Improvement of water-use efficiency of crops using symbiotic microorganisms: A potential sustainable approach to water management in cropping systems

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Abstract

This study focuses on a collection of endophytes comprising plant-living bacteria and yeast isolated from native Salicaceae trees in Washington State of the United States. The article demonstrates the agricultural potential of these endophyte isolates through laboratory and greenhouse experiments conducted under abiotic stress conditions, highlighting their capacity for biological nitrogen fixation (BNF) and other growth-promoting traits. Among the various benefits observed, this article discusses explicitly the enhanced water use efficiency (WUE) observed in host plants that could be utilized in agricultural water management in cropping systems. Elite endophyte strains were carefully selected based on their growth-promoting characteristics, BNF capacity, and in vitro phytohormone production profiling, confirming their ability to synthesize ABA and other hormones. Furthermore, a consortium of these endophytes successfully colonized rice plants and significantly increased biomass under N-limited conditions. In a subsequent study on leaf physiology, rice leaves inoculated with the endophytes exhibited reduced stomatal conductance during the daytime and decreased stomatal density. These responses were likely attributed to increased ABA content in the inoculated plants, resulting in reduced transpiration and improved WUE without compromising

photosynthetic capacity. Even with less opening stomata the inoculated rice plants were able to maintain photosynthetic capacity. This may due to re-assimilation of respiratory CO2 from the endophytes. Additionally, the endophytes facilitated electron transfer and CO2 diffusion in rice leaves, particularly under elevated CO2 conditions. These findings suggest that the enhanced WUE conferred by endophytes is closely related to stomatal responses in the host plant. The effects were particularly pronounced under N-limited, drought stress, and elevated CO2 conditions. However, the specific contribution of increased ABA synthesis, CO2 re-assimilation, or both remains unclear, necessitating further mechanistic investigation.

Keyword: Plant-endophyte symbiosis, Water relations, Photosynthesis, Respiration, Water-use efficiency

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Introduction

Climate change poses a significant challenge to agriculture, affecting crop yields and increasing the demand for food production due to the growing global population (Cai et al., 2015). Sustainable methods are needed to enhance crop resiliency and improve yield while reducing the reliance on chemical inputs and irrigation. Water usage, mainly through irrigation, is a significant concern, and innovative approaches to water use efficiency (WUE) are necessary due to unpredictable precipitation patterns and extreme weather events (Flexas, 2016).

To achieve higher crop sustainability, efforts include increasing plant water use efficiency through genetic manipulation (Franks et al., 2015), drought-tolerant genotype selection (Condon et al., 2004), and canopy structure optimization (Drewry et al., 2014). However, these techniques still need to be developed and may not be practical for large-scale implementation.

Endophytes, microorganisms living within plants, have been found to enhance plant fitness and performance under stressful conditions (Döbereiner, 1992). They offer a promising avenue to increase crop WUE and yields. Previous studies have demonstrated the benefits of endophytes in improving plant fitness under low water availability, including water stress tolerance (Gagne-Bourque et al., 2016；Khan et al., 2015；Khan et al., 2016). The use of endophytic symbioses in agriculture has the potential to supplant current methods and enhance crop resilience.

Endophytic bacteria and yeast were isolated from Salicaceae plants and have shown potential symbiotic traits, such as phytohormone production and N fixation (Doty et al., 2009). Previous studies demonstrated increased biomass and yield potential in rice when inoculated with endophytes (Kandel et al., 2015). However, the physiological benefits and mechanisms underlying these effects have yet to be fully explored.

Some endophytes produce abscisic acid (ABA), a hormone in stomatal control and development. Studies have shown that endophytes producing ABA can trigger stomatal closure

and affect stomatal development, potentially contributing to drought tolerance in host plants (Khan et al., 2016).

Rice is an isohydric species sensitive to water demand during the daytime (Parent et al., 2010). The control of stomatal openings is crucial for managing water resources and photosynthesis. The effects of endophytes on stomatal control have shown conflicting results in different plant species.

A series of greenhouse experiments were conducted using rice to understand endophyte effects on host-plant water relations comprehensively (Rho et al., 2018b). The study investigated physiological attributes such as stomatal responses, water use, and biomass gain upon endophyte inoculation. The effects of different endophyte strains and environmental conditions, such as water deficits, elevated CO2, and light levels, were also examined.

The study also explored the potential role of endophytes in mitigating the down-regulation of photosynthesis under elevated CO2 conditions(Rho et al., 2020). Like rhizobium bacteria in legumes (Ainsworth and Rogers, 2007；Rogers A. et al., 2009), endophytes may help alleviate down-regulation by facilitating carbon (C) and nitrogen (N) metabolic processes.

The respiratory activity of endophytes contributes to their energy requirements, derived from carbohydrates provided by the host plant (Rho et al., 2018a). The release of CO2 during respiration can be re-assimilated by the host plant for photosynthesis (Bloemen et al., 2013), potentially reducing the need for stomatal openings to take up atmospheric CO2. The re-assimilation of respiratory CO2 can benefit plants by providing an additional source of CO2 for photosynthesis.

Further investigations are needed to understand the respiratory behaviors of endophyte-symbiotic plants and the metabolic costs and benefits associated with endophytic symbiosis.

Overall, the study provides insights into the mechanistic impacts of endophyte symbiosis on water relations and photosynthesis in rice plants, offering valuable knowledge for enhancing crop sustainability and water management in agriculture.

Material and Methods

Experiment 1: A greenhouse study using multiple endophyte strains

1.1 Origins and inoculation preparation of endophytes

Nine strains of diazotrophic endophytic bacteria and yeast used in this study were previously characterized by Doty et al. (2009). These strains (WP1, WP5, WP9, WP19, WPB, WW5, WW6, WW7C, and PTD1) were isolated from wild black cottonwood and willow trees at the Snoqualmie River, Western Washington, while PTD1 was isolated from hybrid poplar (Doty et al., 2005). These strains have shown potential diazotrophic activity and positive effects on biomass increase in host plants (Doty et al., 2009；Kandel et al., 2015；Khan et al., 2015；Khan et al., 2016；Knoth et al., 2013；Knoth et al., 2014). We used the same inoculation method developed in prior studies.

The selected endophytes were grown on a N-limited combined carbon medium (NL-CCM) to maintain their N fixation ability. After confirming growth, cell suspension cultures were initiated in flasks with NL-CCM broth. The optical density of the bacterial culture was measured after 3-5 days using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, USA). The final inoculation concentration was adjusted to OD600 = 0.1 (~1 × 107 cells) using sterile deionized water and N-free liquid media (Doty et al., 2009). A mock inoculum was prepared as a control. All procedures were conducted using sterile techniques.

1.2 Preparation of plant and microbial materials

Three bacterial strains (WP5, WPB, and PTD1) were selected to study host plants' water relations, particularly stomatal behaviors. Experiment 1 was conducted from October 15th, 2014, to March 28th, 2015.

The M-206 rice variety (Oryza sativa subsp. japonica M-206, very-early to early-maturing variety) was chosen and surface-sterilized using 3% NaOCl. Seeds were rinsed with sterilized deionized water to eliminate the remaining NaOCl. Although this technique may not remove

all internal microorganisms, it ensures surface cleanliness for studying endophyte effects.

Seeds were planted in 1-gallon pots with horticultural root media (Sunshine Mix #2 Sun Gro Horticulture, Agawam, MA, USA). Four treatment groups (WP5, WPB, PTD1, and CTRL) with ten replicates each were arranged in a randomized complete block design. The greenhouse had controlled temperature, relative humidity (RH), and lighting conditions. After inoculation, plants received a N-free liquid nutrient solution and water.

1.3 Measurement of stomatal conductance

On the 163rd day after germination, at approximately the R3-4 growth stage, diurnal changes in stomatal conductance (gs) were measured at 3-hour intervals from 9 am to 6 pm using steady-state leaf porometers (SC-1, Decagon Devices, Inc., Pullman, WA, USA). The instruments were calibrated on-site before the initial measurements taken at 9 am.

Experiment 2: Growth chamber study with endophyte consortia

To gain mechanistic insights into stomatal conductance responses, we repeated Experiment 1 in the same greenhouse facility from March 23rd to July 20th, 2017. The objective was to test the hypothesis that endophyte consortia producing ABA would increase in vivo ABA concentrations in host plants.

