Survival of SSG, an Endophytic *Burkholderia* Biocontrol Agent, on the Boxwood Leaf Surface[1]

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Abstract

Survival of *Burkholderia cepacia complex* (Bcc)-based biocontrol agents (BCA) has been associated with their field performance for foliage disease control. SSG, a strain of boxwood endophytic Bcc, suppresses a broad spectrum of plant foliage diseases, including boxwood blight, but the control efficacy declines over time. Factors affecting SSG survival on leaf surfaces were investigated to promote the application of the BCA for boxwood blight management. 'Justin Brouwers' boxwood plants were treated with SSG cells at 10^7 to 10^8 colony-forming unit (CFU)·ml^{-1}, maintained in a moist chamber at 10, 20, or 30 C (50, 68, 86 F), and sampled after the inoculum was blow-dried at 0, 3, 6, 12, and 24 h after treatment. The retained cells per leaf at 0 hours was 10^5 to 10^6 CFU, but only less than 10% of the cells survived 24 h after application, irrespective of the wet period and temperature. A wet condition of 12 and 24 h at 20 and 30 C facilitated SSG survival on the second day. Further survival of SSG was affected by temperature but not wetness. Damp conditions and pleasant temperatures can improve bacteria survival and stability and are keys to promoting BCA field applications.

Index words: Biocontrol agent, endophyte, survival on plant surfaces, temperature, wetness.

Species used in this study: Bacterium strain, SSG (*Burkholderia sp*);Plant species: *Buxus sempervirens* L. 'Justin Brouwers'.

Significance to the Horticulture Industry

Boxwood blight is a destructive and fast-growing disease threatening *Buxus* in the nursery, landscape, and plant sale industries. Chemical control remains the most used and effective method in response to the disease outbreak, but the cost and environmental footprints of chemicals also remain a serious concern to growers. Recently identified biocontrol agents for the management of boxwood blight have shown promise to reduce dependence on chemical control. However, the stability of microorganisms for field applications is poorly understood. This research investigated the impact of wet periods and temperatures on the survival of a potent blight biocontrol agent, *Burkholderia* strain SSG, on boxwood plants after the BCA was sprayed on plants. This study would assist those developing formulations to maximize the performance of the biocontrol agent under field conditions.

Introduction

Boxwood blight is a destructive, fast-spreading disease damaging private and public gardens worldwide (LeBlanc et al. 2018). The disease remains the number one problem of boxwood, although it has been around Europe for more than two decades and in the United States for about ten years (Calabro 2018, Gilson 2018, Ivors et al. 2012). Unfortunately, managing the disease mainly relies on repeated fungicide applications. Chemical control is expensive and temperately effective. It also can lead to fungicide resistance, human health effects including cancer, and environmental issues such as biological diversity reduction with harmful chemical residues and contaminated plants, soils, and groundwater (Anonymous 2019, Baudoin et al. 2015, Bush et al. 2016).

A few microorganisms have been identified as biocontrol agents (BCA) for alternative control options in boxwood blight management (Kong 2019, Kong and Hong 2017, Kong and Hong 2020a, Kong and Hong 2020b, Yang and Hong 2018, Yang and Hong 2017). These BCAs provided moderate to excellent plant protection from the disease under controlled conditions. In the laboratory test plants were maintained in a moist chamber for 24 to 48 hours at room temperature after spraying the BCA cell or spore suspension before inoculating with the pathogen. However, it is not clear whether such test conditions that are scarce in reality is necessary for the survival of these microorganisms and whether the leaf wetness period and surrounding temperature may affect BCA survival after treatment.

Developing a stable and effective formulation for the successful transition of BCA from the laboratory to the field requires our understanding of the survival or stability of biocontrol microorganisms after application (Leggett et al. 2011). Except for *Trichoderma koningiopsis* Mb2, a fungus that produces spore having a cell wall, all the recently identified BCAs are gram-negative bacteria. These bacterial BCAs survive poorly compared with the microorganisms producing spores (Mcquilken et al. 1998). It has been reported that the *Burkholderia cepacia* complex (Bcc) survives poorly on the leaf surface (Joy and Parke 1994). However, as a Bcc and a potent boxwood blight suppressor, SSG can survive in the soil for at least 50 days (Kong and Hong 2020b). Whether SSG may be exceptional in survival following foliar application remains unclear.

