	++				
9 April	TT	+	1.1	T T	+	+ +	+	++				
10 April		+		+	+	+	+	+				
11 April					+	+ +	+ +	+				
12 April												
13 April												
14 April		+ +	+ +									
15 April										+		+
16 April					+	+	++	+ +				
17 April 18 April	+	+		+	+	+	+	+	т	+		<u>т</u> т
19 April				T		T				1		T T
20 April												
21 April	+	+								+		
22 April	+	+ +	+	+ +						+	+	
23 April	+	+ +	+	+ +					+	+		
24 April		+ +	+ +	+ +					+	+	+	+
25 April	+	+ +	+	+					+	+	+	+
26 April	+	++	+	++	+	+	+	+			+	
27 April 28 April	+ +	++	++	++						+		
20 April		т	т	г		+				+		
30 April					+	+						
1 May		+			+	+						
2 May									+	+	+	+

Table 3. Occurrence of favourable meteorological conditions (+ +) and marginal meteorological conditions (+ +) for infection of onion downy mildew output as assessed using the infection risk model ^a.

Date	2016				2017				2018			
	Saga	Sasebo	Fukuoka	Kumamoto	Saga	Sasebo	Fukuoka	Kumamo	oto Saga	Sasebo	Fukuoka	Kumamoto
3 May 4 May		+					+					
5 May		+			+	+	+	+				
6 May 7 May		+ +	+	+ + +					+ +	+	+	+
8 May		+		+					+	+	+	+ +
9 May	+	+	+ +	+ +				+				
10 May	+	+	+	+	+	+		+ +				

 Table 3. Cont.

^a See Figure 3 for details of the risk model and Table S2 for the location of the meteorological stations.



Figure 5. Development of onion downy mildew in unsprayed plots in 2016. Arrows indicate the field (day) in which infected plants were placed as an inoculum source.



Figure 6. Development of onion downy mildew in unsprayed plots in 2017.

The 2017 growing season was characterised by unfavourable conditions for disease development (Figure 6). Favourable meteorological conditions occurred sporadically from early to late March (Table 3), and no disease symptoms were observed from late March to mid-April. After the occurrence of favourable meteorological conditions from early to mid-April, disease incidences and severities in Fields B, E, and F increased in late April. Following that, favourable meteorological conditions occurred again in late April, increasing disease severity in Field E and both disease incidence and severity in Field G from early to mid-May.

In 2018, disease incidence and severity were the second-highest after 2016 (Figure 7). Favourable meteorological conditions occurred in early March (Table 3), but no disease symptoms were observed in late March. Disease symptoms were observed in Fields B, H, and I in early April after favourable meteorological conditions occurred from mid- to late March. After that, favourable meteorological conditions occurred in early April, and the disease incidences and severities in Fields B, H, and I and disease incidence in Field G increased from mid- to late April. In addition, the favourable meteorological conditions from mid- to late April subsequently led to increased disease incidence and severity in Field G from late April to early May.



Figure 7. Development of onion downy mildew in unsprayed plots in 2018.

3.2. Prediction Ability of the Infection Risk Model

The variables (the number of days in which the prespecified meteorological conditions were met during the putative infection period) were evaluated for their correlation with subsequent disease development of onion downy mildew (Figure 4). It was revealed that the infection risk model output (favourable and marginal meteorological conditions) was significantly correlated with disease development (odds ratio, 2.48; CI, 1.36–4.52; p = 0.003; Table 4). Daily mean relative humidity (RH \geq 75%) was also significantly correlated, although the odds ratio was notably lower than that of the output of the infection risk model (odds ratio, 1.92; CI, 1.14–3.25; p = 0.015). Other variables, such as daily precipitation, were not significant predictors of subsequent disease development (Table 4).

Table 4. Relationship between the meteorological conditions during the 3-day putative infection period and the probability of disease development of onion downy mildew by binary logistic regression ^a.

Variable		Number of Days ^b	Odds Ratio (95% Confidence Interval)	<i>p-</i> Value (Fisher's Exact Test)
Output of infection risk model	Favourable and marginal meteorological conditions	0.89 ± 0.10	2.48 (1.36-4.52)	0.003
Daily mean relative humidity (RH)	$\begin{array}{l} {\rm RH} \ge 90\% \\ {\rm RH} \ge 85\% \\ {\rm RH} \ge 80\% \\ {\rm RH} \ge 75\% \end{array}$	$\begin{array}{c} 0.21 \pm 0.05 \\ 0.43 \pm 0.08 \\ 0.60 \pm 0.09 \\ 0.97 \pm 0.10 \end{array}$	1.05 (0.40–2.75) 1.20 (0.65–2.22) 1.60 (0.91–2.81) 1.92 (1.14–3.25)	0.924 0.561 0.106 0.015
Daily precipitation (Rain)	$0.5 \text{ mm} \le \text{Rain} < 5.0 \text{ mm}$ $0.5 \text{ mm} \le \text{Rain} < 20.0 \text{ mm}$ $0.5 \text{ mm} \le \text{Rain} < 40.0 \text{ mm}$ $0.5 \text{ mm} \le \text{Rain}$	$\begin{array}{c} 0.34 \pm 0.05 \\ 0.67 \pm 0.07 \\ 0.88 \pm 0.09 \\ 0.95 \pm 0.09 \end{array}$	0.85 (0.28–2.56) 0.98 (0.44–2.17) 1.31 (0.74–2.34) 1.24 (0.71–2.15)	0.768 0.963 0.353 0.450

^a We used field observation data (78 total) of disease development (n = 31) and no disease development (n = 47) from 2016 to 2018 (see Table S3 for details). ^b Number of days in which the event occurred during the 3-day infection period (mean \pm SE).