2.1 Plant material preparation and inoculation

We followed the protocols from Experiment 1 for preparing the plant and microbial samples and conducting the inoculation process. In summary, 32 rice plants were grown in 3-gallon pots in four sunlit chambers. Half of the surface-sterilized plants were inoculated with the nifH endophyte consortium seven days after germination. The plants were cultivated under well-watered and N-limited conditions, and measurements and sampling were carried out over 104 days. The average air temperature, recorded using a data logger (CR1000, Campbell Scientific, Logan, UT, USA), was 21/17 ° C (day/night). The average daily light integral (DLI) was 10.8 mol m−2 d−1 of photosynthetically active radiation (PAR) with a 16/8-hour

photoperiod of supplemental lighting. Air temperature and light intensity were recorded every 15 minutes. The average relative humidity (RH) was 57% during the day and 59% at night.

2.2 Measurement of stomatal conductance

At 66 days after germination, around the V6-7 growth stage, gs of the youngest fully expanded leaves was measured at 12 pm and 6 pm using steady-state leaf porometers (SC-1). The measurement procedure described in Experiments 1 and 2 was applied.

2.3 In vivo ABA assay

Diurnal changes in in vivo ABA content were determined biochemically using the Phytodetek enzyme-linked immunosorbent assay (ELISA) kit (PDK 09347/0096, Agdia, Elkhardt, IN, USA). At 96 days after germination, rice leaf samples were harvested around the R3-4 growth stage at 12 pm and 6 pm. The fully expanded youngest leaves were frozen in liquid N in centrifuge tubes and stored at −80 ° C until further analysis. The samples were ground into a fine powder, and approximately 100 mg of each powder was transferred to a microtube. ABA was extracted using 1 mL of 80% methanol at 4 ° C overnight. The mixture was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was collected. The pellet was resuspended, and the extraction process was repeated with 1 mL of fresh 80% methanol at 4 ° C overnight. After centrifugation, the supernatant was combined with the previous day' s extracts. The pooled supernatant was dried using a vacuum concentrator until approximately 50 μL of liquid remained. The dried extract was diluted with TBS buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM MgCl2, 3 mM NaNO3) to a final volume of 500 μL. The diluted sample was further analyzed for ABA using the Phytodetek ELISA assay kit, and ABA concentrations were measured using a multichannel spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). Each sample (CTRL/MIX) at each time point had eight replicates.

Experiment 3: Water deficit study with endophyte consortia

Experiment 3 aimed to assess endophyte effects on long-term water use efficiency (WUE) under well-watered and water deficit conditions. The trial was conducted from July 14th to October 6th, 2015, using M-206 rice. Differences from previous experiments included endophyte treatment and fertilization/irrigation conditions outlined below. Measured metrics were weekly pot-based transpiration and biomass allocation at harvest.

3.1 Plant material preparation and inoculation/water deficit treatments

The average air temperature (day/night) was 29/20 ° C. The average DLI was 35.8 mol m−2 d−1 of PAR with a 16/8-hour photoperiod. The average RH was 57%/71% (day/night).

Following seed surface sterilization, four rice seeds were planted in 1-gallon pots with horticultural root media (Sunshine Mix #4). Thirty-two pots were prepared and placed in plastic buckets. Half of the samples were inoculated seven days after germination with an endophyte consortium. Each plant received a 2-mL inoculum of the consortium (MIX) using the described technique. The other half received a mock inoculum (CTRL).

Similar to Experiment 1, a randomized complete block design was used. Six pots without plants were used to measure weekly soil evaporation rates. Every week, 200 mL of full-strength N Hoagland solution was supplied, and pots were fully irrigated. After six weeks, half of the pots experienced water deficit (S), while the other half remained non-stressed (NS). The design included non-stressed control (NS_CTRL), non-stressed inoculated (NS_MIX), stressed control (S_CTRL), and stressed inoculated (S_MIX) groups, each with eight replications. Soil water potential was measured using a psychrometer (SC-10).

3.2 Calculation of water use efficiency

Weekly transpiration was recorded until harvest after four weeks of induced water deficits. Total transpiration was calculated. Measured dry weights were divided by total transpiration after 72-hour drying at 70 ° C. Pot-based total transpiration and dry weights were used to calculate WUE.

WUE of productivity = total biomass gain (g)/total transpiration (L/pot).

Experiment 4: CO2 enrichment study with a single endophyte strain

In this experiment, we aimed to investigate the stomatal responses influenced by endophytes under two different atmospheric CO2 concentrations.

4.1 Preparation of plant material and inoculation with endophytes

The host plant species chosen was M-206 rice. Seeds were surface-sterilized with 3% NaOCl solution and incubated in sealed Petri dishes on water agar. After germination, seedlings were transferred to pots filled with horticultural media. Endophyte-inoculated treatment (E+) received 2 mL of the endophyte inoculum applied to the crown of the seedlings, while the mock-inoculated control (E−) received an equal volume of endophyte-free solution. The pots were placed in plastic buckets for easy water and fertilizer supply. Each chamber accommodated both E− and E+ pots and an empty pot for monitoring soil evaporation. Weekly fertilizer supply and full irrigation were maintained.

4.2 CO2 treatment and inoculation

The experiment followed a 2 × 2 factorial design, with two atmospheric CO2 concentrations (ambient CO2, AMB, and elevated CO2, ELE) and two inoculation statuses (E− and E+). Four chambers were used, two for AMB and two for ELE. CO2 concentrations were controlled using air ducts and flowmeters. Each chamber accommodated eight pots and an empty pot for evaporation monitoring. Temperature, humidity, light intensity, and CO2 concentration were recorded. The average temperature was 23/19 ° C, RH was 60/66% (day/night), and the DLI was 9.1 mol m−2 d−1 of PAR.

4.3 Stomatal conductance measurements

At 128 days after germination, gs of the youngest fully expanded leaves was measured at

3-hour intervals using leaf porometers (SC-1). The measurement procedure was consistent with Experiment 1.

4.4 Simultaneous leaf gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence parameters were measured on the second youngest fully expanded leaves. Parameters such as net CO2 assimilation rate (A), gs, transpiration rate (E), and electron transport rate (ETR) were recorded using portable gas analyzers equipped with leaf chamber fluorometers. Intrinsic and extrinsic water-use efficiencies (iWUE and eWUE) were calculated. CO2-response curves (A/Ci curves) were constructed under ambient and low oxygen conditions to estimate photosynthetic parameters. The low-[O2] method was used to estimate leaves' mesophyll conductance (gm)(Bunce, 2009).

Experiment 5: A controlled environment in vitro and in vivo study using a single endophyte strain for respiration measurement

5.1 Leaf respiration measurements

Leaf samples were collected to measure CO2 release and O2 consumption to determine rice plants' respiration rates. One plant was randomly selected per pot 100 days after germination (DAG). The total leaf area of each plant was measured using a leaf area meter. The leaves were then placed in Petri dishes covered with aluminum foil to promote respiration. After dark adaptation, the Petri dishes were inserted into a soil flux chamber of a gas exchange measurement system to measure CO2 efflux. The CO2 release data was divided by leaf area measurements to estimate the area-based respiration rate. Immediately after the CO2 measurements, leaf samples were excised to obtain two leaf disks. The leaf disks were incubated in a chamber of an O2 electrode to measure O2 consumption. The leaf disks were collected, dried, and weighed to calculate a mass-adjusted respiration rate. This rate was then used to adjust the area-based rate by multiplying it by the specific leaf area.

5.2 Quantification of endophytic bacteria in planta

A separate experiment was conducted to quantify endophytic bacteria in rice plants. Surface-sterilized seeds were grown in pots under the same conditions as the main experiment. Half of the seedlings were mock-inoculated, while the other half were inoculated with WP5. The plants were grown under fully irrigated conditions until the panicle initiation stage. Bacterial extraction was performed on 100 DAG. Leaf, stem, and root tissues were separated and surface sterilized. Samples were homogenized and spread on agar plates for bacterial counting.