In this study, we investigated the survival of SSG on boxwood foliage under controlled conditions to better understand the ecology of Bcc BCA. Boxwood plants were

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placed in a closed box at 10, 20, and 30 C after been sprayed with the cell suspension from nutrient agar culture. The plant leaves were rinsed in sterilized distilled water (SDW) to suspend the SSG cells after the inoculum was dried at 0, 3, 6, 12, and 24 h. Survival of the cells on the leaves was determined based on the colony-forming units (CFU) of the SSG suspension. The ultimate goal was to help develop successful formulations and the application of SSG in the field by providing survival information of the BCA on the leaf surface after treatment.

Materials and Methods

Cell culture and suspension. The Bcc SSG strain was isolated from leaves of *Buxus sempervirens* 'Justin Brouwers' (Kong and Hong 2020b). It was recovered from a long storage at -80 C (-112 F) by subculturing on potato dextrose agar (PDA) at 28 C (82 F) for 48 h. A single colony of the recovered culture was then streaked on nutrient agar in 100 cm (39 in) plates and subcultured for another 48 h. For cell suspension preparation, the subculture was suspended in 100 ml sterilized distill water (SDW) and diluted ten times to have a concentration at 10^7 to 10^8 CFU·ml^{-1}. The concentration of the cell suspension presented as CFU was determined by plating 100 µl on the PDA as previously described (Kong 2019).

Plant growth and preparation. Two-year-old *Buxus sempervirens* 'Justin Brouwers' plants grown from liners were used. Liners were transplanted in 9 cm (3.5 in) wide pots filled with general-purpose PRO-MIX BX (Premier Horticulture Inc, Quakertown, PA): pine bark-based potting mix PM2 (Pacific Organics, Henderson, NC) at a 2:1 ratio. Plants were overwintered in a greenhouse and were fertilized with Miracle-Gro water-soluble all-purpose plant food with an NPK ratio of 24-8-16 (Scotts Miracle-Gro Company, Marysville, OH) twice per season and regularly irrigated before use. Plants were transferred from the greenhouse to growth chambers at 10, 20, and 30 C (50, 68, 86 F) a day before use for the SSG survival test. Plants to be tested were randomized in large plastic storage containers 55.9 × 33 × 44.5 cm (22 × 13 × 17.5 in) in each growth chamber before treatment.

Plant treatment and SSG survival test. Besides the three temperatures, five wetting times (0, 3, 6, 12, and 24 h) at each temperature were included for the SSG survival experiments. Fifteen plants, three for each wet period, were used for each temperature in an experiment. Three additional plants were added to each growth chamber in a separate container at different temperatures for an SSG background precheck. These other plants were not included in the time course assays for SSG survival. SSG is a small sage green bacterium on PDA. The background check was to determine whether the bacteria with similar morphology may be present on the plants before SSG treatment so that they can be subtracted after the experiment. For the background check, two leaves from each of three replicate plants were vortexed in 30 ml 0.01% Tween 80 in a 50-ml tube for 5 min to wash off the microbes from the leaves. Then a 50 µl aliquot of the suspension was plated on PDA. Small sage green colonies on the plates were counted after an incubation at 48 h at 28 C (82.4 F).

Freshly prepared SSG cell suspensions at 10^7 to 10^8 CFU·ml^{-1} were used as the inoculum for the SSG survival experiments. For each time point starting with the 24 h wet period, a set of 9 plants, three from each of three growth chambers at different temperatures, were sprayed with 20 ml of the bacteria cell suspension. The sprayed plants were transferred to a moist storage container that contained a layer of water and was lidded to keep the moisture at the same temperature except for those for the wet period of 0 h. The sprayed plants were blown for 15 min at a one-meter distance to allow the bacterial cell suspension to dry using a 91 cm (36 in) 2-speed direct drive drum fan (MODEL HVT-36C, Intertek Group plc, London, United Kingdom) immediately or after an incubation for a specific wet period. Two middle leaves were randomly sampled from branches of each of the blown three replicate plants of the same treatment and placed in 30 ml 0.01% Tween 80 in a 50-ml tube. The sampled plants were then placed back to the same growth chamber in a dry container in the same order of the original design. The sampled six leaves in the tube were vortexed for 5 min to wash off the bacterial cells of inoculum. The cell suspensions from leaf samples at shorter and 24 h wet periods were diluted 100 times to allow counting correctly. Fifty microliters of the diluted or undiluted suspension from sampled leaves were spread on two-replicate 100-mm PDA plates for calculating CFU per leaf. All the treated plants in the dry containers were sampled again after 24 h at 2, 4, and 7 days post-inoculation (dpi) to determine the effects of temperature and wet period on extended survival.