In the ROC curve analysis of the logit model using the output value of the infection risk model (Table S3), the resulting AUC was 0.70 (Figure 8). The resulting sum of sensitivity and specificity was maximised by a probability cut-off value of 0.50 for the logit model, at which point the sensitivity and specificity were 0.84 and 0.55, respectively.



Figure 8. Receiver operating characteristic (ROC) curve for disease development prediction of onion downy mildew from 2016 to 2018 in Saga Prefecture, Japan.

3.3. Effectiveness of Metalaxyl in the Field

Severe outbreaks of downy mildew occurred in Fields A, B, and C (Figures 5 and 9) in fungicide experiments 1-1, 1-2, 2, and 3 conducted in 2016. The lowest disease severities were observed for the chlorothalonil + benthiavalicarb-isopropyl applications with maximum values of 22%, 26%, 19%, and 37% (Figure 9) for experiments 1-1, 1-2, 2, and 3, respectively, while the treatments with chlorothalonil + metalaxyl-M resulted in higher disease severities of 54%, 53%, 34%, and 65% for experiments 1-1, 1-2, 2, and 3, respectively. Chlorothalonil application also resulted in higher disease severities, with the maximum values for experiments 1-1, 1-2, 2, and 3 being 54%, 55%, 39%, and 60%, respectively. The efficacies of the chlorothalonil + metalaxyl-M applications, calculated by comparing disease severity at the final stage of each treatment to that in the non-treated plots, for experiments 1-1, 1-2, 2, and 3 were 20%, 22%, 29%, and 25%, respectively.

In fungicide experiments 4 and 5 (conducted in 2017 and 2018, respectively), the disease incidences and severities increased in late April (Figure 6, Field F; Figure 7, Field H). Mancozeb applications were effective with significant differences in disease incidences and severities compared with the control in experiments 4 and 5 (Table 5). On the other hand, the efficacies of chlorothalonil + metalaxyl-M applications, derived by comparing disease severity at the final stage of each treatment to that in non-treated plots, were 18% and 45% for experiments 4 and 5, respectively, with no significant differences in disease incidences incidences and severities compared to the control in either of the experiments (Table 5).



Figure 9. Effects of fungicide treatments on onion downy mildew in 2016. The arrows indicate the spray dates.

	(Fungic	Date of Invi ide Experiment	estigation 4, Conducted	in 2017)	Date of Investigation (Fungicide Experiment 5, Conducted in 2018)					
Treatment (Active Ingredients)	Disease Incidence (%) ^a		Dis Severi	ease ty (%) ^a	Dis Incide	ease nce (%)	Disease Severity (%)			
	19 April	25 April	19 April	25 April	18 April	26 April	18 April	26 April		
Mancozeb	6.7 ± 3.5	$9.3\pm4.1~^{*,b}$	1.3 ± 0.7	$1.9\pm0.8~{}^{*}$	2.7 ± 0.7 **	$21.3\pm16.3~{}^{*}$	$0.5\pm0.1~{*}$	4.4 ± 3.4 **		
Chlorothalonil +Metalaxyl-M	10.7 ± 3.7	65.3 ± 15.7	2.1 ± 0.7	13.5 ± 3.4	40.7 ± 21.0	77.3 ± 16.7	8.5 ± 4.6	16.3 ± 3.6		
Control	22.0 ± 14.0	76.0 ± 11.1	4.4 ± 2.8	17.1 ± 3.4	80.7 ± 4.4	100.0 ± 0.0	17.9 ± 2.1	28.5 ± 2.5		

Table 5. Effects of fungicide treatments on onion downy mildew in 2017 and 2018.

^a mean \pm SE. ^b Asterisks indicate significant differences from the control (Dunnett's-test: * p < 0.05, ** p < 0.01).

3.4. Metalaxyl Sensitivity Assays

The average germ-tube length of *P. destructor* collected from the 11 fields (Table 6) and grown in distilled water for 24 h ranged from 493 to 1377 μ m. The percentages of germ-tube lengths relative to those of control *P. destructor*, collected from an organic farm (Saga Kawasoe) and then grown in water amended with metalaxyl at 0.32, 1.6, 8, 40, and 200 μ g ai/mL, were less than 50%. As a result, the EC₅₀ value was less than 0.32 μ g ai/mL (Table 6).

Table 6. Percentage of germ-tube length relative to that of control *Peronospora destructor* when grown in water amended with different metalaxyl and metalaxyl-M concentrations.

