Original Paper

Prevention of Powdery Mildew Disease in Tomato Nursery by Improved Hot Water Spraying Device

Nur Akbar Arofathullah*, Ani Widiastuti**, Yufita Dwi Chinta***, Tomoyuki Kobayashi****, Sayuri Tanabata***** and Tatsuo Sato*****

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

**Faculty of Agriculture, Universitas Gadjah Mada,

***Graduate School of Environmental Science, Division of Biosphere Science, Hokkaido University,

**** Fukushima Agricultural Technology Centre,

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

トマト育苗場における温湯散布—うどんこ病に対する予防効果とその機作—

ヌル アクバル アロファトゥラ *・アニ ウィディアストウティ **・

ユフィタ ドウイ チンタ ***・小林智之 ****・七夕小百合 *****・佐藤達雄 *****

* 東京農工大学連合農学研究科, ** ガジャ・マダ大学農学部, *** 北海道大学大学院環境科学院,

**** 福島県農業総合センター, ***** 茨城大学農学部附属国際フィールド農学センター

In plant nurseries, reducing the frequency of chemical application is becoming a challenge owing to the appearance of hardly controllable pathogens, spread of diseases, and demand by farmers. This study was therefore conducted to develop a practical, alternative fungal control strategy against powdery mildew by using hot water spray in a tomato nursery. The expected effects of hot water spray treatment are induced resistance and disinfection. Gray mold was used as an experimental model to determine the conditions for practical application of hot water spray for inducing resistance to plant fungi by heat shock treatment. Hot water dipping of tomato seedlings at 50°C for 20 s induced resistance against gray mold and increased the expression of some pathogenesis-related genes, *viz.*, pathogenesis-related protein 1a (*PR1a*), basic intracellular β -1,3-glucanase (*GluB*), and basic intracellular chitinase (*Chi9*). A prototype of a towable hot water sprayer was developed, and its performance was tested in the field. When hot water was sprayed at 57°C $\pm$ 2°C while moving at a speed of 0.5 m/min, the leaf temperature of certain parts of the seedlings reached approximately 50°C for 20 s, thereby inhibiting powdery mildew. Moreover, it was not necessary to heat the whole plant at the target condition because heat shock is known to induce systemic resistance. These results suggest that hot water spraying might be an effective technique to prevent powdery mildew in tomato nursery.

Key Words : alternative protection, gray mold, powdery mildew, heat shock-induced resistance, nursery.

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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

茨城大学農学部国際フィールド農学センター

Center for International Field Agriculture Research &

Education, College of Agriculture, Ibaraki University,

Ami, Inashiki, Ibaraki 300-0331, Japan

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

1. Introduction

During recent years, the number of nursery specialists has been increasing in Japan. The supply of seedling (nurseries) from nursery producers helps to reduce labor force and production cost for the vegetable growers. Grafting is necessary for providing some desirable traits to nursery plants. The use of mechanical systems can accelerate the production of grafted nursery plants. Vegetable growers can systematically obtain grafted seedlings from nursery producers on schedule, as the cost of seedlings is comparable to that of raising seedlings on their own. Meanwhile, providing pathogen-free seedlings is an absolute requirement for nursery producers to prevent pathogens from spreading to areas where they are transported. Chemical fungicide use is not expected for making pathogen-free seedlings. There are two main reasons why nursery producers should reduce chemical use. One is the emergence of chemical-resistant pathogens. In the case of tomato, chemical resistance has already been reported in some major pathogens such as powdery mildew, gray mold (Rodríguez et al., 2014), and leaf mold. Overapplication of fungicides that contain the same chemical component is considered a major cause of pathogen resistance. Another reason is that vegetable growers request nursery producers to minimize the use of chemicals on seedlings. As the upper limit of chemical application for every registered crop is determined by the Japanese Agricultural Chemical Regulations, vegetable growers are accountable for the number and amount of chemical application in their production. Therefore, nursery producers are forced to balance pathogen-free seedling production with reduced chemical application.

Powdery mildew was not a serious disease in tomato because farmers were applying fungicides against leaf mold until leaf mold-resistant varieties were released commercially in Japan. These fungicides are thought to be effective against powdery mildew as well. Recently, because most of the new tomato varieties are leaf-mold resistant, farmers have been tending to refrain from fungicide

application. As a result, since 2000, powdery mildew has re-emerged as a highly infectious disease of tomato in Japan (Matsuda et al., 2001).

