Research Paper

Hot Water Spray Over Strawberry Plants Effectively Controls the Occurrence of Strawberry Powdery Mildew in Everbearing Strawberry Production

Dinar Mindrati FARDHANI*, Yuuki OHTA**, Nur Akbar AROFATULLAH***, Agung Dian KHARISMA*,
Junjira SATITMUNNAITHUM***, Sayuri TANABATA***, Isao OGIWARA****,
Ani WIDIASTUTI***** and Tatsuo SATO***

* United Graduate School of Agriculture, Tokyo University of Agriculture and Technology

** Riastar Farm Co. Ltd, Kawasaki, Yonesaki, Rikuzentakata, Iwate

*** Center for International Field Agriculture Research and Education, College of Agriculture, Ibaraki University

**** Faculty of Agriculture, Tokyo University of Agriculture and Technology

***** Faculty of Agriculture, Universitas Gadjah Mada

四季成り性イチゴにおける温湯散布のうどんこ病発生抑制効果

ディナール ミンドラティ ファルダニ*・太田祐樹**・ヌル アクバル アロファトゥラ***
アグン ディアン カリスマ*・ジャンジラ サティンマンナイタム***・七夕小百合***

荻原 勲****・アニ ウィディアストゥティ*****・佐藤達雄***

* 東京農工大学連合農学研究科, ** リアスターファーム

*** 茨城大学農学部附属国際フィールド農学センター, **** 東京農工大学農学部

***** ガジャ・マダ大学農学部

Extensive use of sterol demethylation inhibitor (DMI) fungicides against strawberry powdery mildew has resulted in DMI-resistant genotype emergence. In this study, we examined the preventive effect of hot water spraying (HWS) on the occurrence of powdery mildew in everbearing strawberry, predominantly infected by DMI-resistant powdery mildew fungi from 2016 to 2018. Hot water of $54 \pm 1^\circ\text{C}$ was sprayed on Natsuakari for 20 s to achieve the target leaf temperature of 50°C . Disease occurrence under the chemical fungicide (F) + HWS treatment was less severe than that under the F treatment until the end of the cultivation period in February 2018. However, the preventive effect of HWS was not observed in already infected leaves. The incidence of DMI-resistant genotype was 0% at the start, and it increased to 62% and 100% in the infected fruits and leaves (including F+HWS) in September and November 2016 until the end of cultivation, respectively. On the contrary, disease severity increased between August 2017 and February 2018 under the F treatment, whereas only a slight infection was detected under the F+HWS treatment. Thus, HWS could induce resistance in strawberry against powdery mildew fungi, including DMI-resistant fungi, and effectively control the occurrence of powdery mildew in long-term everbearing strawberry cultivation.

Key Words : chemical resistance, demethylation inhibitor, heat shock-induced resistance, long-term cultivation, practical test

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Corresponding author

佐藤達雄 Tatsuo SATO

〒300-0331 茨城県稲敷郡阿見町阿見 4668-1

Ami, Inashiki, Ibaraki 300-0331, Japan

E-mail : tatsuo.sato.strawberry@vc.ibaraki.ac.jp

1. Introduction

Everbearing strawberries are cultivated in cooler climatic regions in Japan, such as the coastal regions in Iwate Prefecture. Currently, there is a need to establish preventive measures against powdery mildew in strawberry, as it is a common and persistent problem in strawberry cultivation. The fungus *Podosphaera aphanis* (Wallr.) U. Braun & S. Takam causes severe infection in the aerial parts of strawberry plants, resulting in the loss of yield and reduction in fruit quality (Koitabashi et al., 2011). In Japan, demethylation inhibitor (DMI) fungicides are frequently used to control powdery mildew in strawberry. These fungicides specifically inhibit sterol biosynthesis via fungal cytochrome P450 sterol 14 α -demethylase (CYP51) (Kelly and Kelly, 2013). However, continuous DMI application has induced mutations in *CYP51* gene reducing the disease control efficacy of the fungicides (Erickson and Wilcox, 1997). The Fungicide Resistance Action Committee (FRAC, 2019a) reported that DMI fungicides have a medium risk of inducing pathogen resistance. The sensitivity of powdery mildew fungi to DMI fungicides has decreased at the field level in Japan (Nakano et al., 1992). Nakayama (2007) identified a single point mutation in codon 461 of *CYP51* gene in DMI-resistant powdery mildew fungi on strawberry and developed a polymerase chain reaction (PCR)-based method to detect DMI-resistant genotype. In certain instances, the cessation of DMI fungicide application has made powdery mildew difficult to control in strawberry cultivation. Both DMI resistance and legal fungicide application rate limits have aggravated the problem of powdery mildew infection. In 2007, the Japanese Government published a regulation to restrict the number of chemical fungicide applications per cultivation period regardless of the total application duration (Ministry of the Environment, Government of Japan, 2007). For extended cultivation periods, approved non-chemosynthetic fungicides with no application limit and a few fungicides with different modes of action can be applied. Even June-bearing strawberry varieties are at the risk of powdery