				Percent (%) of Gerr	n-Tube L	ength Relat	ive to Tha	at of Conti	ol (Total l	Number N	leasured)				
Geographic			Metal	axyl (µg	ai/mL)				Metalaxyl-M (µg ai/mL)							
Origin	200	40	8	1.6	0.32	0	EC ₅₀ ^a	200	40	8	1.6	0.32	0.064	0	EC ₅₀	
Saga, Honjou	_ b	29.0 (12)	59.7 (30)	88.8 (13)	109.9 (16)	100 (30)	13.1	-	_	-	-	-	-	-	-	
Saga, Kawasoe	25.4 (30)	23.2 (24)	34.9 (30)	38.3 (30)	38.8 (30)	100 (29)	< 0.32	26.9 (30)	33.2 (30)	22.7 (30)	33.8 (30)	20.8 (30)	58.5 (30)	100 (29)	< 0.32	
Saga, Kubota	-	-	-	-	15.5 (9)	100 (11)	< 0.32	-	18.6 (6)	26.6 (6)	24.2 (7)	30.6 (13)	13.3 (5)	100 (11)	< 0.064	
Kashima 1	-	19.5 (24)	39.3 (15)	61.9 (22)	73.6 (23)	100 (30)	3.1	-	-	-	-	-	-		-	
Kashima 2	-	63.3 (31)	120.0 (27)	130.2 (14)	133.2 (14)	100 (10)	>40	75.1 (30)	119.3 (18)	106.6 (19)	113.5 (28)	122.7 (19)	127.4 (23)	100 (10)	>200	
Kashima 3	90.3 (30)	120.0 (17)	133.5 (23)	138.8 (21)	78.7 (29)	100 (22)	>200	28.3 (30)	118.7 (30)	109.3 (23)	132.1 (27)	113.7 (30)	116.5 (22)	100 (22)	>40	
Kanzaki	52.7 (30)	71.1 (19)	74.0 (17)	82.6 (20)	79.6 (21)	100 (30)	>200	-	-	-	-	-	-	-	-	
Miyaki 1	-	85.7 (31)	109.9 (12)	120.9 (17)	113.4 (22)	100 (29)	>40	-	-	-	-	-	-	-	-	
Miyaki 2	107.8 (12)	104.0 (17)	110.0 (12)	106.4 (18)	99.7 (19)	100 (21)	>200	-	-	-	-	-	-	-	-	
Shiroishi 1	56.7 (8)	69.5 (4)	56.8 (5)	109.7 (7)	149.9 (6)	100 (7)	>200	-	-	-	-	-	-	-	-	
Shiroishi 2	70.4 (6)	133.9 (6)	124.5 (5)	110.7 (4)	139.5 (7)	100 (5)	>200	-	-	-	-	-	-	-	-	

^a EC₅₀ = effective concentration that reduces germ-tube elongation by 50%. ^b—indicates missing data.

In contrast, the relative germ-tube lengths of *P. destructor*, collected from Shiroishi 1 (from where some of the inoculum plants used in fungicide experiments 1-1, 1-2, and 3 were collected) and then grown in water amended with metalaxyl at 0.32, 1.6, 8, 40, and 200 μ g ai/mL, were greater than 50%, that is, the EC₅₀ value was greater than 200 μ g ai/mL (Table 6). The EC₅₀ values for metalaxyl on *P. destructor*, collected from Shiroishi 2 (adjacent to the field where fungicide experiment 2 was conducted), Kashima 3, Kanzaki, and Miyaki 2, were also greater than 200 μ g ai/mL. Overall, the EC₅₀ values for metalaxyl and metalaxyl-M on *P. destructor* collected from 8 out of the 11 fields and 2 out of the 4 fields, respectively, were greater than 10 μ g ai/mL (Table 6). Thus, less-metalaxyl-sensitive strains of *P. destructor* were identified in Saga Prefecture.

4. Discussion

In the disease survey fields, the symptoms of downy mildew caused by secondary infections appeared earlier in early maturing onion varieties than in the medium-latematuring onion varieties in 2016, 2017, and 2018 (Figures 5–7). Several studies have reported that host-plant age and size can affect the incidence of onion downy mildew, as shown below; thus, even in favourable environments for disease development, it has been observed that onset occurred at 9 weeks after transplanting [39] or that disease development only occurred late in the growing season [40]. Furthermore, in a study examining the effects of planting date on onion plants, the size of plants when the first symptoms of downy mildew were observed was approximately 25 cm in length [41]. In addition, inoculation with P. destructor sporangia at different growth stages in onion plants showed that host plants became more susceptible to downy mildew when bulb enlargement began [6]. The major secondary-infection periods of onion downy mildew in the three years of this study ranged from early March to early April for early maturing onion varieties and from late March to late April for medium-late-maturing onion varieties (Figures 5-7). We observed that the main infection periods in both varieties corresponded to the time of onion bulb enlargement, which agrees with the results of the above-described inoculation experiments [6].

In 2016, disease symptoms caused by secondary infections developed from late March to early April in Field A, where the primary-infected plants were placed as an inoculum source on 2 March, and in Field B with no primary-infected plants (Figure 5). The symptoms caused by secondary infections developed from mid- to late April in Field C, where the primary-infected plants were placed as an inoculum source on 30 March, and in Field D, where the primary-infected plants were not placed. Naturally occurring primaryinfected plants with systemic symptoms were confirmed in the field adjacent to Field B in March but not in Field D. The presence or absence of primary-infected plants (inoculum source) may have led to the difference in the periods of symptom development caused by secondary infections. In 2017, we could not detect the symptoms of downy mildew caused by secondary infections until mid-April, even in Field F, where the primary-infected plants were placed on 23 March (Figure 6). In Field B, the same onion varieties were cultivated in the same place in the 2017 season following the 2016 season (Tables 1 and S1), and the disease symptoms caused by secondary infections were detected in late April (Figure 6). Thus, the disease symptoms in 2017 developed approximately one month later than in 2016. The main reason for this may be attributable to the sporadic occurrences of favourable meteorological conditions for P. destructor infection in March 2017 (Table 3).