Induction of disease resistance by physical means is proposed as an alternative protection technique. In tomato, Sato et al. (2005) reported that heat shock treatment by hot water dipping induced resistance against gray mold, via increase in salicylic acid (SA) content and pathogenesis-related gene expression. A similar effect has been reported after hot water dipping of strawberry plant at 50°C for 20 s (Widiastuti et al., 2013b; Sato et al., 2018); barley at 50°C for 30–60 s (Schweizer et al., 1995); cucumber at 40°C for 120 s (Yoshino et al., 2011); melon at 50°C for 20 s (Widiastuti et al., 2011); and *Arabidopsis* at 45°C for 120–180 s (Kusajima et al., 2012). Widiastuti et al. (2013b) suggested that heat shock-induced resistance (HSIR) has multiple signaling pathways involving systemic acquired resistance (SAR) that is mediated by SA. SAR is expected to confer broad-spectrum resistance against various pathogens in different crops. To clarify the activation of HSIR, some defense-related genes were used as expression markers, such as pathogenesis-related protein 1 gene in *Arabidopsis* (Kusajima et al., 2012) as well as peroxidase, glucanase, and chitinase 1 genes in melon (Widiastuti et al., 2011). In such treatments, only a part of the plant is subjected to the shock condition, and HSIR is triggered systemically.

Hot water spraying has been suggested as a practical means of activating HSIR and directly inhibiting pathogen growth (Yamagishi et al., 2009; Ogawara et al., 2012; Yoshino et al., 2012; Sato et al., 2018). The prototype of hot water sprayer device has been developed in our previous study (Yoshino et al., 2012), and it was proven to effectively protect cucumber against gray mold as well as induce the accumulation of SA and the expression of peroxidase gene in the leaves. However, the implementation of sprayer prototype under field condition was laborious as the sprayer should be manually operated and was not designed to spray to multiple seedlings simultaneously.

The purpose of this research is to evaluate an

improved design of hot water spraying device based on boom configuration for the induction of disease resistance in tomato. To confirm the effectiveness of hot water treatment, activation of defense response by hot water dipping were performed against gray mold as a model experiment. The intervention of HSIR was confirmed by expression analysis of marker genes. Subsequently, a prototype of a hot water sprayer was developed and tested against powdery mildew in a tomato nursery.

2. Materials and Methods

1) Hot water treatment for inducing resistance against pathogen in tomato

Seedlings of tomato (*Solanum lycopersicum* L.) cultivars Momotaro and Natsunokoma were grown in pots with culture soil at room temperature with a 16:8 h (light: dark) photoperiod for 20 days. Natsunokoma has been used in a previous study for the initial investigation of HSIR (Sato *et al.*, 2005). On the contrary, Momotaro was used to confirm HSIR in different varieties. Two-leaf stage seedlings were used for the inoculation test. The aerial parts of seedlings were dipped upside down in water heated in a water bath to 50°C for 20 s (HWD). As a positive control, a plant activator, 1,2-benzisothiazol-3(2H)-one-1,1-dioxide (BIT; 5 ml of 1 mg/ml, Wako Pure Chem Industries, Osaka, Japan) was sprayed on the aerial parts of seedlings at the same time as the HWD treatment. BIT has been known to activate SAR via an SA-mediated signaling pathway. Non-treatment (NT) was used as the negative control. Gray mold inoculum was prepared and inoculated at 24 h after HWD or BIT treatment on December 9, 2013 according to Yoshino *et al.* (2011). Gray mold is caused by *Botrytis cinerea*, a polyphagous, saprophytic fungus, and used as an inoculum because of the difficulties in handling powdery mildew fungus, which is an obligate parasite with limited occurrence. Contrarily, the inoculum of gray mold can be used any time, because it can be cultured on agar medium (Choquer, 2007). Furthermore, the HSIR of tomato was discovered using gray mold (Sato *et al.*, 2005), and its mode of action has a close relationship with that of SAR against a broad

spectrum of pathogens (Schweizer, 1995; Widiastuti *et al.*, 2011; Widiastuti *et al.*, 2013b). The diameter of the disease lesions was measured 2 days after inoculation. Each treatment was applied to 5 plants and repeated 3 times.