mildew infection and inadequate fungicide-induced protection although their harvest period is only approximately 0.5 y. It is challenging to establish effective disease control measures without any practical alternative techniques for everbearing strawberries throughout the cultivation periods.

Heat disinfection with hot water has been proposed as an alternative powdery mildew control method in strawberry cultivation (Okayama et al., 1997; Koitabashi et al., 2002). Hot water spraying (HWS) has been proven to be an effective powdery mildew control method. It has been tested as a temporary treatment measure in nurseries (Konishi et al., 2010) and practical fields (Yamagishi et al., 2009). Ogawara et al. (2012) and Sato et al. (2018) reported that powdery mildew could be continuously controlled in June-bearing strawberries with weekly HWS and chemical applications. HWS may also activate heat shock-induced resistance (HSIR) (Konishi et al., 2010; Widiastuti et al., 2013b). Arofathullah et al. (2019) developed a traction-type hot water sprayer based on the prototype of Sato et al. (2018) for use in a tomato nursery. In their system, the appropriate spraying speed was regulated via a flat directional nozzle, and the efficacy of the system against powdery mildew was empirically validated.

Induction of HSIR via heat treatment involves systemic acquired resistance (SAR) mechanism (Widiastuti et al., 2013b). SAR could be activated by chemicals that mimic natural signaling compounds from pathogens (Kohler et al., 2002; Deepak et al., 2006). Reminiscent of HSIR, chemical inducers do not have a direct effect on pathogens (Watanabe, 1977). Ishii et al. (1999) described that the application of chemical inducers of disease resistance could protect plants against pathogens without the emergence of SAR-resistant genotype. In this concept, HSIR may be a component of systemic protection against chemical-resistant pathogens and minimize the risk of resistance, because there are no reports of HSIR-resistant genotype development following the induction of heat shock defense responses.

In the present study, we investigated the seasonal changes in DMI-resistant powdery mildew occurrence in everbearing strawberry production and assessed

the validity of fungicide selection based on personnel observations. We also made certain modifications to the hot water sprayer developed by Arofathullah et al. (2019) and examined the efficacy of HWS to establish an effective disease control system for long-term everbearing strawberry cultivation.

2. Materials and Methods

2.1 Experimental site

The experiments were conducted from 2015 to 2018 in a strawberry production field in Rikuzentakata City, Iwate Prefecture, Japan, where frequent occurrences of powdery mildew have been reported by farmers. Strawberry plants were cultivated in several large-scale greenhouses.

2.2 Production of everbearing strawberries

Strawberry plants were cultivated twice in succession. The first cultivation period was from April 2015 to February 2017. The second cultivation started in March 2017 after implementing the disease-prevention measures and ended in February 2018. The everbearing strawberry variety Natsuakari (Okimura et al., 2011) was hydroponically grown in a greenhouse constructed with a wooden frame and plastic film. Coconut fiber in plastic cultivation cases (14 m × 0.3 m × 80 mm) was used as a substrate. The plastic cases were placed 0.9 m above the ground level. Strawberry seedlings were arranged in a two-row zigzag pattern during the third week of April 2015, with a spacing of 230 mm between plants and 1.1 m between rows. Each row consisted of two bed lines. There were 121 plants per line (7.91 plants/m²).

The surface of the substrate was covered with white thermal insulation. European honeybees (Doredore, Marutotokai Shoji Inc., Aichi, Japan) were used as pollinators throughout the cultivation period. Hydroponic fertilizer (Tank Mix F & B; OAT Agrio, Tokyo, Japan) was applied at the rate of 0.7 dS/m⁻¹ at transplantation and 1.0 dS/m⁻¹ after root establishment. The timing of nutrient solution application was set as follows: 1 min per 30 min in the summer and 2 min per 1–2 h in the winter.