5.3 Estimation of microbial respiratory CO2 in planta

The assumption that rice provides sufficient carbohydrates for endophytic respiration was supported by measuring the soluble sugar content in the rice sap. The microbial respiratory CO2 release in planta (Rmic) was estimated by multiplying the bacterial density (CFU), the respiration rate (R), and the fresh weight (FW) of each tissue. Using a portable gas exchange measurement system, these estimates were compared to A measured by gas exchange measurements. Combined with the in vitro bacterial respiration survey data set, the estimates of microbial respiratory CO2 release in planta (Rmic) was determined using the following equation.

$$R_{mic}(\mu mol\ g^{-1}s^{-1}) = CFU\ (cells\ FW\ g^{-1}) \times R\ (\mu mol\ s^{-1}g^{-1}) \times FW(g)$$

Statistical analysis

Statistical analysis was conducted using R version 3.2.2 for all experiments. The specific procedures varied depending on the design of each experiment. Experiment 1 used a contrast matrix to compare control plants with three single-strain inoculated plants, with a blocking effect included in the model. For Experiment 2, a simple t-test procedure was applied at each time point to identify significant differences between control and inoculated plants. Experiment

3 employed a 2 × 2 factorial design with blocking effects on the experimental plot. Two-way ANOVA was used to analyze the response variables. In Experiment 4, a split-plotted CO2 treatment design was used. The chamber effect was found to be non-significant, and therefore, a two-way ANOVA was applied to the variables corresponding to the factorial design. The number of replications for Experiments 1, 2, 3, and 4 were eight, ten, eight, and eight, respectively.

Experiment 4 used a mixed-effect model due to the split-plot design and random effects. The N treatment was set as a random effect, and the inoculation treatment and CO2 treatment were considered fixed effects. The chamber effect was tested as a random effect in Experiment 4-2 and was determined to be insignificant. Two-way ANOVA was performed using a linear mixed-effect model regression, and Tukey’ s HSD method was used for within-group separation.

In Experiment 5, a standard two-sample t-test procedure was employed to test the statistical significance of differences in all measures. Blocking effects were removed in the analysis.

All statistical analyses were conducted using R version 3.2.2 (R Core Team, 2020).

Results and Discussion

Decreases in stomatal conductance during daytime by endophytes

The daytime decreases in gs were observed in E+ plants inoculated with multiple strains of bacteria (PTD1/WP5/WPB). These strains resulted in an average 27% decrease in gs at 12, 3, and 6 pm in Experiment 1 (Fig. 1), with significance levels of $P = 0.124$, 0.005, and <0.001, respectively. At noon, there were no significant differences in gs and in vivo ABA content between E− and E+ rice leaves (Fig. 2). However, at 6 pm, there was a significant decrease in gs ($P = 0.043$) and an increase in in vivo ABA concentrations ($P = 0.006$) in E+ rice leaves (Fig. 2). Overall, endophyte inoculation caused a nearly three-fold increase in in vivo ABA

concentrations in rice leaves.

Similarly, in Experiment 4, inoculating a single strain endophyte resulted in a significant daytime decrease in gs. There was an average 18% decrease in gs at 12, 3, and 6 pm (Fig. 4) with significance levels of P = 0.037, 0.013, and 0.081, respectively. No statistical differences were found in other time points between AMB and ELE conditions. At 9 am, there were no differences in measurements between E− and E+ plants (P = 0.195, Fig. 4). During the peak time of the photosynthetic gas exchange (12-3 pm), the differences in gs became more pronounced, showing 20 to 21% decreases in E+ plants. High CO2 levels reduced gs by 29% across both E− and E+ treatments (Fig. 4).

Consistent patterns of afternoon decrease in gs were observed in Experiments 1, 2, and 4 (Fig. 1, Fig. 2A, and Fig. 4). These experiments, conducted under different environmental conditions, showed similar patterns of decreased gs in the afternoon.

Two potential mechanisms explain the afternoon reduction in stomatal conductance. Firstly, it could be attributed to the effects of endophyte ABA production. ABA is a critical hormone in stomatal control, and the inoculated plants may have had higher ABA levels due to the additional ABA provided by the endophytes, leading to faster stomatal closure. Additionally, endophytes may induce faster circadian clock responses to environmental cues, enabling more efficient water use by the host plants. The two-fold increase in WUE observed in Experiment 3 under water deficit conditions supports this hypothesis (Fig. 3C).

The second explanation involves microbial respiration and the recycling of CO2 by plants. As gs decreased in the afternoon, CO2 supply from the atmosphere would drop in E+ plants. However, the increases in WUE suggest that E+ plants could maintain photosynthetic CO2 assimilation with less CO2 through stomata. It is possible that respired CO2 by endophytes in the intercellular spaces of leaves could be readily available for the Calvin cycle, avoiding the need for diffusion over longer distances. Previous studies have shown the re-assimilation of respired CO2 by plant tissues, highlighting the significance of respired CO2 sources in the assimilation process.

In contrast to our findings with bacterial and yeast endophytes, a meta-analysis by Auge et al. (2015) reported an average 24% increase in gs by mycorrhizae under water-stressed conditions. The mechanisms underlying these two symbiotic interactions differ, with mycorrhizae aiding in water absorption from the rhizosphere to enhance drought tolerance. At the same time, endophytes, as observed in this study, contribute to water conservation mainly by reducing gs.

Increases in WUE of hosts

We observed increases in biomass and WUE in rice plants under both non-stress (NS) and water deficit stress (S) conditions (Fig. 3). The water deficit treatment significantly affected all three measures: a 62% decrease in biomass ($P< 0.001$, Fig. 3A), an 85% decrease in total transpiration ($P< 0.001$, Fig. 3B), and a 221% increase in WUE of productivity ($P = 0.002$, Fig. 3C) for all plants (CTRL and MIX combined). E+ plants exhibited a 16% increase in biomass compared to E− plants under water deficit treatments ($P = 0.039$, MIX in Fig. 3A). The effect of endophytes in reducing total transpiration was more pronounced under the S treatment compared to the NS treatment ($P = 0.009$, interaction effect – INT – in Fig. 3B), with decreases of 30% and 22% in S and NS, respectively ($P = 0.096$ and <0.001).

The endophyte treatment significantly increased the WUE of the combined NS and S plants (84% increase, $P = 0.047$, Fig. 3C), primarily due to decreases in total transpiration (26% decrease, $P< 0.001$, Fig. 3B) rather than increases in biomass (16% increase, $P = 0.039$, Fig. 3A). The effectiveness of endophyte treatment was more pronounced under S, with WUE increases more than two-fold compared to NS (116% vs. 52% in Fig. 3C).

The alterations in stomatal development and diurnal behaviors, accompanied by plasticity in cell water relations, give host plants an advantage in water conservation during the daytime, particularly under high light and warmer conditions when evapotranspiration demand is high. Although stomata were closed and the supply of atmospheric CO_2 to the intercellular spaces was reduced, photosynthetic CO_2 assimilation was not affected by endophyte inoculation. This

advantage will likely accumulate over the entire growth period, indicating that it can have a more significant impact if there are more sunny days than cloudy and overcast days.

The decreases in cumulative total transpiration of the inoculated plants were more significant under water deficit conditions (Fig. 3B). The beneficial effects of endophytes on host plants under stress conditions have been reported in numerous studies. These microorganisms appear to activate the defense mechanisms of plants by signaling stress response pathways even before the stress is imposed, thereby enhancing the host's ability to cope with various stress conditions (Pandey et al., 2012).

Alleviation of photosynthetic down-regulation by endophytes under elevated CO2 conditions

The A/Ci curve analysis revealed that the ELE treatment led to photosynthetic down-regulation at the panicle initiation stage in E− plants under both high N (HN) and low N (LN) conditions (Fig. 5). However, the A/Ci curves of E+ plants showed higher asymptotes compared to E− plants.

In E− plants, the FvCB photosynthetic biochemistry parameters (Vc,max, Jmax, and TPU) of the A/Ci curves were decreased by the ELE treatment compared to E+ plants under ambient (AMB) conditions (Table 1). Under HN conditions, E− plants exhibited decreases of 10% in Vc,max, 3% in Jmax, and 21% in TPU in response to the ELE treatment. Under LN conditions, E- plants showed a 16% decrease in Jmax and a 2% decrease in TPU. In contrast, E+ plants did not show reductions in these parameters. Under ELE conditions, E+ plants had increases of 5% in Vc,maxand 14% in Jmax compared to E− plants under HN conditions. The increases were more pronounced under LN conditions, with E+ plants showing a 33% increase in Vc,max, a 7% increase in Jmax, and a 22% increase in TPU. These increases in parameters in response to endophyte inoculation were all significant (Table 1, $P < 0.01$). Significant interaction effects of INOC×CO2 were observed for Jmax and TPU ($P = 0.031$ and $P = 0.010$, respectively).