2) Expression analysis of induced resistance marker genes

Seedlings of the tomato cultivar Momotaro with approximately the same leaf size were subjected to HWD, as described above. Relative changes in gene expression were measured by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from each leaf disk at 3 days after treatment by using a commercial extraction kit (RNAiso Plus; Takara Bio Inc., Shiga, Japan), and mRNA was reverse transcribed. Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with an anchored-oligo (dT)18 primer was used according to the manufacturer's instructions. The synthesized first-strand cDNA was used as the template for quantitative RT-PCR (qRT-PCR) on a Thermal Cycler Dice Real Time System (TP850; Takara Bio Inc.), according to the manufacturer's manual. Three genes encoding pathogenesis-related proteins: pathogenesis-related protein 1a (*PR1a*), basic intracellular β -1,3-glucanase (*GluB*), and basic intracellular chitinase (*Chi9*) were used as expression markers. The expression levels in each sample were normalized to the expression level of actin. The corresponding primer sequences were as follows: actin (U60480), forward 5'-cctatgttggtgatgaagctcagtc-3' and reverse 5'-tggtcttcgggagcaacacgaa-3'; *GluB* (M80608), forward 5'-tcttgccccatttcaagttc-3' and reverse 5'-tgcacgtgtatccctcaaaa-3'; *Chi9* (Z15140), forward 5'-ctccaatggtcttccacat-3' and reverse 5'-gaaattgctgtcttctctgc-3'; and *PR1a* (AJ011520), forward 5'-acaagctcaaaactcccctca-3' and reverse 5'-tcaaaagccggttgatttcaaga-3'. Three technical replicates of qRT-PCR were used for each biological replicate.

3) Development of a hot water sprayer for tomato seedlings

A hot water sprayer prototype was designed

Fig. 1. Schematic diagram and complete view of the developed hot water sprayer
A: plane of the structure, B: back elevation, C: nozzle angle, D: complete view
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, p: nozzle, q: bench, r: tomato seedling, s: spray area, t: effective duration for heating the seedlings, which should be more than 20 s + heating time.

(Fig. 1A and B) as follows. We set a cart (b) towed with a winch (n) through a wire rope (m) at 0.5 m/min (o) on a curtain rail (a). A cold-water hose (d) and a hot-water hose (e) were hung on the rail (g). A propane gas boiler (f) was used for heating water. The two water hoses were connected to a mixing valve (h) to maintain a temperature of 57°C

± 2°C, measured using a checking thermometer (j). The spray water amount was set at 3.5 L/min discharged through 15 nozzles via the main valve (k) monitored using a pressure gauge (i). Nozzles (p) were placed at every 10 cm in a boom (l). For effective spraying, flat, wide-angle non-mist chip nozzles (N-KAL-15R; Yamaho Industry Co., Ltd., Wakayama, Japan) were installed. The specifications of these nozzles are as follows: nozzle hole diameter, 1.5 mm; average particle diameter, 670 μm at 0.2 MPa; ejection amount, 0.99 L/min at 0.1 MPa; and zone of dispersion, 90°, based on the manufacturer's catalog. Tomato seedlings (r) were placed on the nursery bench (q). The nozzle height was adjusted to 5 cm above the tallest seedlings (c). To ensure uniform duration of exposure to high temperature at a certain point on the leaves, the flat nozzle was set along the moving direction, perpendicular to the ground (Fig. 1C). The leaf temperature was measured by thermocouple (wire diameter: 0.3 mm) recorded by data logger (GL-200A; Graphtec Corporation, Yokohama, Japan). The sensing parts of the thermocouple were attached behind the leaves by surgical tape.

4) Practical effect of hot water spray (HWS)

The performance of the sprayer for protection against powdery mildew in tomato was evaluated by inoculation test. Two-leaf-stage seedlings of the cultivar Rinka 409 were used for the HWS experiment in the greenhouse as this variety spread rapidly in five years during this study period. Any difference in tolerance against powdery mildew was not found out among Momotaro, Natsunokoma, and Rinka 409 varieties in the preliminary test. The HWS treatment was carried out as described above from October 8, 2015 onwards weekly, in a greenhouse. The conidia of powdery mildew were collected from infected leaves of tomato maintained in the greenhouse. Conidial suspension (2×10^4 conidia/ml) was sprayed on whole plants until run-off, immediately after the first HWS. Non-treated seedlings were used as negative control (NT). Disease severity was estimated by the extent of leaf area showing a lesion at 27 days after inoculation

(November 4) according to the following scale: 0, healthy leaf; 1, less than 5%; 2, 5% or more; 3, 25% or more; and 4, more than 50%. The disease index (DI) was calculated as follows: $DI = [\sum (n \times v) / 4 \times Z] \times 100\%$, where n is the lesion score as ranked, v is the number of samples in the score category, and Z is the total number of samples. The experiment was repeated thrice, with all leaves from five plants per replicate.