Similar to 2016, favourable meteorological conditions occurred in early March 2018 (Table 3), but, unlike in 2016, no disease symptoms were observed in late March, which may be attributed to the delayed growth of onion plants in early spring 2018, as the mean temperature from December 2017 to February 2018 was 5.4 °C (in Saga City; Table S2), which was lower than the mean temperature of 7.6 °C from December 2015 to February 2016 (Table S6). In warm winters, onion plants grow faster, and the risk of infection by *P. destructor* increases [6]. In 2016, the early appearance of the disease symptoms was attributed to the occurrences of favourable meteorological conditions in early March; furthermore, onion plants became more susceptible to *P. destructor* in early March, as they grew faster owing to the warm winter.

In seasons with the greatest risk of onion downy mildew, sporulation–infection events estimated using disease forecasting models tended to occur on consecutive days in New Zealand [42]. In the present study, the characteristic meteorological conditions for *P. destructor* infection frequently occurred in 2016 in early and late April. Furthermore, it should be noted that early April (with high-risk meteorological conditions) coincided with the period of disease development (risk of prolific sporulation) in early maturing onion varieties, while late April (with high-risk meteorological conditions) coincided with the period of disease development (risk of prolific sporulation) in both early and medium–late-maturing onion varieties. The occurrence of the sequence of weather conditions that

characterise sporulation–infection periods and maintain the continuity of infection cycles is critical for onion downy mildew epidemics [8]. March–April 2016 was one such sequence, wherein disease development and favourable meteorological conditions for sporulation–infection were synchronised and repeatedly occurred at intervals approximately equal to the latent period of *P. destructor*, leading to the severe outbreak of downy mildew.

In the present study, the dates of downy mildew infection were estimated via an infection risk model (Figure 3) using data from meteorological stations (Figure 1). In Japan, a similar model of rice leaf blast caused by *Pyricularia oryzae* [43] has been widely used for rice production. Due to spatial variability, when meteorological conditions are on the cusp of infection risk according to the model, favourable conditions may occur at some meteorological stations, but not at others. Accordingly, Koshimizu [43] concluded that it is better to macroscopically estimate infection of entire areas that include $\geq 2-3$ meteorological stations and share similar meteorological-topographical conditions rather than estimating infections around each station. When applying the onion downy mildew risk model, here, there was only a single meteorological station (Saga City) monitoring RH at hourly intervals in Saga Prefecture; thus, as is the case with the rice leaf blast model, there was a risk of overlooking the occurrence of favourable (or marginal) meteorological conditions. To address this limitation, data from three other meteorological stations located in the same northern Kyushu district as Saga Prefecture were also employed (Figure 1; Table S2).

In the binary logistic regression analysis (Figure 4), the output of the infection risk model (favourable and marginal meteorological conditions in Figure 3) based on the data from the four meteorological stations was significantly correlated with disease development (Table 4). Daily mean RH \geq 75% was also significantly correlated, although the odds ratio was lower than that of the risk model output. All other RH variables (RH \geq 80%, RH \geq 85%, and $RH \ge 90\%$) were insignificant in relation to disease development. High humidity favours sporulation and infection [5]; thus, the reason(s) why the more humid conditions (e.g., $RH \ge 80\%$) were not significantly associated with disease occurrence may be related to the limited number of days meeting this criterion (Table 4). Furthermore, the number of rainy days was not significantly associated with disease development in the present study either (Table 4). It has been reported that optimal meteorological conditions for P. destructor sporulation and infection were obtained when there was considerable rainfall the day before and the day of sporulation is cloudy, with or without occasional light rain [6]. Elsewhere, Palti [22] reported that rain had variable effects on *P. destructor*, where prolonged rainfall appeared to remove spores from the leaves, while brief rain showers often produced wetness periods too short in duration for infection, as spore survival was low during such periods. Accordingly, further studies are needed to clarify the precise effects of rainfall on P. destructor infection.

As already described, numerous infection risk models of onion downy mildew have been developed [9–11]. Some components assumed in these models, such as variable temperature conditions for sporulation, were simplified in the present study; however, novel variables, such as the relationships of wind speed and RH with sporulation, infection, and spore survival, were incorporated (Figure 3). In this study, we incorporated the criterion of sporangia survival when daytime RH was \geq 55%; however, it has been reported that temperatures also affected survival [18,21]. Accordingly, further studies are needed to also incorporate temperature conditions into the risk model.

The AUC based on the ROC curve of the logit model was 0.70 (Figure 8), suggesting that the risk model was acceptable for predicting the probability of disease. Notably, this model was developed using the output value of the infection risk model over three days (using the mean of the four meteorological stations) as the input variables (Figure 4; Table S3). Model ability was evaluated using the total number of days of occurrence with favourable or marginal conditions for infection output by the model. Accordingly, the effective use of the model output requires further analyses regarding how to quantify the differences in risk levels between favourable and marginal conditions.