The down-regulation of C3 photosynthesis typically involves decreases in the initial slope

(Vc,max) and the asymptote (Jmax) of the A/Ci curve, as observed in our results (Fig. 5). This down-regulation starts with the accumulation of non-structural carbohydrates (NSCs) and starch in chloroplasts of source tissues exposed to long-term elevated CO2. This accumulation leads to a reduction in Rubisco turnover rates and ultimately decreases the content and activity of Rubisco and associated enzymes involved in CO2 assimilation in the Calvin-Benson cycle as a negative feedback response (Drake et al., 1997). Interestingly, E+ plants showed higher FvCB C3 photosynthetic parameters values, with higher Vc,max, Jmax, and TPU than E− plants under ELE conditions, regardless of N levels (Table 1). This pattern of A/Ci curves resembles the photosynthetic responses of legumes to ELE conditions reported by Ainsworth and Rogers (2007). They found that legumes showed fewer down-regulation symptoms compared to other C3 crop species in studies using FACE facilities worldwide, as evident from A/Ci curves and parameterizations. This phenomenon was attributed to the ability of legumes to utilize N derived from BNF to sustain Rubisco content and capacity under ELE conditions, as well as the increased sink strength provided by nodules formed by symbiotic rhizobium bacteria in legume roots. Ainsworth et al. (2004) experimentally demonstrated the source-sink relationship between plant hosts and symbiotic bacteria under elevated CO2 levels by manipulating the sink strength of the plant. They found that a decrease in sink capacity due to the absence of nodules resulted in significant down-regulation of photosynthesis. Furthermore, endophytes can fix N, which can be utilized for creating sink tissues and promoting biomass production (Kim et al., 2003).

In this context, a possible explanation for mitigating down-regulation in plants inoculated with N-fixing endophytes can be drawn from the legume-rhizobium symbiosis analogy. First, the BNF by endophytes is a well-established trait of plant-endophyte interactions, as supported by our previous study with Salicaceae endophytes (Knoth et al., 2014). Although we did not estimate the amount of N in the leaves originating from endophytes in the present study, the higher chlorophyll content in E+ plants compared to E- plants indicates an increase in leaf N status (Table 1). Leaf chlorophyll content is strongly correlated with leaf N content in

rice. SPAD units are commonly used as indicators of leaf N status. Therefore, the increase in chlorophyll content may explain the overall improvement in photosynthetic performance in the presence of N-fixing endophytes. Second, although the biological sink strength of endophytes has not been quantified, they actively consume carbohydrates provided by the plant host. Considering that other symbiotic associations (such as rhizobium bacteria and mycorrhizal fungi) can cost the host plant 5-20% of total carbohydrates, it is likely that endophytes also drain a significant amount of carbohydrates from the host, serving as active biological sinks. Host plants can allocate more extensive carbon reserves when abundant environmental substrates are available, particularly under ELE conditions.

Increases in ETR and gm under elevated CO2 with endophyte inoculation

The ETR was higher in E+ plants compared to E− plants only under the ELE treatment, and endophyte inoculation increased ETR by 20% and 28% in HN and LN conditions, respectively (Table 1). No changes in ETR were observed under AMB conditions in either N regime. The INOC×CO2 interaction effect was significant.

Although the specific mechanisms underlying the increases in ETR in response to endophyte inoculation under ELE conditions are challenging to determine with our data, the response was consistent under both N-sufficient and N-limited conditions. Woodward et al. (2012) found that symbiotic tomato plants with fungal endophytes exhibited increased photochemical efficiency (ΦPSII) compared to non-symbiotic plants. The increase in ΦPSII can explain the increased ETR since the relationship between the two is positively linear (Baker, 2008). This suggests that more ATP and NADPH were produced in the light reaction of photosynthesis per unit of absorbed light energy in symbiotic plants compared to non-symbiotic plants, and these energy-rich molecules are consumed in the process of CO2 fixation, as seen in the increases in Amax (Table 1, Fig. 5).

Considering that plants under elevated CO2 will experience limitations in photosynthesis due to decreased regeneration of RuBP (Ainsworth and Rogers, 2007), the increases in ETR

resulting from endophyte inoculation, along with other relevant PSII activities, are promising results.

Mesophyll conductance (gm) showed contrasting responses to endophyte inoculation depending on CO2 levels (Table 1), and the INOC×CO2 interaction effect was marginally significant (P = 0.053). Under ELE conditions, E− plants exhibited significant reductions in gm, with decreases of 29% and 70% under HN and LN conditions, respectively, while E+ plants showed an 18% increase with HN and a 27% decrease with LN. In response to endophyte inoculation under ELE conditions, plants showed increases in gm of 39% and 142% under HN and LN conditions, respectively. This was associated with increases in the ratio of intercellular to ambient CO2 concentration (Cc/Ci) of 4% and 58% under HN and LN conditions, respectively (Table 2).

Higher gm facilitates the diffusion of CO2 through the chloroplast walls and other layers along the pathway from the atmosphere to the site of carboxylation, allowing for a better supply of CO2 to the photosynthetic machinery (Flexas et al., 2008).

Increases in gm were observed only under ELE conditions, coinciding with ETR increases (Table 1). The coordinated mechanisms of photosynthetic electron transport in PSII, together with the supply of bicarbonate to the thylakoid space, have been described and reviewed by Govindjee et al. (1993) and van Rensen and Klimov (2005). Under ELE conditions, plants produce more carbohydrates due to increased carboxylation substrate supply. Microorganisms residing in the intercellular spaces of host plants consume photoassimilates through respiration, releasing CO2 that can readily dissolve into bicarbonate ions (Rho et al., 2018a). This may enhance the light-harvesting process in PSII, leading to an increase in ETR. Upregulating the activities of the PSII complex can also suppress stomatal opening without affecting CO2 assimilation, resulting in increased WUE, as observed in our results (Table 1). With more CO2 and NSCs under ELE conditions and, consequently, more microbial release of respiratory CO2, symbiotic plants have a better chance of having more internal CO2 available for assimilation. As a consequence, gm could be increased by this signal and further stimulate the entire

assimilation process, as indicated by the increases in Amax in our data.

Further increases in WUE with endophyte inoculation under elevated CO2 and low-N conditions

Overall, there were no significant changes in intrinsic water use efficiency (iWUE) in E+ plants in response to the endophyte inoculation. However, a significant increase was observed in the effective water use efficiency (eWUE) ($P = 0.028$, Table 1). The 58% increase in the ratio of intercellular to ambient CO2 concentration (Cc/Ci) is consistent with this increase in eWUE ($P = 0.096$).

Under HN conditions, the endophyte inoculation did not change eWUE in plants. However, under LN conditions, a significant 20% increase in eWUE was observed in E+ plants ($P = 0.045$).

Furthermore, we found that the response of WUE in E+ plants was influenced by N supply, with more significant increases observed under LN conditions compared to HN (Table 1). In contrast, Rho et al. (2018b) showed that Salicaceae endophytes increased WUE by reducing stomatal aperture during the afternoon while maintaining photosynthetic capacity under AMB conditions. Under ELE conditions, endophytes appear to modulate internal leaf components in the presence of abundant resources, such as carbohydrates. More fundamental approaches at the molecular scale are required to gain a mechanistic understanding of these responses.

Endophyte inoculation under AMB conditions did not significantly alter physiological characteristics at the leaf level (Table 1). This aligns with the findings of Rogers Alistair et al. (2012), who observed increased biomass. However, no effects on photosynthetic parameters such as A, gs, and photosynthetic WUE (i.e., either iWUE or eWUE) in Enterobacter-inoculated Populusdeltoides cuttings. The authors suggested that the productivity increases were more related to increases in leaf area at the whole-plant physiological scale. Although some parameters showed varying effects, E+ plants in our study displayed similar responses to

E− plants under AMB conditions.

Nevertheless, several photosynthetic parameters showed significant INOC×CO2 interactions, includingAmax, ETR, Jmax, TPU, gm, and Cc/Ci (Table 1). This indicates that endophyte inoculation enhanced these photosynthetic properties, which may be more efficient under ELE conditions.

Estimating microbial respiratory CO2 from endophytic bacteria in rice

A series of in planta and in vitro assays were conducted to estimate the effects of the bacterial endophyte strain WP5 on rice plants. WP5, isolated initially from native poplar trees in Washington State, was identified as Rahnella sp. through 16S rRNA sequence analysis. In planta assays were performed using rice as a C3 model crop, while pure cultures of WP5 were grown in MG/L media plates for in vitro assays.

Results from in planta respiration measurements showed that E+ plants exhibited higher rates of CO2 release (Rc) and O2 consumption (Ro) compared to E− plants, as measured by a gas exchange measurement system and a Clark-type electrode, respectively (Fig. 6). However, there was a significant difference between the two methods of measurement ($P< 0.001$). The Ro method detected a significant 159% increase in respiration rate in endophyte-inoculated plants ($P = 0.004$), while the 24% increase observed using the Rc method was insignificant ($P = 0.215$).