All labor was consigned to official agricultural

workers. Heating and ventilation temperature thresholds in the greenhouse were set in the range of 8–28°C. An artificial heater was automatically activated when the ambient air temperature dropped to <8°C. Ventilation was manually applied when the ambient air temperature increased to >28°C. Runners and old leaves were removed at appropriate times. Deformed and rotten fruits were also removed during routine management. Lateral buds were left intact. All other management practices were performed according to the local conventions.

2.3 Control of powdery mildew

The following two treatments were tested: fungicides alone (F) and fungicides combined with HWS (F+HWS). Both treatments were implemented in the same greenhouse to assess the effects of HWS. No control (without treatment) plots were set up, as they often become the pathogen source and interfere the disease evaluation.

Throughout the cultivation period, the farm personnel visually inspected the plants, selected fungicides that they considered the most appropriate, and periodically applied them in accordance with the Agricultural Chemicals Regulation Law (Ministry of the Environment, Government of Japan, 2007). All applications were recorded to evaluate the decisions of the personnel. There are no legal restrictions on the number of applications of sodium hydrogen carbonate, copper, potassium hydrogen carbonate mixture, or other fungicides with comparable modes of action. Azoxystrobin, fludioxonil, flutianil, benomyl, iminoctadine, diethofencarb, thiophanate, cyflufenamid, pyribencarb, and penthiopyrad were also applied in March, May, July, August, September, and October 2017. Pyribencarb and benomyl belong to the high resistance risk group (FRAC, 2019a).

2.4 Hot water spraying system

A hot water sprayer developed by Arofathullah et al. (2019) was modified and tested in the present study (Fig. 1A). The nozzle position was selected to ensure bilateral coverage of strawberry plants (Fig. 1B). The distance between each flexible metal tube and the water pipe was 10 cm, which was wider than

Fig. 1 Schematic representation of the hot water sprayer modified from that of Arofatullah et al. (2019).
A: Overall structure of the hot water sprayer. a: rail; b: cart; c: height-adjusting pantograph; d: cold water hose; e: hot water hose; f: propane gas boiler; g: curtain rail; h: mixing valve; i: pressure gauge; j: thermometer; k: main valve; l: boom; m: wire rope; n: winch; o: speed controller.
B: Back view of the hot water sprayer. a: rail; b: cart; c: height-adjusting pantograph; k: main valve; l: boom; p: flexible tube; q: nozzle; r: bench; s: strawberry.
C: Nozzle water spray range. q: nozzle; s: strawberry; t: effective duration for heating plants, which should be >20 s + heating time; u: spray range.
D: Experimental strawberry cultivation and treatment setup in a greenhouse.

the space between plants in the zigzag rows. In this way, two nozzles could spray hot water on the entire leaf surface (Fig. 1C). A flexible metal tube was inserted between each nozzle and the spray boom to facilitate the adjustment of nozzle position according to strawberry plant growth without rearranging the main body of the system (Fig. 1D). HSIR was triggered when ≥ 1 leaf per plant was treated at the optimum temperature (Widiastuti et al., 2013b). To ensure the optimal treatment (50°C for 20 s), the sprayer was controlled using a winch through a wire rope at a 0.5 m/min traction speed and $54 \pm 1^{\circ}\text{C}$ for 20 s.

Thermocouple wires of diameter 0.3 mm were attached underneath eight randomly selected leaves of the same plant. Leaf temperature was monitored using the GL-200A data loggers (Graphtec Corp.,

Yokohama, Japan). HWS treatment was started in the first week of July 2015 and was applied weekly. HWS was suspended during August 2016 and February 2017 because of technical difficulties in the hot water sprayer and strawberry replanting, respectively.

2.5 Occurrence of powdery mildew and molecular determination of DMI resistance

The effect of HWS on powdery mildew occurrence was evaluated monthly from November 2015 to February 2018; disease index (DI) was used for this purpose. To evaluate the severity of spontaneously occurring powdery mildew, the leaves and fruits of randomly selected plants were sampled and disease severity was scored monthly from November 2015 according to the lesion area as follows: 0, healthy

leaves or fruits; 1, <10%; 2, $\geq 10\%$; 3, $\geq 30\%$; and 4, >50%. The DI was calculated as follows:

$$DI = [\sum (n \times v) / N \times Z]$$

where, n is the lesion score class, v is the number of samples in the score class, N is the highest score, and Z is the total number of samples. Plants that had less than five leaves or fruits were excluded from the evaluation. Four biological replicates were used for the F and F+HWS treatments. Each of these replicates consisted of 10 and 5 plants during the first and second cultivation periods, respectively.