Statistics. A randomized complete block design was used for the SSG survival experiment that was run four times. Specifically, plants in each growth chamber at different temperatures were randomized by wetting time period, 3 plant pots per block and 3 blocks per treatment. At SSG treatment, plants for the same wet period at different temperatures were pooled and inoculated with the bacterial cell suspension. After the treatment, plants were placed back to their original container in the same growth chambers for incubation with the inoculum except for those for the 0 h wet period, which were blown and sampled immediately. Sampled plants with dried inoculum were arranged in the same order in a dry container of the same growth chamber for further sampling. The treatment CFU data were analyzed with the Real Statistics Resource Pack installed in Microsoft Excel (Microsoft Corporation, Washington, USA) using add-ins (Zaiontz 2020). One Factor ANOVA was used to analyze the homogeny of the data from the different experiments. Homogenous experiments were pooled for Two Factor ANOVA for determining the variance significance of treatments. Tukey's HSD test was used for the follow-up analysis separating the variances and determining the level of significance at $\alpha = 0.05$. Regression between the survival with temperatures and wet periods was done with the Data Analysis function in Microsoft Excel.

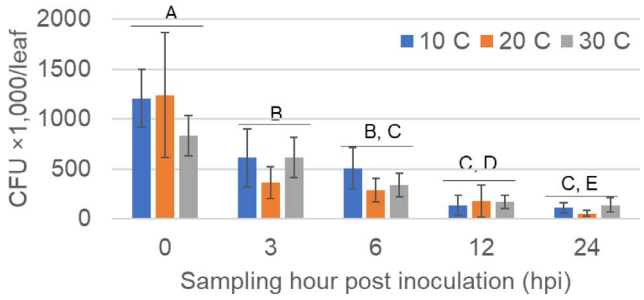

Fig. 1. Survival of SSG on boxwood leaves at different temperatures within 24 h after application. Plants were sprayed with SSG cell suspension at 10^7 to 10^8 CFU·ml^{-1}, placed in a growth chamber at 10, 20, 30 C, and sampled at 0, 3, 6, 12, and 24 h when the inoculum was blow-dried. The SSG colony-forming unit (CFU) per leaf was calculated based on colony counts of suspended bacteria from the sampled leaves. Each column is a CFU average from six leaves of four independent experiments. Bars indicate standard errors, and letters on the top of three columns of each time point indicate a significant level at α=0.05 of the difference among the sampling points. The samples with the same letter are not significantly different.

Results and Discussion

Morphologically similar SSG bacteria were not detected in the background check for all experiments. Therefore, the SSG counts of the experiments were all from the inoculated SSG. The initial concentration of SSG used for plant treatment was 10^7 to 10^8 CFU·ml^{-1}. Due to the immediate, observable runoff of the bacterial suspension from the leaves at spraying, our focus was mainly on the remaining SSG cells on the plant leaves immediately after the inoculated SSG suspension had dried. The retained SSG cells on the plant at zero-hour post-inoculation (hpi) ranged between 8×10^5 to 1.2×10^6 per leaf. There were no significant differences among the experiments within 24 h after application ($P = 0.2467$); therefore, all four experiments were pooled for the survival analysis. SSG CFU per leaf declined significantly with extended sampling time (Fig. 1). About half of the bacteria population was gone after 3 to 6 h, and only 10% was left after 24 h.

The decline was independent of temperature. SSG survived similarly on the plants at 10, 20, or 30 C. The CFU difference between temperatures was not significant ($P = 0.5411$), and no correlation was present between the temperature and bacteria survival ($P = 0.2343$).

To determine the impact of wetness on SSG survival, three of the four survival experiments were expanded. Plants sampled at each wet period of these experiments were sampled again at 1, 2, 3, 4, and 7 dpi to investigate CFU per leaf after the inoculum was dried up. Data from these experiments were pooled as there were no significant differences among the experiments ($P = 0.4283$, 0.9824, 0.0982, 0.2091 at 1, 2, 3, and 4 dpi, respectively). There was a positive correlation between the bacteria survival and the wet period ($R^2 = 0.6512$; $P = 0.0233$) at 24 h or 1 dpi. The decline from the 0 to 6 h wetting time was very substantial compared to wetting times of 12 and 24 h (Fig. 2). Compared to 0 to 28 CFU per leaf without a wet period (samples were immediately taken after the inoculum was blow-dried), 40,000 to 100,000 CFU per leaf was detected for samples with a 24 h wet period (Fig. 2). Similarly, CFU recovered from those with a wet period of 12 h was 13,000 to 15,000 CFU, while that recovered from plants with a shorter wet period was hundreds of times less. Therefore, previous leaf wetness extended SSG survival on the plant.