In the ROC curve analysis (Figure 8), the cut-off value identified by Youden's index was 0.50. This represents the level where the average number of occurrence days within a 3-day period is <1. Although the repeated occurrence of favourable conditions for infection by *P. destructor* increases the risk of outbreaks, even conditions lasting only 1–2 days can lead to rapid disease development, as seen in 1955 in Japan [6]. Such characteristics of this disease (rapid increases in pathogen population and development of epidemics) are considered to be associated with its low cut-off value, where the sensitivity (proportion of disease development correctly classified as such) and specificity (proportion of no disease development correctly classified as such) were 0.84 and 0.55, respectively. The relatively low specificity value (0.55) may be due to the fact that the logit model was here solely based on meteorological risk factors, while it ignored pathogenic risks.

Recently, an infection risk model was developed for downy mildew of cucurbits based on binary logistic regression using average daily temperature and number of hours with RH > 80% over a 24 or 48 h period [44,45]. These studies also found that the RH and temperature data required for risk model development could be obtained from weather stations. In previously described infection risk models of onion downy mildew [9–11], hourly values of leaf wetness were used as an important component of infection; however, here, highhumidity duration data (RH \geq 80%) were used in their place (Figure 3). Accordingly, the accuracy of the risk model here proposed may be lower than those of previous efforts; notably, it was still found to be acceptable according to the ROC curve analysis (Figure 8). Further, here, this risk model can easily provide estimates of daily infection risk using meteorological station data; thus, this simplified model (Figure 3) can likely be useful in certain instances.

Onion downy mildew can only be effectively controlled when spraying begins in the first sporulation–infection period [9]. In Ontario, Canada, several properly timed fungicide applications were reported to be required to control onion downy mildew in season, with applications always preceding infection for best results [46]. Similarly, timely fungicide applications were also reported to be necessary prior to infection in western Oregon, USA [47]. Accordingly, most of the experimental procedures in the present study also commenced prior to disease appearance (Tables 1 and 2).

Preventive application of mancozeb has been demonstrated as an effective technique against onion downy mildew [15,48-50], and mancozeb has been found to be vastly superior to chlorothalonil in controlling the fungus [15,48]. Araújo et al. [50], Raziq et al. [51], and Araújo and Resende [52] demonstrated that mancozeb + metalaxyl (or metalaxyl-M) was an effective treatment with respect to reducing onion downy mildew. Previously, however, these metalaxyl fungicides have only been evaluated when used in combination with the highly effective mancozeb. In fungicide experiments 1-1, 1-2, 2, and 3 (conducted in 2016), chlorothalonil and chlorothalonil + metalaxyl-M applications against onion downy mildew were ineffective (Figure 9). In fungicide experiment 4 in 2017 and fungicide experiment 5 in 2018, mancozeb applications were effective against onion downy mildew, but chlorothalonil + metalaxyl-M applications were not (Table 5). In an experiment conducted in an onion field in Saga Prefecture in 1981, the percentages of diseased leaves were 0.5% and 51.3%, and the efficacies derived by comparing non-treated plots were 99% and 41% in the plots sprayed four times with metalaxyl and seven times with chlorothalonil, respectively [53]. In contrast, the efficacies ranged between 18% and 45% in the plots sprayed 3–5 times with chlorothalonil + metalaxyl-M in the fungicide experiments conducted in 2016, 2017, and 2018 (Figure 9; Table 5), indicating that the field performance of metalaxyl fungicide had declined.

In the laboratory experiments, the percentages of germ-tube lengths relative to those of control *P. destructor* collected from an organic farm (Saga Kawasoe) and then grown in water amended with metalaxyl and metalaxyl-M at 0.32, 1.6, 8, 40, and 200 µg ai/mL were less than 50%. However, the germ tubes were slightly elongated even at 40 and 200 µg ai/mL, and the relative germ-tube lengths were approximately 20–30% in this strain of *P. destructor* (Table 6). This slight growth of the germination tube can be attributed to the fact that

metalaxyl fungicides do not inhibit the early stages of the disease cycle [34]. A previous report suggested that the percentages of colony growth relative to those of the control of metalaxyl-sensitive and metalaxyl-M-sensitive isolates of *Phytophthora capsici* when grown on media amended with metalaxyl and metalaxyl-M, respectively, at 10 and 100 μ g ai/mL were approximately 10–40% [54]. Therefore, it is considered that the strain of *P. destructor* collected from organic farms in this study was sensitive to metalaxyl fungicides.

In contrast, the percentages of the germ-tube lengths relative to those of control *P. destructor* grown in water amended with metalaxyl fungicides at 0.32, 1.6, 8, 40, and 200 μ g ai/mL were greater than 50% in certain strains. Overall, less-metalaxyl-sensitive strains of *P. destructor* were detected in Saga Prefecture in this study (Table 6). Here, the sensitivities of *P. destructor* to metalaxyl and metalaxyl-M collected were assessed for 11 and 4 fields, respectively. In the future, it is necessary to increase the number of points for collecting diseased plants, while continuing to monitor the susceptibility of this pathogen to metalaxyl fungicides.

Although there have been numerous reports on the resistance of downy mildew to metalaxyl (e.g., grapevine downy mildew) [12,13], few studies have reported on metalaxyl resistance in onion downy mildew [15–17]. The first metalaxyl-resistant isolate of *P. destructor* was detected in Australia from a commercial onion field in 1989 [15]. It was considered that the overuse of metalaxyl during 1980–1988 contributed to the development of phenylamide resistance in *P. destructor*, although the strategy of using proprietary mixtures of phenylamide plus a protectant to prevent or delay resistance has been used since 1982 [15].