For the genetic diagnosis of DMI resistance, 207 powdery-mildew fungus-infected samples from both treatments were collected and examined. Conidia and a part of the mycelia were collected from infected strawberry leaves and fruits from April 2016 to February 2018. Total DNA was extracted using a commercial DNA extraction kit (NucleoSpin Plant II; TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Two kinds of primer sets were used to amplify the target region involving the 1,532nd base in codon 461 and identify the DMI-resistant genotype with *1532F* (5'-GCTCTCCCTACACAAAATGTC-3') and *1532R-T* (5'-CATCGATGTCTCCCTGCTCT-3') and a susceptible wild type genotype with *1532F* and *1532R-C* (5'-CATCGATGTCTCCCTGCTCC-3') (Nakayama, 2007). PCR was performed with 30

cycles at 95°C for 120 s, 95°C for 15 s, 64°C for 60 s, and 72°C for 60 s using a 96-well thermal cycler (Applied Biosystem Verity Thermal Cycler; Thermo Fisher Scientific, Waltham, MA, USA). The final extension was performed after amplification at 72°C for 5 min using DNA polymerase (KOD FX Neo; Toyobo Co. Ltd, Osaka, Japan), according to the manufacturer's instructions.

2.6 Yield evaluation

All fruits were harvested twice weekly until January 2018. Only marketable fruits each weighing >6 g were counted. Total yield of all 121 experimental plants per treatment plot was determined. We could not include yield estimate replicates owing to the lack of research force in the disaster area.

3. Results and Discussion

3.1 Improvement in the hot water sprayer

The addition of a flexible metal tube was an improvement to the previous model, which enhanced product application efficacy. The new model was fitted with eight nozzles covering eight strawberry plants simultaneously. The effectiveness of this configuration was validated in the previous study (Arofathullah et al., 2019).

Figure 2 shows the typical change trends in leaf

Fig. 2 Changes in leaf temperature during hot water spraying. Leaf temperature was measured in eight randomly selected leaves (1–8). Hot water spraying was started at 0 s on strawberry leaves that emerged first. The spraying condition were as follows: temperature, $54 \pm 1^\circ\text{C}$; traction speed, $0.5 \text{ m}^3/\text{min}$; water application rate, $3.5 \text{ L}/\text{m}^2$.

Fig. 3 Effects of fungicide application and hot water spraying on the seasonal changes in powdery mildew occurrence in strawberry and chronological detection of putative DMI-resistant genotype of powdery mildew on strawberry by PCR. Samples were collected from both treatment regimens (fungicide only and fungicide plus HWS). Percentage of mutant samples was calculated by comparing the number of resistant samples identified with the total number of samples collected. F: fungicide only treatment, negative control. F+HWS: combined fungicide and hot water spraying treatment. The error bars indicate standard error (n = 4).

temperature. Only leaf numbers 2, 3, and 8 were exposed to the target leaf temperature of 50°C for 10 s possibly because they were located in the same line under the nozzle. Even leaves treated at higher temperatures for super optimal periods, they were in good condition without any damage. Konishi et al. (2010) reported that crown rot and gray mold were controlled in June-bearing strawberries under the same conditions.

3.2 Fungicide application history

In this study, 23 fungicides, including five DMIs, were applied throughout the cultivation period. In the first year of the study, triflumizole was co-applied with cyflufenamid in the second week of May. Since 2003, this mixture has been approved for use in strawberry cultivation in Japan to prevent the early emergence of DMI-resistant genotype (Haramoto et al., 2006). DMI fungicides were reapplied in the fourth week of September and the third week of October and November when powdery mildew symptoms appeared. In 2015, prophylactic agents including organic copper, live *Bacillus subtilis*, and *Talaromyces flavus* were sprayed at least once a month. Between January and April of the second year, when the disease progressed severely