A similar decline trend was observed at 2 dpi ($R^2 = 0.97$, $P = 0.4813$) and 3 dpi ($R^2 = 0.86$, $P = 0.3166$), although there were no significant linear regressions between the decline and the wet period. SSG survival on boxwood at extended dpi was inferior. Most CFU per leaf, even with 12 or longer hours of previous wetness, was less than 500 (Fig. 2). The effect of previous wetness was no longer important on extended survival for SSG ($P > 0.5038$).

SSG survived similarly at 10, 20, and 30 C at the first two days after application (Fig. 2). The differences between the tested temperatures ($P = 0.3441$) and the interactions between these temperatures and wetness periods ($P = 0.1092$) were insignificant. This result indicated that SSG survival within 2 dpi was not affected by temperature.

In contrast, temperatures affected the extended survival of SSG after two days. The survival at 30 C at 3 dpi was significantly better than at 10 and 20 C ($P = 0.018$ and $P = 0.025$, respectively). A similar trend was observed for SSG survival at 4 dpi (Fig. 2), although there was no difference among the temperature ($P = 0.2263$). Very few SSG was recovered at 7 dpi (Fig. 2), indicating that neither temperature nor previous wetness was important for long term survival.

The endophytic Bcc SSG from boxwood has been shown many traits as an outstanding biocontrol agent, including wide-spectrum pathogen suppression and plant growth promotion (Kong and Hong 2020a, Kong and Hong 2020c, Kong and Hong 2020b, Kong et al. 2020). This study investigating SSG survival after plant treatment adds new insights into SSG ecology and its application.

Most gram-negative bacteria survive poorer than gram-positive bacteria because they do not produce heat- and desiccation-resistant spores (Mcquilken et al. 1998). Poor field performance of Bcc-based biocontrol agents has been reported on foliage diseases due to the lack of survival of the bacterial cells on the plant leaf surfaces (Joy and Parke 1994). However, how "poor is poor" has not been defined. This study quantified the survival of SSG, a member of Bcc, on plant surfaces after application through the time-course experiments. Our data show that more than 90% of SSG cells can die off just one day after application on foliage, and only one ten thousandths can survive for four days and almost none for a week, even if they were wetted for 24 h and kept at the optimal temperature after application. It was not expected that SSG was such a short-term survivor on boxwood plants because it survived in potting mix for at least 50 days as a residue after being used as a biosanitizer for diseased leaf debris (Kong and Hong 2020b). However, since SSG was isolated from boxwood leaves (Kong and Hong 2020b), the result is not surprising. As an endophyte, SSG may avoid dehydration and get nutrients in the plant tissue through interactions with the plant as rhizosphere microorganisms (Santoyo et al. 2016).