Metalaxyl was introduced in Japan around 1985 and was applied at approximately 200 μ g ai/mL for onion protection. Later, around 2010, metalaxyl was replaced with metalaxyl-M and applied at approximately 40–80 μ g ai/mL. Metalaxyl fungicides have always been applied in combination with contact fungicides such as mancozeb or chlorothalonil for resistance management. However, as a result of using metalaxyl fungicides, which generally carry a high risk of resistance development in pathogens, for approximately 30 years, it appeared that the less metalaxyl-sensitive strains of *P. destructor* were distributed in Saga Prefecture, and it is necessary to consider chemical controls that do not depend on metalaxyl fungicides. This study found that the major secondary-infection periods of onion downy mildew corresponded to the periods of onion bulb enlargement from early March for early maturing onion varieties and late March for medium–late-maturing varieties. Therefore, it is crucial to spray effective fungicides (for example, preventive mancozeb spraying), independently or combined, around each major infection period to control the disease. The output values of the infection risk model developed in this study can help inform how to use the other effective fungicides (e.g., spray intervals).

5. Conclusions

In March 2016, the symptoms of downy mildew caused by secondary infections appeared earlier owing to a warm winter. Furthermore, there was synchronous repeated disease development and favourable meteorological conditions for infection in early and late April, resulting in the development of polycyclic epidemics. These characteristic meteorological conditions were major factors contributing to the severe disease outbreak in Saga, Japan, in 2016. The emergence of *P. destructor* strains less sensitive to metalaxyl, the most effective traditional fungicide against this onion pathogen in the past ~30 years, may be an additional predisposing factor fuelling outbreaks.

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/horticulturae8070578/s1, Table S1: Further explanation of location, onion cultivation, and disease assessment in the disease survey fields; Table S2: Meteorological stations located in the northern Kyushu district where the data used in this study were obtained; Table S3: Decision logic regarding whether the disease development of onion downy mildew has occurred, the date obtained by back-calculating the latent period from the putative date of disease development, and the number of days of occurrence of favourable meteorological conditions for infection output by the risk model during the 3-day putative infection period; Table S4: Latent periods of *P. destructor* under each temperature condition used in this study; Table S5: Further explanation of list of the fungicides included in fungicide experiments 1–5; Table S6: Temperature and precipitation during the period when the onion plants were cultivated in the main field.

Author Contributions: S.-i.S. conceived and designed the study; S.-i.S. and S.W. performed the research study; S.-i.S. analysed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partly supported by grants from NARO Bio-oriented Technology Research Advancement Institution Project (the special scheme project on regional development strategy).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Jun-ichirou Yamaguchi (Saga Prefectural Upland Farming Research and Extension Centre), Nobuya Tashiro, Akira Kawaguchi (NARO Western Region Agricultural Research Centre), and Shiro Okumura (Fukuoka Industrial Technology Centre) for their helpful advice and discussion. We also thank the staff of Prefectural Agricultural Research Centre and Agricultural Technology Support Centre in Saga, Japan, for their technical assistance and encouragement.

Conflicts of Interest: The authors declare no conflict of interest.