under the F treatment, bicarbonate was sprayed consecutively with fenarimol and triflumizole. All samples collected in April 2016 were determined as wildtype (Fig. 3). Until April, the rationale for fungicide selection by the farm personnel suggested that they thought powdery mildew could still be controlled with DMI. Therefore, DMI-based myclobutanil was sprayed in May to reduce disease severity, which was efficacious under the F treatment until June. As the severity of powdery mildew increased in July, DMI-based difenoconazole was reapplied. Figure 3 shows the occurrence of minimal powdery mildew infestation under the F+HWS treatment until July 2016 suggesting that HWS may have suppressed the infection. After September, the symptoms recurred under both treatments, but slightly declined thereafter until the end of 2016. Eight of the 13 (62%) samples collected in September 2016 were DMI resistant (Fig. 3), whereas the incidence of DMI-resistant genotype increased to 100% in all samples (including F+HWS) from November 2016 to the end of cultivation (Fig. 3). Nevertheless, disease severity apparently differed between the F and F+HWS treatments, suggesting that HWS may have effectively suppressed powdery mildew, including

fungicide resistance.

The farm personnel sprayed therapeutic and prophylactic agents such as bicarbonate and organic copper from October 2017 until the end of cultivation as disease severity was high. Five high-risk fungicides with different modes of action and DMI-based myclobutanil were consecutively sprayed from the planting of new strawberries in February 2017 until August 2017. The application of high-risk fungicides in the absence of resistance management may rapidly promote pathogen resistance (FRAC, 2019b). The appearance of powdery mildew escalated until the end of cultivation.

DMI fungicides were applied 12 times throughout the cultivation period. Quinone outside inhibitor (QoI) fungicides such as pyribencarb and azoxystrobin were used as alternative fungicides as they have different modes of action.

A tentative fungicide application schedule was developed at the start of cultivation. However, the infection spread faster than expected, possibly because of invasion from neighboring farms. On the contrary, only DMI- and QoI-based fungicides showed therapeutic efficacy. Therefore, DMI fungicides were frequently and sometimes exclusively reapplied during cultivation. Even when DMI fungicides are administered thrice per growing cycle, the risk of occurrence of DMI-resistant powdery mildew fungi increases (Erickson and Wilcox, 1997). Bals and Gilles (1986) reported a reduction in the susceptibility of powdery mildew to triazoles after repetitive applications between 1975 and 1985. Nakano et al. (1992), based on field trials, reported that the susceptibility of powdery mildew fungi to DMI has been decreasing in Japan. Moreover, various DMI fungicide analogs against powdery mildew have been sold under different trade names, and this could further complicate fungicide application management. Farmers invest 17 working hours per day on an average for field management during the busiest season (Kumudini and Hasegawa, 2009) when farm personnel do not have sufficient time to evaluate fungicide selection. With limited knowledge of the active ingredients in fungicides, farmers tend to select fungicides by trade name

and target pest. Therefore, repeated applications of the same types of DMI fungicides could increase a selection pressure for DMI resistance. Furthermore, certain genotypes may have been relatively less susceptible to the chemicals such as triforine, fenarimol, and myclobutanil used in the present study (Nakano et al., 1992; Sombardier et al., 2010).

3.3 Efficacy of hot water spray against powdery mildew incidence

During the first cultivation in April 2015, powdery mildew appeared under both F and F+HWS treatments and persisted until December 2015. However, the apparent severity was lower under the F+HWS treatment than under the F treatment until the end of 2016 (Fig. 3). Powdery mildew infection under the F treatment was the most severe in April 2016 (DI 11.25). Thus, HWS suppressed the occurrence of the disease. However, powdery mildew infection increased under the F+HWS treatment after August 2016 as HWS was suspended at this time, because the direct therapeutic efficacy of HWS was not recognized. Figure 3 shows that the DI under the F+HWS treatment after August 2016 did not rapidly decrease after infection. Sato et al. (2018) reported that HSIR was only prophylactic and not therapeutic. Moreover, the field-level duration of induced resistance was <1 week in the June-bearing varieties. Yamagishi et al. (2009), Masunaga et al. (1998), and Okayama et al. (1997) reported that hot water and heat treatments were only transiently efficacious, possibly because the conidia persist after HWS. Therefore, HWS must be reapplied weekly. In the present study, powdery mildew infection under the F+HWS treatment was less than that under the F treatment.

The incidence of powdery mildew was observed under the F treatment from September 2017 until the end of the cultivation period and decreased only in October. This indicated that chemical application failed to control the disease spread. The appearance of powdery mildew under the F+HWS treatment was less severe than that under the F treatment until the end of the cultivation period in February 2018. Thus, HWS might help reduce

fungicide application frequency. Ogawara et al. (2012) reported that weekly HWS for 20 s at 65–67°C reduced the rate of chemical fungicide application to approximately 30%.