Estimates of microbial respiratory CO2 release demonstrated that endophytic microbes, such as WP5, can contribute a substantial amount of CO2 to the system (Fig. 7). The total respiration estimate for WP5 was 0.143 μmol CO2 g−1 s−1, which was similar to the actual photosynthetic assimilation rate of 0.127 μmol CO2 g−1 s−1.

The hypothesis for this study was derived from previous research indicating plants' re-assimilation of respired CO2. Bloemen et al. (2013) demonstrated that the upper leaf tissues can re-assimilate a portion of CO2 respired by root tissues. Similarly, Busch (2020) showed that photorespired CO2 in C3 plants can be incorporated into photosynthetic assimilation

processes. These findings provided the foundation for the prediction made in this study.

The estimated density of bacterial microbiota population on leaf surfaces is typically around 1 × 106-107 cells cm-2. Our microbial count information showed a similar range of microorganisms in the host plants. Based on our results, we estimated the bacterial respiration in rice plants to be 0.143 μmol CO2 g−1 s−1, which could contribute significantly to the plants' CO2 assimilation/production cycle. Although some respired CO2 is lost during mass transport in root and stem tissues, approximately 20% of transported CO2 could be re-assimilated. This suggests that an additional 0.071 μmol CO2 g−1 s−1 from microbial respiration could potentially be available for photosynthesis in leaves, accounting for approximately 57% of the total assimilation (Fig. 7). The difference in in planta respiration rates between E− and E+ plants observed by the Rc method (24%) was not as pronounced as that observed by the Ro method (159%). This indicates that some portion of the respiratory CO2 may reenter the photosynthetic assimilatory pathways and be incorporated into the plant before release, resulting in decreased detectable CO2 release in E+ plants measured by the Rc method.

Further empirical evidence, such as employing the 13CO2 method to differentiate photo-assimilates, is needed to verify this hypothesis.

Conclusion

In summary, our research aimed to assess the symbiotic impacts of Salicaceae endophytes on the eco-physiology of rice plants and evaluate the potential of using symbiotic microorganisms to enhance crop WUE for building sustainable cropping systems. Our focus was on carbon metabolism and water relations. The experiments were designed based on current knowledge of plant-microbe interactions.

The water relations study (Rho et al., 2018b) revealed that endophyte inoculations led to alterations in water relations and improved WUE in rice plants. Previous characterization

of Salicaceae endophytes demonstrated their potential to produce ABA, a phytohormone that regulates stomatal responses. We observed decreased stomatal conductance and density in endophyte-inoculated rice plants, reducing transpiration and water consumption. Despite these changes, the photosynthetic capacity and biomass of the inoculated plants remained unaffected, leading to a significant enhancement in WUE.

In the photosynthesis study (Rho et al., 2020), we investigated the effects of endophyte inoculations on photosynthetic performance under elevated CO2 conditions. C3 plants typically experience down-regulation of photosynthesis in response to long-term exposure to elevated CO2, resulting in reduced biomass gain. With their ability to fix atmospheric N, we hypothesized that Salicaceae endophytes would mitigate this down-regulation. Indeed, endophyte-inoculated rice plants displayed improved photosynthetic enzyme activities, increased Rubisco capacity, and enhanced internal CO2 diffusion for carboxylation, particularly under N-limited conditions. Consequently, these improvements increased WUE in the inoculated plants, especially under elevated CO2.

Furthermore, our respiration study (Rho et al., 2018a)explored the possibility of re-assimilation of endophytic microbial respiratory CO2. We found that endophyte inoculations increased the respiration rates of the host plants. In vitro, characterization of microbial respiration revealed a positive correlation between microbial respiration rates, microbial cell numbers, and carbohydrate supplies. The density of endophytic bacteria in plant tissues was significantly higher in the inoculated plants. Based on these findings, we estimated that around 57% of the CO2 assimilated by photosynthesis was re-assimilated from microbial respiratory CO2. This re-assimilation could compensate for the reduced uptake of atmospheric CO2 due to stomatal closure induced by endophytes.

Overall, our study provides insights into the physiological mechanisms underlying the symbiotic impacts of Salicaceae endophytes on rice plants (Fig. 8). The findings highlight the potential of harnessing endophytic symbioses to improve crop WUE and contribute to sustainable cropping systems.

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Table 1 Descriptive and inferential statistics of photosynthetic parameters at the operational points of the CO2 response curves (Fig. 5). The means of four and eight replicated responses are provided with the standard errors of the means in parentheses. F-statistics of two-way ANOVA test results for inoculation effect (INOC), [CO2] effect (CO2), and their interaction effects on various photosynthesis characteristics are presented with statistical significance codes. The corresponding P-values of the F-statistics are presented in parentheses. Adapted from Rho et al. (2020).

N	CO2	INOC	n	Amax μmol CO2 m-2 s-1	ETR μmol CO2 m-2 s-1	Vcmax μmol CO2 m-2 s-1	Jmax μmol CO2 m-2 s-1	TPU μmol CO2 m-2 s-1
HN	AMB	E−	4	25.46	128.1	103.3	135.6	11.77
				(0.476)	(5.246)	(2.570)	(10.94)	(0.851)
		E+	4	25.17	127.8	113.9	149.9	10.75
				(0.029)	(5.017)	(7.645)	(5.545)	(0.321)
	ELE	E−	4	26.36	103.8	93.11	131.5	9.308
				(0.481)	(2.431)	(3.539)	(3.155)	(0.364)
		E+	4	32.23	124.9	108.12	155.2	11.35
				(2.437)	(9.380)	(6.902)	(10.90)	(0.816)
LN	AMB	E−	8	10.85	84.66	35.65	89.90	5.979
				(0.797)	(7.882)	(3.059)	(4.460)	(0.326)
		E+	8	11.14	85.53	43.96	86.40	6.525
				(0.940)	(5.339)	(5.147)	(5.405)	(0.362)
	ELE	E−	8	14.23	91.77	36.50	75.50	5.854
				(0.663)	(6.745)	(2.529)	(2.553)	(0.162)
		E+	8	17.71	117.3	47.58	96.13	7.284
				(1.348)	(7.909)	(5.825)	(5.819)	(0.392)
INOC				9.441**	4.910*	9.780**	8.923**	6.989*
df = 1				(0.004)	(0.032)	(0.003)	(0.005)	(0.011)
CO2				34.29***	2.308	0.118	0.060	0.101
df = 1				(< 0.001)	(0.136)	(0.733)	(0.807)	(0.752)
INOC x CO2				6.310*	4.518*	0.233	5.022*	6.562*
df = 1				(0.016)	(0.039)	(0.632)	(0.030)	(0.014)

gs mol H2O m-2 s-1	gm mol CO2 m-2 s-1	Ci/Ca unitless	Cc/Ci unitless	E mmol H2O m-2 s-1	iWUE mol CO2 mol-1 H2O	eWUE mol CO2 mol-1 H2O	SPAD unitless
0.593	0.190	0.783	0.543	6.517	0.0046	0.393	41.57
(0.091)	(0.023)	(0.032)	(0.028)	(0.329)	(0.0007)	(0.017)	(1.157)
0.636	0.160	0.799	0.461	7.143	0.0042	0.369	42.77
(0.047)	(0.007)	(0.016)	(0.028)	(0.335)	(0.0004)	(0.018)	(0.510)
0.612	0.135	0.883	0.713	5.851	0.0047	0.473	40.75
(0.095)	(0.009)	(0.020)	(0.020)	(0.702)	(0.0009)	(0.061)	(1.016)
0.627	0.188	0.873	0.742	5.783	0.0052	0.558	43.35
(0.041)	(0.027)	(0.009)	(0.013)	(0.044)	(0.0004)	(0.044)	(1.187)
0.191	0.166	0.744	0.732	2.259	0.0058	0.484	38.42
(0.016)	(0.031)	(0.014)	(0.066)	(0.191)	(0.0003)	(0.022)	(1.465)
0.177	0.150	0.717	0.642	2.069	0.0065	0.540	38.62
(0.021)	(0.049)	(0.011)	(0.056)	(0.139)	(0.0003)	(0.028)	(1.071)
0.164	0.050	0.784	0.433	1.709	0.0097	0.844	37.36
(0.026)	(0.014)	(0.022)	(0.076)	(0.116)	(0.0011)	(0.033)	(1.515)
0.155	0.121	0.736	0.684	1.815	0.0121	1.013	41.85
(0.018)	(0.022)	(0.020)	(0.069)	(0.204)	(0.0010)	(0.070)	(2.562)
0.006	1.134	2.898°	1.006	0.133	2.627	5.176*	3.294°
(0.939)	(0.294)	(0.096)	(0.323)	(0.717)	(0.112)	(0.028)	(0.076)
0.315	5.456*	12.09**	0.032	11.63**	29.25***	74.17***	0.315
(0.577)	(0.025)	(0.001)	(0.857)	(0.001)	(< 0.001)	(< 0.001)	(0.577)
0.012	3.988°	0.664	5.594*	0.009	1.254	2.325	1.928
(0.912)	(0.053)	(0.420)	(0.024)	(0.9 26)	(0.269)	(0.135)	(0.172)

Statistical significance codes: o, *, **, and *** for P < 0.10, 0.05, 0.01, and 0.001 levels, respectively.