References

- 1. Survilienė, E.; Valiuškaitė, A.; Raudonis, L. The effect of fungicides on the development of downy mildew of onions. *Zemdirbyste* **2008**, *95*, 171–179.
- 2. Afifi, M.; Zayan, S. Development of forecasting system for onion downy mildew in Egypt. J. Prod. Dev. 2009, 14, 37–50. [CrossRef]
- 3. Parkunan, V.; Gitaitis, R.D.; Dutta, B.; Langston, D.B.; Ji, P. An epidemic of downy mildew caused by *Peronospora destructor* on Vidalia sweet onions in Georgia in 2012. *Plant Health Prog.* **2013**, *14*, 54. [CrossRef]
- Van der Heyden, H.; Dutilleul, P.; Charron, J.-B.; Bilodeau, G.J.; Carisse, O. Factors influencing the occurrence of onion downy mildew (*Peronospora destructor*) epidemics: Trends from 31 years of observational data. *Agronomy* 2020, 10, 738. [CrossRef]
- 5. Yarwood, C.E. Onion downy mildew. *Hilgardia* 1943, 14, 595–691. [CrossRef]
- 6. Demizu, T. Epidemiological and therapeutic studies on the downy mildew of onions. Bull. Osaka Agric. Exp. Stn. 1963, 4, 1–95.
- Gilles, T.; Phelps, K.; Clarkson, J.P.; Kennedy, R. Development of MILIONCAST, an improved model for predicting downy mildew sporulation on onions. *Plant Dis.* 2004, 88, 695–702. [CrossRef]
- 8. Hildebrand, P.D.; Sutton, J.C. Weather variables in relation to an epidemic of onion downy mildew. *Phytopathology* **1982**, *72*, 219–224. [CrossRef]
- 9. Jesperson, G.D.; Sutton, J.C. Evaluation of a forecaster for downy mildew of onion (*Allium cepa* L.). *Crop Prot.* **1987**, *6*, 95–103. [CrossRef]
- 10. De Visser, C.L.M. Development of a downy mildew advisory model based on downcast. *Eur. J. Plant Pathol.* **1998**, *104*, 933–943. [CrossRef]
- 11. Friedrich, S.; Leinhos, G.M.E.; Löpmeier, F.-J. Development of ZWIPERO, a model forecasting sporulation and infection periods of onion downy mildew based on meteorological data. *Eur. J. Plant Pathol.* **2003**, *109*, 35–45. [CrossRef]
- Gisi, U. Chemical control of downy mildews. In *Advances in Downy Mildew Research*; Spencer-Phillips, P.T.N., Gisi, U., Lebeda, A., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2002; pp. 119–159.
- 13. Gisi, U.; Sierotzki, H. Fungicide modes of action and resistance in downy mildews. *Eur. J. Plant Pathol.* **2008**, 122, 157–167. [CrossRef]
- 14. Fungicide Resistance Action Committee. FRAC Code List 2022. Available online: http://www.frac.info (accessed on 17 June 2022).
- 15. O'Brien, R.G. Control of onion downy mildew in the presence of phenylamide-resistant strains of *Peronospora destructor* (Berk.) Caspary. *Aust. J. Exp. Agric.* **1992**, *32*, 669–674. [CrossRef]
- O'Brien, R.G.; Galea, V.; MacManus, G. VG402: Development of an Onion Downy Mildew Forecasting System. Horticulture Australia Ltd. 1997. Available online: https://ausveg.com.au/app/data/technical-insights/docs/VG402.pdf (accessed on 2 February 2022).
- Wright, P.J. Resistance of *Peronospora destructor* (onion downy mildew) to metalaxyl fungicides. In Proceedings of the 57th New Zealand Plant Protection Society's Annual Conference, Rydges Le Grand. Hamilton, New Zealand, 10–12 August 2004; p. 347. [CrossRef]
- Sutton, J.C.; Hildebrand, P.D. Environmental water in relation to *Peronospora destructor* and related pathogens. *Can. J. Plant Pathol.* 1985, 7, 323–330. [CrossRef]
- 19. Gilles, T. Forecasting downy mildew diseases. In *Advances in Downy Mildew Research*; Spencer-Phillips, P.T.N., Jeger, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2004; Volume 2, pp. 35–67.