3. Results and Discussion

1) Hot water treatment for inducing resistance against pathogen in tomato

The diameter of the lesions was as follows: 11.4 mm in HWD, 6.8 mm in BIT, and 15.4 mm in NT for Momotaro; and 11.1 mm in HWD, 6.5 mm in BIT, and 15.0 mm in NT for Natsunokoma (Fig. 2). Compared to NT, HWD at 50°C for 20 s caused a significant reduction in the severity of infection in both varieties, although induced resistance in HWD was weaker than that in BIT. Resistance was induced against gray mold under the same condition (50°C for 20 s) as in melon (Widiastuti *et al.*, 2011) and strawberry (Widiastuti *et al.*, 2013b). Thus, the condition of hot water treatment (50 °C for 20 s) obtained in the present study is suggested as a common condition for inducing resistance in various crops.

2) Expression analysis of induced resistance marker genes after HWD

Tomato *PR1a* gene expression is upregulated during the invasion of root-knot nematode and treatment with exogenous SA (Lavrova *et al.*, 2017), whereas *GluB* and *Chi9* expression in tomato leaves is induced after treatment with methyl jasmonate and ethylene, as well as wounding (Wu and Bradford, 2003). Therefore, in the present study, *PR1a*, *GluB*, and *Chi9* were used as marker genes of HSIR to fungal infection in tomato. The expression of all tested genes increased 3 days after HWD (Fig. 3). This suggested that HWD activated the plant defense system in tomato in the same manner as that by HSIR, which was first reported in cucumber by Stermer and Hammerschmidt (1987). Also, our

Fig. 2. Effect of hot water dipping and BIT treatment on inducing resistance against gray mold in two tomato cultivars, Momotaro and Natsunokoma.

A: Gray mold lesion diameter; B: photograph of lesions on Momotaro; C: photograph of lesions on Natsunokoma.

a: NT, b: HWD, c: BIT

Different letters in each treatment indicate significant differences by Tukey's honestly significant difference test ($n = 3$, $P < 0.05$).

previous study in melon showed that HSIR has a non-specific mechanism in plant stress response, as it upregulates many defense-related genes in plants and functions against common pathogens (Widiastuti *et al.*, 2011). Furthermore, Yoshino *et al.* (2011) and Widiastuti *et al.* (2013a) reported that partial treatment of the first leaves with HWD could suppress disease symptoms in untreated leaves, which indicates the activation of systemic resistance mechanism. Based on these results, the HS treatment of only a part of a plant is sufficient to induce defense response in the untreated parts. Therefore, hot water spraying from the upper part of plants can be used as a practical means for inducing

Fig. 3. Changes in the relative expression level of pathogenesis-related genes in the tomato cultivar Momotaro.

A: *PR1a*, B: *GluB*, C: *Chi9*

Gene-expression levels in the first leaf at 3 days after HWD were quantified by qRT-PCR and normalized to actin expression. Vertical bars indicate the standard error (n = 6).

resistance.

3) Development of hot water sprayer

For practical application of hot water in a greenhouse, a novel hot water sprayer was designed in the present study (Fig. 1D). In this model, multiple nozzles were installed in a straight line, perpendicular to the ground in the forward direction, to cover multiple seedlings at the same time (Fig. 1B, C). The distance between each nozzle was set at 10 cm, which was equivalent to or shorter than the distance between seedlings grown in plastic pots of 9 cm diameter. Thus, one or more leaves could be treated with hot water; an even treatment of the entire seedling was not required because HSIR is a systemic reaction. One leaf is sufficient to induce systemic resistance in the entire plant, as has been demonstrated in cucumber (Yoshino et al., 2011), melon (Widiastuti et al., 2013a), and strawberry (Widiastuti et al., 2013b). This sprayer was rolled up on a rail using an electric winch installed at the end of the nursery bench. To attain the optimum condition (50°C for 20 s), a temperature higher than 50°C is required because of heat loss due to vaporization. Moreover, heating time must be added to a 20-s duration of the target leaf temperature. In other words, at least one part of the seedling must fall under the moving spray area of hot water during HWS + 20 s (Fig. 1C-t). As a result of the preliminary test (data not shown), the speed of the sprayer's horizontal movement was set at 50 cm/min.