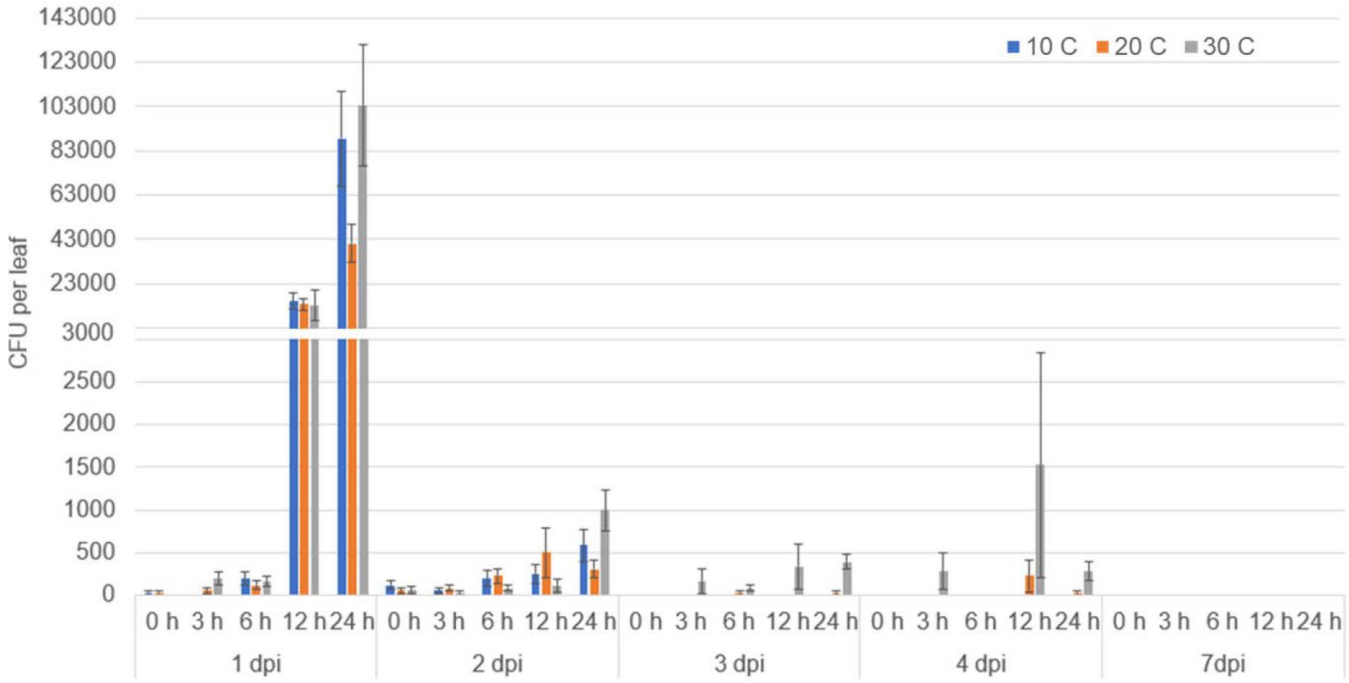

Fig. 2. Effects of wet leaf period and temperature on survival of SSG at 0 to 24 h after application. Plants were sprayed with an SSG cell suspension at 10^7 to 10^8 CFU·ml^{-1}, placed in a growth chamber at 10, 20, 30 C, and sampled at 1, 2, 4, and 7 days post-inoculation (dpi) after the inoculum was blow-dried at 0, 3, 6, 12, and 24 h. The SSG colony-forming unit (CFU) per leaf was calculated based on colony counts of suspended bacteria from the sampled leaves. Each column is a CFU average from six leaves of three independent experiments. Bars indicate standard errors.

This study identified leaf wetness as an important factor of SSG survival on the leaf surface. SSG in the suspension survives thousands of times better in a wet period of 12 h or longer after plant application regardless of the decline of its population with time progression (Fig. 1 & 2). Therefore, preventing bacterial cell dehydration or keeping leaf wetness is critical to promote Bcc-based biocontrol agent survival after application. Identifying effective formulants for the agent formulation is urgently needed, while scheduling applications according to the weather forecast for wet and cloudy conditions may improve SSG survival, promoting the efficacy of the BCA in the field. Many adjuvants can slow evaporation, aid in coverage over the leaf surface, break surface tension caused by waxes and oils, attract and bind water to the surface to prevent rapid drying and create films on the surface that hold in moisture (Burges ,998; Gossen et al. 2008), which may have potential and needs to be researched.

Comparatively, the temperature is not as crucial as leaf wetness. The impact of temperature on SSG survival after the application was not significant until three days after application, when most of the cells had already died off. It is unclear why the higher temperature was suitable for SSG when the SSG suspension applied on the leaves had dried out (Fig. 2). One possibility is that the bacterium thrives at a higher temperature. Meanwhile, a higher temperature increases plant photosynthesis, transpiration, and respiration, leading to oxygen and water level exchanges and the opening of the plant's stomata. As a result, SSG may gain moisture, nutrients, or even entry into plant tissue and survive better.

Other factors affecting SSG survival remain to be understood. SSG survives well in the potting mix where irrigation can maintain adequate moisture, the rhizosphere can provide nutrients, and there is little light exposure (Kong and Hong 2020b, Santoyo et al. 2016). Endophytes originate from the soil, where they assemble, colonize and enter plants (Trivedi et al. 2020). Certainly, SSG may survive better if it can enter the plant. However, since the majority of the bacterial cells died within a day, identifying successful formulations is key to ensuring maximum survival of SSG on leaf surfaces. Further investigating the impact of light on SSG survival is warranted. If the light is found to contribute to SSG survival, using adjuvants that protect it from UV light (Ravel 1999) may aid SSG efficacy in field applications.

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