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Induction of disease resistance against *Botrytis cinerea* in tomato (*Solanum lycopersicum* L.) by using electrostatic atomized water particles

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ABSTRACT

This study examined the effect of electrostatic atomized water particles (EAWP) on the disease resistance of tomato against *Botrytis cinerea*. Disease development of *B. cinerea* was suppressed by EAWP pretreatment prior to pathogen inoculation. Treatment of tomato plants with EAWP resulted in the upregulation of the nitrate reductase (*NR*) gene, which led to the accumulation of nitric oxide (NO) and *S*-nitrosylated proteins in the cells. In addition, the salicylic acid (SA)-dependent chitinase 3 (*CHI3*) gene was rapidly induced in leaves pretreated with EAWP after the leaves were inoculated with *B. cinerea*. Light microscopy showed browning of the epidermal cells surrounding the inoculation sites and inhibition of fungal hyphal development in the cells. These results suggest that pretreatment of tomato leaves with EAWP inhibits the development of *B. cinerea* in the early stages of infection by regulating the redox flux via the NO- and SA-mediated signal transduction pathways.

Introduction

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a ubiquitous pathogen that causes gray mold disease, is necrotrophic, and attacks over 200 crop hosts worldwide. The pathogen mainly infects fruit, vegetables, and ornamental crops and can particularly affect both postharvest and greenhouse production [1,2]. Although the only effective means of controlling the disease is the application of synthetic fungicides, it is difficult to control because synthetic chemicals often lead to the development of fungicide resistance. Therefore, alternative methods for controlling the disease are required.

Electrostatic atomization is a method used to produce fine liquid droplets with diameters between ten and several hundred micrometers and a relatively narrow size distribution [3]. As shown in Fig. 1, water is dewed by a Peltier element in the discharge electrode (device) by applying a voltage between the discharge electrode and the ground electrode. Compared to other atomization techniques, electrostatic atomization has a number of advantages such as the easy handling of droplets by applying an electric field and avoiding the coalescence of droplets due to the electric charge of the same polarity on the droplets and a narrow size distribution of the generated droplets [4]. Electrostatic atomized water particles (EAWP) that are produced by the electrostatic atomization of moisture contain reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, as well as nitric oxide (NO), nitrate, and nitrite ions [5–7].

Plant defense mechanisms against necrotrophic pathogens, including *B. cinerea*, are complex, and despite extensive studies, their biochemical bases are not yet fully understood. Both ROS and NO are involved in plant immune systems [8,9]. They act as early signals during tomato cells-*B. cinerea* interactions, and they are part of the molecular mechanisms underlying tomato resistance to *B. cinerea* [10]. Kulye Mahesh et al. [11] have presented a model showing the roles of NO and ROS in disease resistance to necrotrophic pathogens. NO bursts play an important role in the disease resistance to necrotrophic pathogens, including *B. cinerea*, whereas ROS bursts have a negative role in resistance or a positive role in the expansion of disease lesions by necrotrophic. Thus, these studies have led us to hypothesize that EAWP containing NO and ROS may induce defense responses in tomato plants against *B. cinerea*.

NO is a key redox-signaling molecule. In plants, it is mainly generated by two enzymes: NO synthase (NOS) [12] and nitrate

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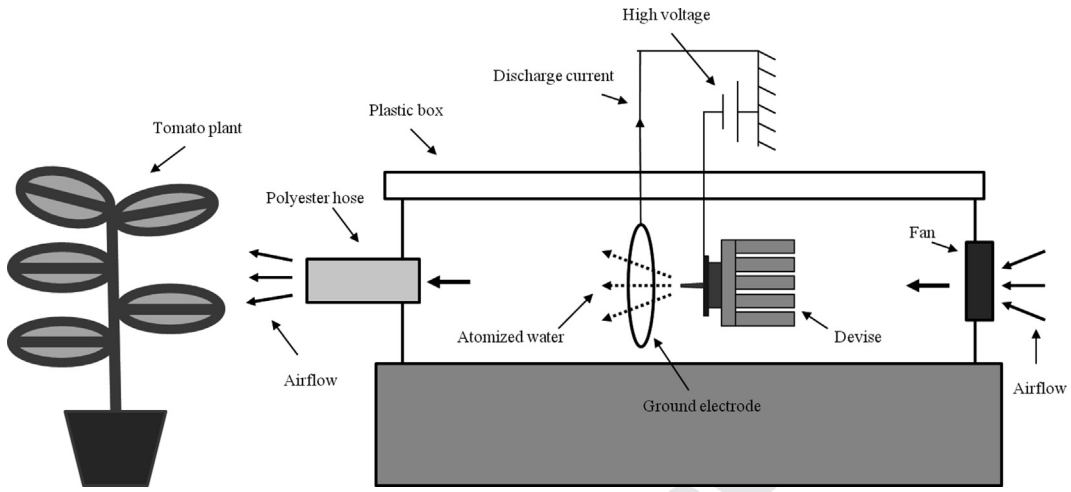

Fig. 1. A sketch of the apparatus used to generate regulated air flow containing electrostatic atomized water particles (EAWP).

reductase (NR) [13]. The NOS protein catalyzes the oxidation of L-arginine to L-citrulline and NO, and NR catalyzes the reduction of nitrite to NO [14]. NR has been identified as a source of NO in *Arabidopsis* [13], and homologous genes have been identified in other plant species [15]. The *S*-nitrosylated protein consists of the covalent addition of an NO moiety to a protein cysteine thiol, forming *S*-nitrosothiol (SNO) [16]. This reversible-protein modification plays a role in many physiological responses, including plant immunity by modulating enzyme activity [17–19], protein localization [20], and protein–protein interactions [21]. Additionally, cellular SNO is involved in both SA accumulation [22] and the expression of SA-dependent genes [23].