Defense response induction by HWS suppresses powdery mildew development by activating the endogenous pathogen resistance mechanisms in the host plant, suggesting that the SAR mechanism plays a role in HSIR (Widiastuti et al., 2013a). Similarly, Ishii et al. (1999) described the host defense-associated mechanisms of benzothiadiazole-induced disease resistance against Japanese pear rust and scab control. Pathogens resistant to these chemical inducers were not detected in these studies. As HWS and chemical inducers have similar action mechanisms, the risk of occurrence of HSIR- or SAR-resistant pathogens could be excluded. Therefore, HSIR could be efficacious for chemical-resistant pathogen control in crop protection.

3.4 Strawberry yield

The first strawberry cultivation period started in June 2015 and the second period began in April 2017. The total strawberry harvest under the F and F+HWS treatments was 1,084 and 1,108, 735 and 703, and 1,819 and 1,811 g/plant in the first, second, and both cultivation periods, respectively (Table 1). Statistical significance could not be tested as there were no replicates. However, no major yield loss was observed. Sato et al. (2018) reported that weekly HWS did not affect June-bearing strawberry yield throughout the cultivation period. Despite its relatively higher powdery mildew incidence, the yield under the F treatment was the same as that under the F+HWS treatment. The farm personnel

removed all diseased fruits as soon as they were found. This practice might have had a thinning effect that increased individual and total fruit weights especially under the F treatment. Further long-term cultivation studies using everbearing strawberry varieties are required to elucidate the influence of HWS on yield.

4. Conclusions

Here, HWS showed a potential to control powdery mildew in everbearing strawberry, although there was no evidence of the direct effects of HWS on powdery mildew infection. However, short-term successive application of hot water over the strawberry plants could not be achieved; this could result in the reappearance of powdery mildew, as the efficacy of the treatment lasts for a short duration. Therefore, HWS must be applied weekly for effective disease suppression even against DMI-resistant genotype that reappeared during long-term everbearing strawberry cultivation. A combination of fungicide application and HWS should be further investigated to ensure powdery mildew control. From the perspective of hot water cost, a propane gas boiler is the most recommended option because the exhaust could serve as a source of carbon dioxide enrichment in the greenhouse. This secondary effect can reduce cost. Overall, HWS is practically applicable, and it has several benefits as a component of systematic protection against chemical-resistant pathogens in strawberry propagation.

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Table 1 Yield of marketable fruits under the fungicide only (F) and fungicide plus HWS (F+HWS) treatments.

Period	F (g/plant)	F+HWS (g/plant)
Jun 2015–Jan 2017	1,084	1,108
Apr 2017–Jan 2018	735	703
Total	1,819	1,811

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要旨

イチゴうどんこ病に対するステロール脱メチル化阻害殺菌剤 (DMI 剤) の過剰使用は DMI 剤耐性菌出現の原因となっている。本研究では、DMI 剤耐性菌が優占する四季成り性イチゴの生産圃場において、牽引式温湯散布装置による温湯散布がうどんこ病感染防止に及ぼす効果を調査した。四季成り性品種「なつあかり」に対し $54^{\circ}\text{C} \pm 1^{\circ}\text{C}$ で 20 秒間、温湯を噴霧したところ、うどんこ病に対する全身抵抗性の誘導条件である葉温 50°C 、10 秒の目標温度が達成された。農薬と温湯散布を組み合わせた場合、2018 年 2 月の栽培終了まで発病度は農薬散布のみの場合より小さかった。しかし既に感染した葉での治療効果は認められなかった。DMI 剤耐性菌の出現は、初期は 0% であったが、2016 年 9 月、11 月にはそれぞれ 62、100% に達し栽培終了まで継続した。一方で農薬のみでは発病度は 2017 年 8 月から 2018 年 2 月まで上昇したが、温湯散布も行った場合、発病は極めて少なかった。これらのことから、温湯散布はイチゴに DMI 剤耐性菌を含むうどんこ病に対する抵抗性を誘導したのと考えられた。温湯散布は四季成り性イチゴの長期栽培において DMI 剤耐性菌を防除する効果的な手法となりうる。

キーワード

実証試験, 熱ショック誘導抵抗性, ステロール脱メチル化阻害剤, 長期栽培, 薬剤耐性