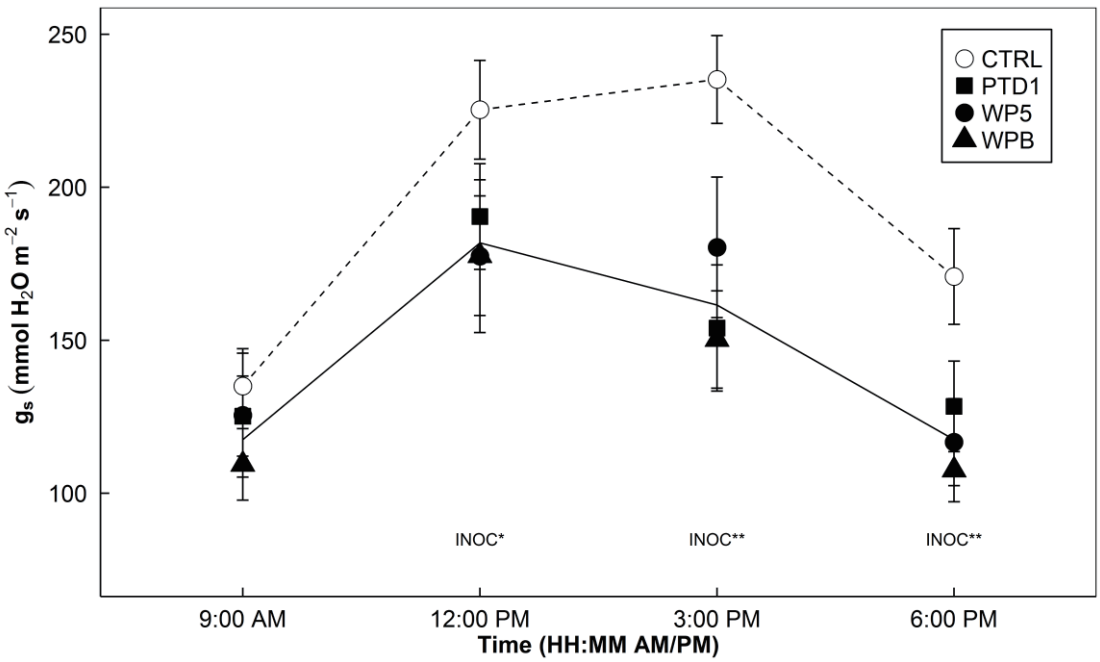

Fig. 1 Diurnal patterns of stomatal conductance (gs) of rice leaves on 163 days after germination in a greenhouse bench experiment (Experiment 1). Open symbols indicate mean gs of control groups, whereas closed symbols indicate mean gs of single strain-inoculated groups (square/circle/triangle = PTD1/WP5/WPB, individually). Error bars of the means represent ± 1 S.E. of replicated samples (n = 10). Single strain endophyte inoculation effect (INOC) is provided at P< 0.05 (*), 0.01 (**) levels. Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison. Dotted and solid lines highlight mean responses of CTRL and INOC plants over time. Adapted from Rho et al. (2018b).

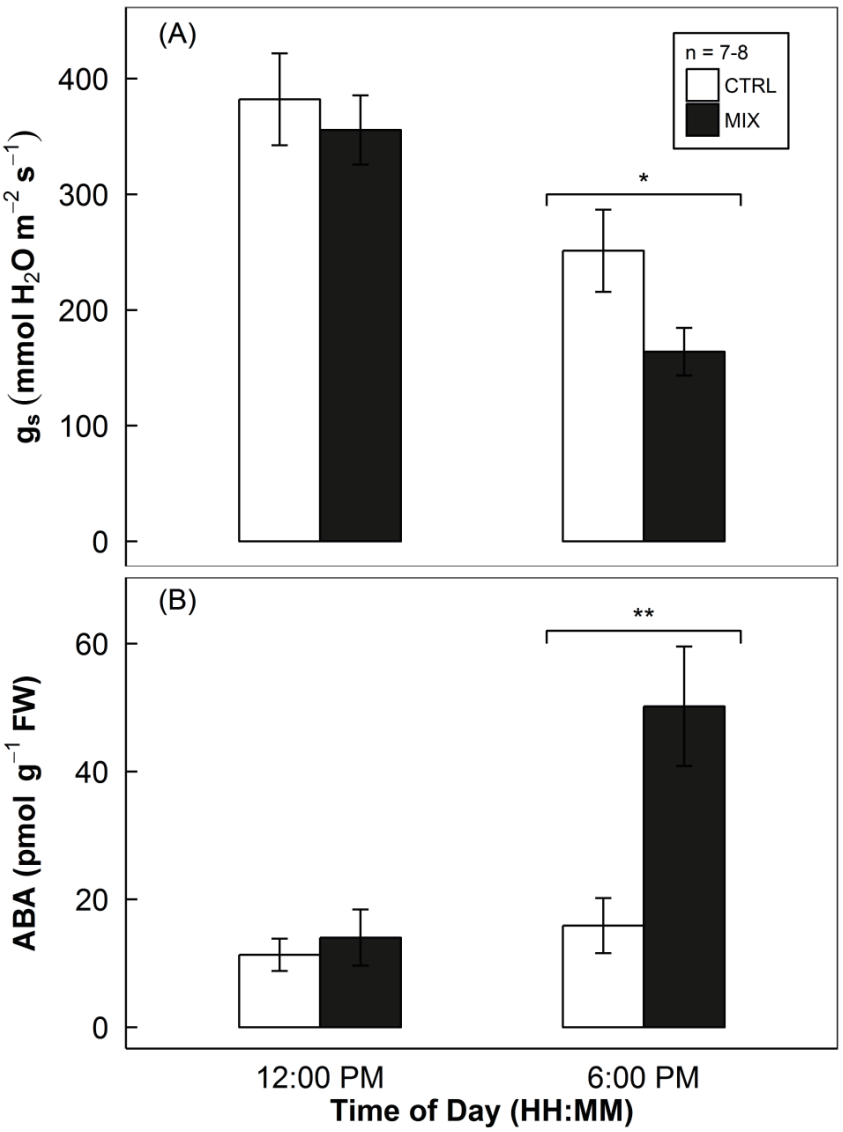

Fig. 2 Stomatal conductance of rice leaves at round V7-8 stage (top panel, A) and in vivo ABA concentrations (bottom panel, B) of rice leaves harvested at around R3-R4 stage in a greenhouse sunlit chamber experiment (Experiment 4). Open and closed bars indicate mean responses of mock-inoculated controls (CTRL) and endophyte consortium-inoculated (MIX) plants, respectively, provided with error bars as ± 1 S.E. of the means (n = 7-8). Endophyte inoculation treatment effect (INOC) is provided at $P< 0.05$ (*) and 0.01 (**) levels at each time point. Adapted from Rho et al. (2018b).