- OEPP/EPPO. Guidelines on good plant protection practice: ALLIUM CROPS. Bull. OEPP/EPPO Bull. 2001, 31, 211–230. [CrossRef]
- 21. Bashi, E.; Aylor, D.E. Survival of detached sporangia of *Peronospora destructor* and *Peronospora tabacina*. *Phytopathology* **1983**, *73*, 1135–1139. [CrossRef]
- 22. Palti, J. Epidemiology, prediction and control of onion downy mildew caused by *Peronospora destructor*. *Phytoparasitica* **1989**, 17, 31–48. [CrossRef]
- DEFRA. Forecasting Diseases of Allium Crops: Project Report HH1742SFV; Department for Environment, Food and Rural Affairs: London, UK, 2002; pp. 11–18. Available online: http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More& Location=None&Completed=0&ProjectID=7930 (accessed on 2 February 2022).
- 24. Leach, C.M. Effect of still and moving moisture-saturated air on sporulation of *Drechslera* and *Peronospora*. *Trans. Br. Mycol. Soc.* **1985**, *84*, 179–183. [CrossRef]
- 25. Su, H.; van Bruggen, A.H.C.; Subbarao, K.V.; Scherm, H. Sporulation of *Bremia lactucae* affected by temperature, relative humidity, and wind in controlled conditions. *Phytopathology* **2004**, *94*, 396–401. [CrossRef]
- 26. Scherm, H.; van Bruggen, A.H.C. Response surface models for germination and infection of *Bremia lactucae*, the fungus causing downy mildew of lettuce. *Ecol. Modell.* **1993**, *65*, 281–296. [CrossRef]
- 27. Scherm, H.; van Bruggen, A.H.C. Weather variables associated with infection of lettuce by downy mildew (*Bremia lactucae*) in coastal California. *Phytopathology* **1994**, *84*, 860–865. [CrossRef]
- 28. Ogura, Y. General Meteorology, 2nd ed.; University of Tokyo Press: Tokyo, Japan, 1999.
- 29. Brischetto, C.; Bove, F.; Languasco, L.; Rossi, V. Can spore sampler data be used to predict *Plasmopara viticola* infection in vineyards? *Front. Plant Sci.* 2020, *11*, 1187. [CrossRef]
- Brischetto, C.; Bove, F.; Fedele, G.; Rossi, V. A weather-driven model for predicting infections of grapevines by sporangia of *Plasmopara viticola. Front. Plant Sci.* 2021, 12, 636607. [CrossRef] [PubMed]
- 31. Turechek, W.W.; Wilcox, W.F. Evaluating predictors of apple scab with receiver operating characteristic curve analysis. *Phytopathology* **2005**, *95*, 679–691. [CrossRef] [PubMed]
- Mandrekar, J.N. Receiver operating characteristic curve in diagnostic test assessment. J. Thorac. Oncol. 2010, 5, 1315–1316. [CrossRef] [PubMed]
- 33. Youden, W.J. Index for rating diagnostic tests. Cancer 1950, 3, 32–35. [CrossRef]
- 34. Schwinn, F.J.; Staub, T. Oomycetes fungicides: Phenylamides and other fungicides against Oomycetes. In *Modern Selective Fungicides*, 2nd ed.; Lyr, H., Ed.; Gustav Fischer: Jena, Germany, 1995; pp. 323–346.
- Ankita; Chandel, S.; Sharma, R.; Meena, V.K. Epidemiological studies of downy mildew of onion. *Int. J. Curr. Microbiol. App. Sci.* 2020, 9, 1–12. [CrossRef]
- Nuninger, C.; Watson, G.; Leadbitter, N.; Ellgehausen, H. CGA 329351: Introduction of the enantiomeric from of the fungicide metalaxyl. In Proceedings of the Brighton Crop Protection Conference: Pest and Disease, British Crop Protection Council. Farnham, UK, 18–21 November 1996; Volume 1, pp. 41–46.
- 37. Bliss, C.I. The method of probits. Science 1934, 79, 38–39. [CrossRef]
- Kanda, Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* 2013, 48, 452–458. [CrossRef]
- 39. Marcuzzo, L.L.; Carvalho, J.; Nascimento, A. Epidemiological aspects of onion downy mildew in the region of Alto Vale do Itajaí, Santa Catarina State, Brazil. *Summa Phytopathol.* **2017**, *43*, 218–222. [CrossRef]
- 40. González, P.H.; Colnago, P.; Peluffo, S.; Idiarte, H.G.; Zipitría, J.; Galván, G.A. Quantitative studies on downy mildew (*Peronospora destructor* Berk. Casp.) affecting onion seed production in southern Uruguay. *Eur. J. Plant Pathol.* 2011, 129, 303–314. [CrossRef]
- Maeso, D. Enfermedades del cultivo de cebolla. In *Tecnología Para la Producción de Cebolla*; Arboleya, J., Ed.; INIA Boletín de divulgación 88: Montevideo, Uruguay, 2005; pp. 151–188.
- Chynoweth, R.W.; Beresford, R.M.; Henshall, W.R.; Wright, P.J. Use of disease forecasting models for control of onion downy mildew in New Zealand. In *Advances in Downy Mildew Research*; Spencer-Phillips, P.T.N., Jeger, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2004; Volume 2, pp. 91–97.
- 43. Koshimizu, Y. A forecasting method for occurrence of rice leaf blast with AMeDAS data. *Bull. Tohoku Natl. Agric. Exp. Stn.* **1988**, 78, 67–121.
- Neufeld, K.N.; Keinath, A.P.; Ojiambo, P.S. A model to predict the risk of infection of cucumber by *Pseudoperonospora cubensis*. *Microb. Risk Anal.* 2017, 6, 21–30. [CrossRef]
- Neufeld, K.N.; Keinath, A.P.; Ojiambo, P.S. Evaluation of a model for predicting the infection risk of squash and cantaloupe by Pseudoperonospora cubensis. Plant Dis. 2018, 102, 855–862. [CrossRef] [PubMed]
- Celetti, M. Increased risk of downy mildew on onions. In *Hort Matters*; Ontario Ministry of Agriculture and Food and Ministry of Rural Affairs: Guelph, ON, Canada, 2015. Available online: http://www.omafra.gov.on.ca/english/crops/hort/news/hortmatt/ 2015/16hrt15a2.htm (accessed on 26 February 2022).
- Pscheidt, J.W.; Ocamb, C.M. Onion (Allium cepa)-Downy Mildew. In *Pacific Northwest Plant Disease Management Handbook*; Oregon State University: Corvallis, OR, USA, 2022; Available online: https://pnwhandbooks.org/plantdisease/host-disease/onionallium-cepa-downy-mildew (accessed on 26 February 2022).

- 48. Smith, R.W.; Lorbeer, J.W.; Abd-Elrazik, A.A. Reappearance and control of onion downy mildew epidemics in New York. *Plant Dis.* **1985**, *69*, 703–706. [CrossRef]
- 49. Mo, C.Y.; Lee, J.H.; Ko, S.J.; Yang, K.Y. Control efficacy of several fungicides against downy mildew of onion at nursery seedling stage. *Res. Plant Dis.* 2016, 22, 208–212. [CrossRef]
- 50. Araújo, E.R.; Resende, R.S.; Alves, D.P.; Higashikawa, F.S. Field efficacy of fungicides to control downy mildew of onion. *Eur. J. Plant Pathol.* **2020**, *156*, 305–359. [CrossRef]
- 51. Raziq, F.; Alam, I.; Naz, I.; Khan, H. Evaluation of fungicides for controlling downy mildew of onion under field conditions. *Sarhad J. Agric.* **2008**, *24*, 85–91.
- 52. Araújo, E.R.; Resende, R.S. Propineb efficiency in controlling downy mildew of onion under field conditions. *Australas. Plant Dis. Notes* **2020**, *15*, 34. [CrossRef]
- 53. Matsuzaki, M.; Kan, M. Effects of control on rot of onion bulbs during storage through fungicide application during the growing season. *Kyushu Plant Prot. Res.* **1982**, *28*, 70–73. [CrossRef]
- 54. Parra, G.; Ristaino, J.B. Resistance to mefenoxam and metalaxyl among field isolates of *Phytophthora capsici* causing Phytophthora blight of bell pepper. *Plant Dis.* **2001**, *85*, 1069–1075. [CrossRef] [PubMed]