Changes in leaf temperature are shown in Fig. 4. Because the sprayer was moving on the rail and the sensing parts of the thermocouple were attached randomly on any leaf of a seedling, there was a time lag among the sensing points. Only leaf no. 4, which was in-line with the nozzle, exceeded the required condition for inducing resistance. No damage was observed in the treated parts after treatment, although longer duration (20 s <) and higher temperature (50°C) conditions were recorded. If the spray temperature is higher than 50°C, the temperature recorded may be higher than the expected internal temperature (50°C) of the leaf.

Fig. 4. Changes in leaf temperature during hot water spraying
 Spraying condition: applied temperature, $57^{\circ}\text{C} \pm 2^{\circ}\text{C}$; amount of water, 3.5 L/min.
 Leaf temperature was measured in four randomly selected leaves (1–4).
 Hot water spraying was started at 0 s on the earliest emerging parts of tomato seedlings.

4) Practical effect of HWS

The severity of powdery mildew in HWS was significantly lower than that in NT seedlings (Fig. 5). The results of tomato HWS confirmed that partial achievement of optimum conditions in the whole plant succeeded in preventing powdery mildew. The same effect has been reported in strawberry under the same conditions (Sato *et al.*, 2018). Meanwhile, hot water should be sprayed once a week (total, thrice until disease observation) because the duration of HSIR has been found to be less than 1 week in both melon (Widiastuti *et al.*, 2013b) and strawberry (Widiastuti *et al.*, 2013a). It is possible that the powdery mildew spores remained after HWS, and therefore, HWS treatment was repeated owing to the short duration of HWS effect. The results obtained from the present study suggested that HSIR and direct effect of hot water contributed to an integrated effect of inducing plant resistance and impairing pathogen growth, as observed in strawberry (Yamagishi *et al.*, 2009).

The present study had a few problems to be solved. A propane gas boiler was used in the experiments. For practical use and further development of the hot water sprayer in tomato

Fig. 5. Effect of hot water spraying on disease index of powdery mildew.

Disease severity was estimated by the extent of leaf area showing a lesion at 27 days after inoculation. Disease index data shown are means of both 1st and 2nd leaves from four plants per replicate, with a total three replicates. Asterisk indicates a significant difference according to a two-tailed unpaired *t*-test ($n = 3$, $P < 0.05$). Vertical bars indicate standard error.

nurseries, reducing gas consumption should be addressed. Also, the exhaust gas from the boiler can be used for carbon dioxide enrichment in a greenhouse for increasing yield, therefore increasing cost efficiency of HWS treatment.

The limitations notwithstanding, the present study highlights that HWS could be an effective technique to prevent powdery mildew by only hot water in a tomato nursery and ensure reduction in the frequency of chemical application. Further studies are required to investigate the efficacy of this technique to prevent mildew in other crop plants.

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摘要

野菜育苗農家では、薬剤耐性菌の出現、病害拡散の防止、生産農家の要望のため農薬散布の削減

が課題になっている。そこで本研究ではトマト育苗農家で実用的に供しうるうどんこ病代替防除技術として、温湯散布（熱ショック）技術の開発を行った。温湯散布処理では誘導抵抗性および熱による消毒が期待される。温湯散布処理による糸状菌に対する抵抗性誘導の実用的な適用条件を決定するための実験モデルとして灰色かび病が用いられた。トマトの苗を 50℃ で 20 秒間温湯浸漬すると灰色かび病に対する抵抗性が誘導され、塩基性細胞内 β -1,3- グルカナーゼ、塩基性細胞内キチナーゼおよび病原感染特異的たんぱく質 PR1a の各病原感染特異的遺伝子の発現量が増加した。牽引可能な温湯散布装置を試作しその性能を生産現場で評価したところ、0.5 m /min で移動しながら 57℃ ± 2℃ の温湯を噴霧すると苗の葉の一部の温度は約 20 秒間、50℃ に達しうどんこ病が抑制された。さらに、熱ショックは全身的に抵抗性を誘導することが知られており、植物体全体を目的温度で加熱する必要はない。これらの結果から温湯散布はトマト育苗場でのうどんこ病防除に効果的である可能性が示唆された。

キーワード

代替防除、灰色かび病、育苗圃、熱ショック誘導抵抗性、うどんこ病