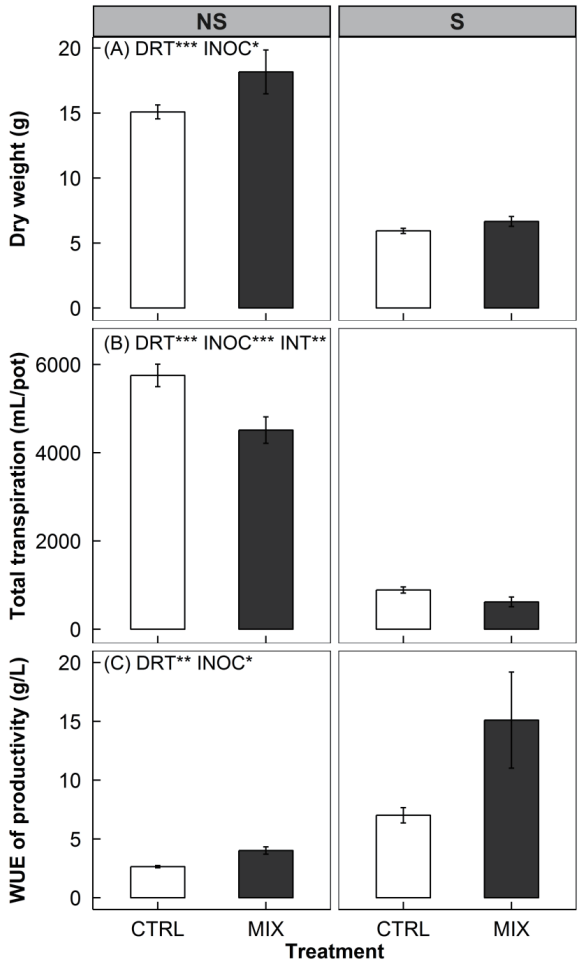

Fig. 3 Total biomass (top panels, A), total transpiration over time (middle panels, B), and water use efficiency (WUE) of productivity (bottom panels, C) of rice without (left panels, NS) and with (right panels, S) water deficits at harvest in a greenhouse bench experiment (Experiment 3). Open and closed bars indicate means of mock-inoculated controls (CTRL) and endophyte consortium-inoculated (MIX) plants, provided with error bars as ± 1 S.E. of the means (n = 8). Two-way ANOVA test results of the treatment effects are placed on each panel. Water deficit treatment effect (DRT), endophyte inoculation treatment effect (INOC), and interaction effect (INT = DRT x INOC) are provided at $P < 0.05$ (*), 0.01 (**), 0.001 (***) levels. Adapted from Rho et al. (2018b).

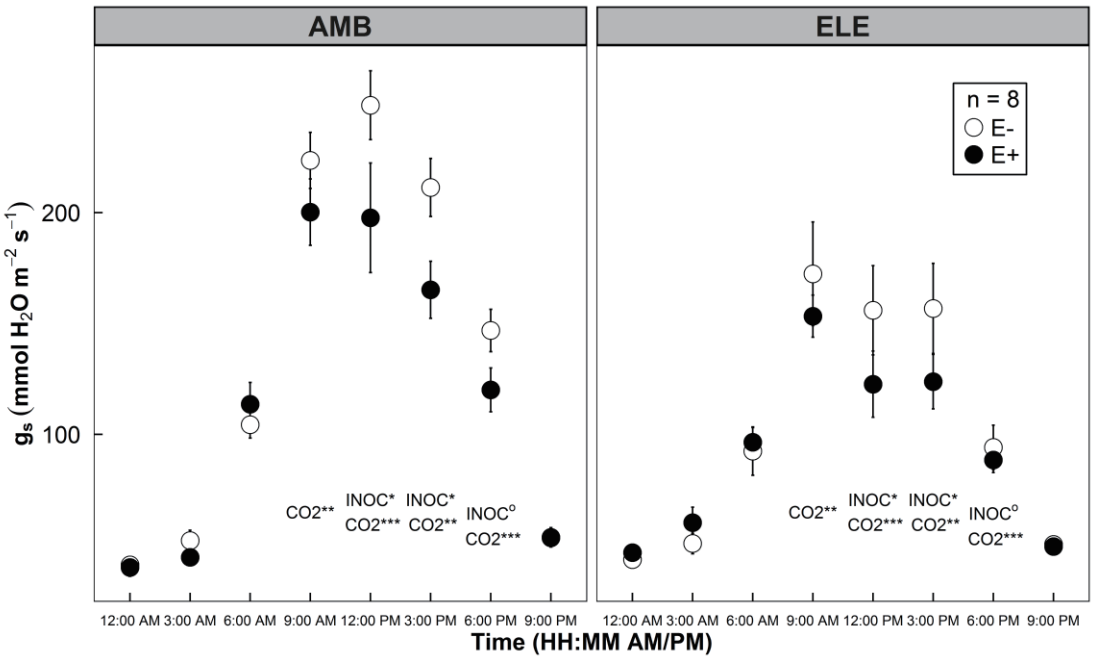

Fig. 4 Diurnal patterns of stomatal conductance (gs) of rice leaves grown under two atmospheric CO2 conditions: ambient (AMB, app. 400 ppm on the left panel) and elevated (ELE, app. 800 ppm on the right panel) in a sunlit chamber experiment (Experiment 2). Open symbols indicate mean gs of control groups (E−), whereas closed symbols indicate mean gs of WP5 inoculated groups (E+). Error bars of the means represent ± 1 S.E. of replicated samples (n = 8). Two-way ANOVA test results are indicated at each time point. CO2 treatment effect (CO2) and endophyte inoculation treatment effect (INOC) are provided at P< 0.10 (o), 0.05 (*), 0.01 (**), 0.001 (***) levels. Adapted from Rho et al. (2018b).

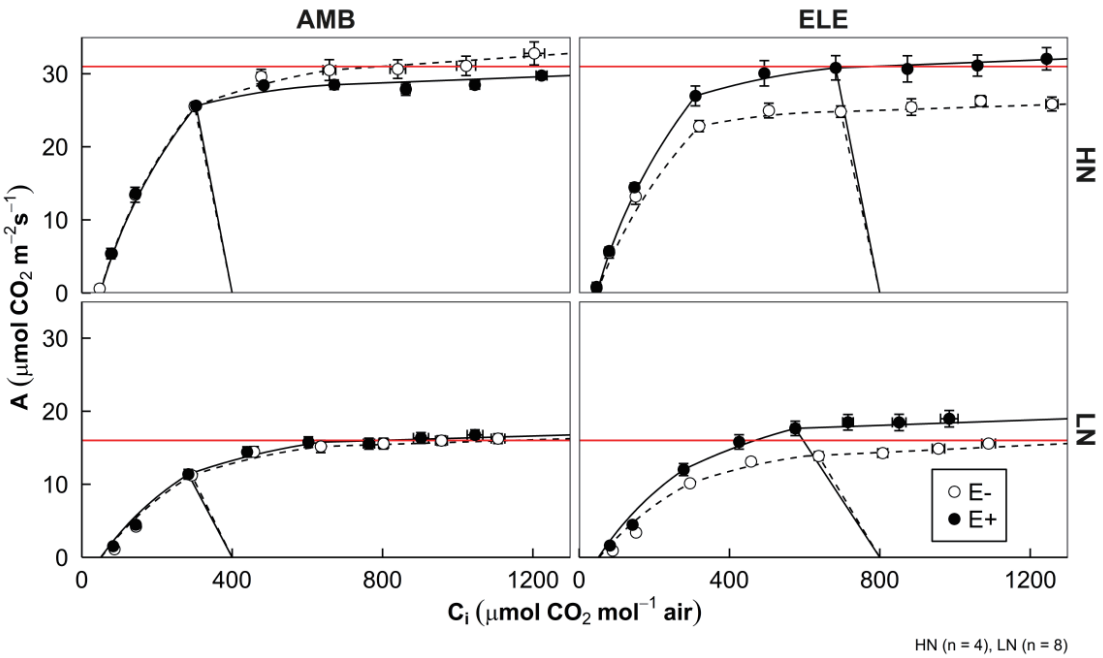

Fig. 5 CO2 response (A/Ci) curves of rice leaves at the panicle initiation stage following growth under ambient (AMB, ~400 ppm) and elevated [CO2] (ELE, ~800 ppm). The top panels show the responses of plants grown under high-N conditions (HN, n = 4) and the bottom panels show plants grown under low-N conditions (LN, n = 8). E–, mock-inoculated control plants; E+, endophyte-inoculated plants. Data are means (± SE). The solid horizontal lines indicate the asymptotes of the curves for E– plants under AMB conditions. Adapted from Rho et al. (2020).