In the present study, we investigated the effect of EAWP on disease resistance in tomato plants against *B. cinerea* and discussed the mechanisms underlying EAWP induced defense responses.

Materials and methods

Plant materials and treatments

Tomato (*Solanum lycopersicum* L.) 'Momotarou' (Takii, Kyoto, Japan), 'Moneymaker' SA-deficient salicylate hydroxylase (NahG), and wild-type seeds were sterilized in 5% sodium hypochlorite for 10 min, rinsed, and then germinated for 5–7 days on moistened filter paper at 25 °C. After germination, the seedlings were transferred to plastic pots containing a mixture of vermiculite and perlite (1:1 mixture) and grown at 25 °C for 5 weeks under a 12-h photoperiod, with a photon flux density of 150 μmol photons m^{-2} s^{-1}.

A device (Electrostatic atomizer, Panasonic, Osaka, Japan) that generates EAWP was fitted into a plastic box (20 × 14 × 5 cm) which was attached to a fan (2.5 × 2.5 cm) and a polyester hose (1.5 cm diameter) was placed in a mini greenhouse (70 × 50 × 160 cm). Tomato plants were exposed to a regulated air flow containing EAWP from the plastic box that contained the EAWP-generating device (Fig. 1). The concentration of hydrogen peroxide in the EAWP was measured as described by Yamauchi et al. [7], and was regulated by controlling the wind speed and the treatment time per hour.

Fungal material and infection method

B. cinerea was isolated from tomato leaves that exhibited the typical symptoms of gray mold. The fungal isolate was maintained on potato dextrose agar (PDA) medium (Daigo, Tokyo, Japan) at 4 °C. Conidial spores were harvested by flooding the surface of 10–14-day-old PDA plates incubated at 25 °C with 50 mL of sterile, distilled water. For the disease development assessment and the gene expression analysis, droplets of 5 μL of spore suspension (5×10^5 spores mL^{-1}) in 1/2 potato dextrose broth (PDB) (Difco, Detroit, MI, USA) were deposited on the leaves of 5-week-old tomato plants. Inoculated plants were kept at 100% relative humidity for 3 days at 25 °C under a 12-h photoperiod with a photon flux density of 30 μmol photons m^{-2} s^{-1}. Lesion diameter was measured three days after incubation. To determine whether EAWP reduces gray mold in tomato plants, 5-week-old tomato plants were treated with EAWP (66 ng H_2O_2 cm^{-3} h^{-1}) for 7 days and were subsequently challenged with *B. cinerea*. The diameter of the developing fungal lesion was measured 3 days post-inoculation.

RNA extraction and quantitative RT-PCR

Tomato plants were treated with EAWP for 7 days and inoculated with *B. cinerea*. Quantitative RT-PCR analysis was performed at 1, 3, and 6 h post-inoculation (hpi). Leaves were frozen in liquid nitrogen and stored until use. Total RNA was prepared with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). The total RNA concentration was determined using a Biospec-nano (Shimadzu, Kyoto, Japan). Samples containing 1 μg of RNA were used for the RT, using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions, in a final volume of 20 μL. For the quantitative real-time PCR reactions, 1 μL of the 1:1 diluted cDNA was amplified in a 20-μL reaction in an optical 96-well plate, with three technical replicates for each sample, using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). The gene-specific primers designed to amplify 70–150-bp fragments from each gene were: glutathione peroxidase (*GPx*, Y14762), thioredoxin peroxidase 1 (*TPx1*, AY281152), thioredoxin (*Trx*, AF261142), nitrate reductase (*NR*, HQ616893), nitric oxide synthase 1 (*NOS1*, DQ539436), chitinase 3 (*CHI3*, Z15141), chitinase 9 (*CHI9*, Z15140), β-1,3-glucanase A (*GLUA*, M80604), β-1,3-glucanase B (*GLUB*, M80608), and the reference gene *Actin* (FJ532351). The following sequences were tested: *GPx* forward 5′-CATTGAAGATATCCAGCAAATGGT-3′, reverse 5′- CCATTCACGTCA ACCTTGTCA-3'; *TPx1* forward 5′-AGCATGTGCCTGGCTTTATTG-3′, reverse 5′-TTTTGCCCAGGCCTTCATC-3′; *Trx* forward 5′-AGGGCTC ATCCCGATACAAA-3′, reverse 5′-AGCAAAGAGCAATGGATTCCA-3′; *NR* forward 5′-CACCCAGAGAAGCCAACAAAG-3′, reverse 5′-AGCAAGTC AAGCACCTCAACCT-3′; *NOS1* forward 5′-TGACCAGGAGACATACGAG