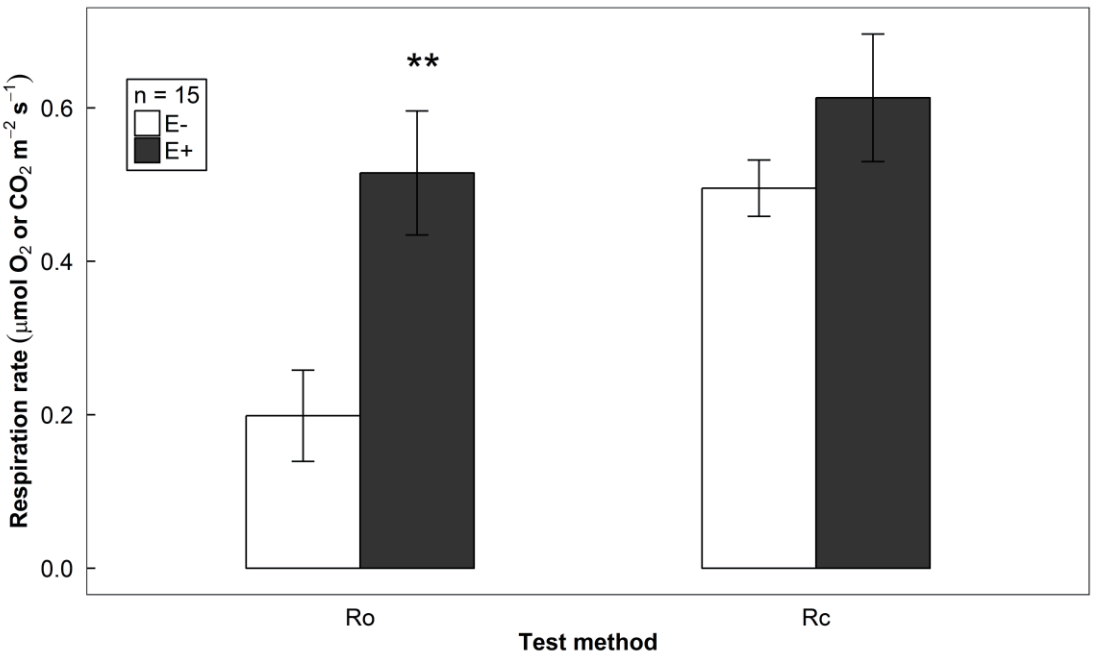

Fig. 6 In planta respiration rates of rice leaves at the panicle initiation stage. Respiration rates were determined by measuring the consumption of O2 (Ro) and the release of CO2 (Rc). Bars present the mean responses of mock-inoculated control (E−, open) and endophyte-inoculated (E+, closed) plants. WP5 (Rahnella sp.) was used to inoculate the plant samples. Error bars show ± 1 S.E.M. (n = 15). Using a 2-way ANOVA test, highly significant differences by method and by method × inoculation interaction were found at $P < 0.001$. Within each method, a t-test was used to detect a statistical significance in the differences between E- and E+ plants. Asterisks indicate significant difference between E− and E+ plants at the $P < 0.01$ level. Adapted from Rho et al. (2018a).

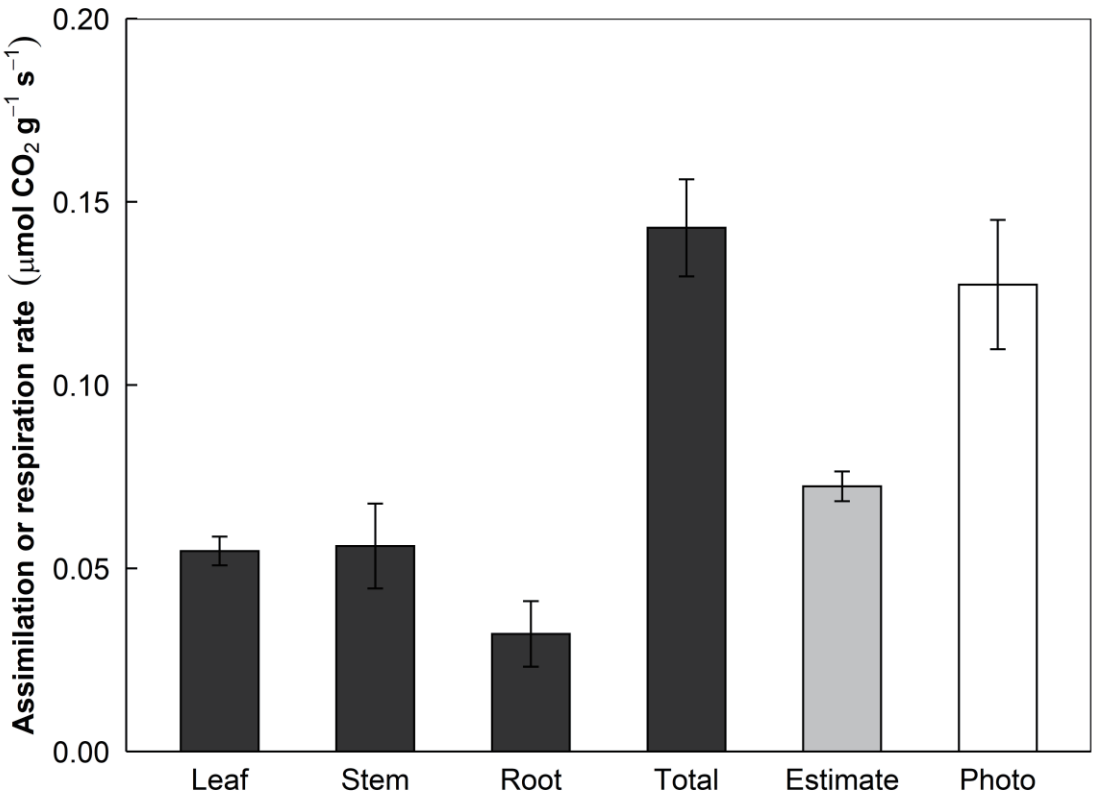

Fig. 7 Estimates of endophytic microbial respiratory CO2 release in planta separated by tissue (Leaf, Stem, Root, and Total, black bars). The estimated total possible microbial respiratory CO2 for re-assimilation is provided (Estimate, gray bar). The data are compared with photosynthetic CO2 assimilation of the leaves (Photo, white bar). Bars indicate the mean responses of WP5 (Rahnella sp.) endophyte-inoculated rice plants. Error bars indicate ± 1 S.E.M. (n = 4). Adapted from Rho et al. (2018a).

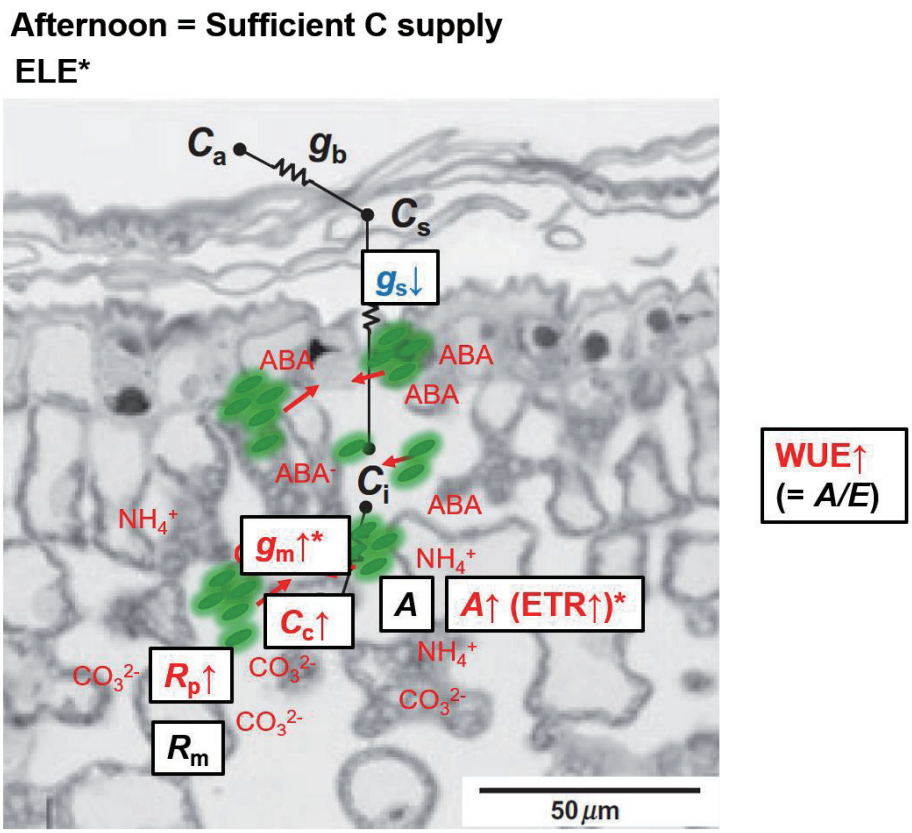

Fig. 8 A hypothetical model to explain the underlying mechanisms centered on leaf level transpiration, photosynthesis, and respiration of the host plant with endophyte inoculated. The microscopic image on the background is adapted and edited from Flexas et al. (2008).