# Salicaceae Endophyte Impacts on Physiological Functions of Host Plants: Water Relations, Photosynthesis, and Respiration in Rice (*Oryza sativa*)

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## Abstract

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Endophytes are bacteria, fungi, and yeast that live inside plants. Endophytes provide fitness benefits to the host plant while receiving carbohydrates in return. A wide range of bacteria and yeast endophyte strains was isolated from native Salicaceae trees growing in a riparian area. Previous studies on these Salicaceae endophyte isolates have shown their symbiotic traits in various host crops across taxa. Biological di-nitrogen fixation, phytohormone production, and an enhancement of drought stress tolerance in plants were important beneficial functions of these endophyte isolates. However, their impacts on the physiology of the host plant had not been examined in-depth. The focus of the present study was on water relations, photosynthesis, and respiration of the host plant to better understand symbiotic associations in the plant eco-physiological context. Select Salicaceae diazotrophic endophytes were inoculated into rice as a model $C_3$ plant, and the effects on the physiology were assessed in a series of greenhouse experiments. The inoculated plants showed reduced stomatal conductance in the afternoon than

control plants. Stomatal density of the inoculated plants was also lower than that of control plants. The accumulation of leaf ABA during the afternoon was facilitated in the inoculated plants. The stomatal responses of the inoculated plants led to decreases in transpiration, and further to increases in water use efficiency of the plants. The endophyte inoculation alleviated down-regulation of the host plant photosynthesis to elevated $CO_2$. Moreover, the inoculated plants showed the improvements in photosynthesis compared to control plants. The improvements featured increases in electron transport rate of the photosynthetic light reactions and increases in internal $CO_2$ conductance of the $CO_2$ diffusion pathways in leaves. The inoculated plants showed increases in respiration rates. *In vitro* respiration rates of the microbes were positively correlated to the concentrations of carbohydrate supply and the number of the microbes on growing media. The *in planta* and the *in vitro* assay results provided an estimation of microbial respiratory $CO_2$ release in the host plant. The estimate was approximately 15% of total assimilated $CO_2$ through photosynthesis. This suggests microbial respiratory $CO_2$ could be a significant amount and possibly reenter the photosynthetic $CO_2$ assimilatory pathways. The stomatal closure, the photosynthetic improvements, and the respiration responses together imply the possibility of the re-assimilation and partially explain the increases in water use efficiency of the plant. Further investigation will be required to confirm the re-assimilation hypothesis with convincing empirical evidence. The key to uncover future significant findings will be an understanding of source-sink relations and carbon-nitrogen relations in plants with endophytes – the resource exchanges between the host plant and endophytes.

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April 20th 2018
At the loving desk of the Grad Office, Merrill Hall, Center for Urban Horticulture in Seattle…

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# CHAPTER 1

## Meta-analyses of Endophyte Effects on Plant Biomass Gain under Environmental Stresses and Physiological Functions

This chapter serves as literature review of the dissertation, consisting of the following two sub-chapters:

**CHAPTER 1A: Do endophytes Promote Growth of Host Plants under Stress? A Meta-analysis on Plant Stress Mitigation by Endophytes**

**CHAPTER 1B: Endophyte Effects on Photosynthesis and Water Use of Plant Hosts: A Meta-analysis**

# CHAPTER 1A

## Do Endophytes Promote Growth of Host Plants under Stress? A Meta-analysis on Plant Stress Mitigation by Endophytes

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## Abstract

Endophytes are microbial symbionts living inside plants and have been extensively researched in recent decades for their functions associated with plant responses to environmental stress. We conducted a meta-analysis of endophyte effects on host plants' growth and fitness in response to three abiotic stress factors: drought, nitrogen deficiency, and excessive salinity. Ninety-four endophyte strains and 42 host plant species from the literature were evaluated in the analysis. Endophytes increased biomass accumulation of host plants under all three stress conditions. The stress mitigation effects by endophytes were similar among different plant taxa or functional groups with few exceptions; eudicots and $C_4$ species gained more biomass than monocots and $C_3$ species with endophytes, respectively, under drought conditions. Our analysis supports the effectiveness of endophytes in mitigating drought, nitrogen deficiency, and salinity stress in a wide range of host species with little evidence of plant-endophyte specificity.
**Key words:** bacteria/fungi/yeast, drought/nitrogen/salinity stress, effect size, endophytes, meta-analysis, plant biomass

## Introduction

A growing body of literature has reported benefits of microbial mutualists on plants under a wide range of environmental conditions. One group of these microorganisms is known as endophytes (Dobereiner 1992). Endophytes have drawn attention of plant scientists as a potential means to mitigate plant stress under a rapidly changing climate where plants will encounter water deficit, frequent flooding, extreme temperatures, nutrient deficiencies, excessive salinity, and other environmental stresses (Rosegrant *et al.* 2009).

Recent studies of the plant-endophyte interactions have shown the role of the endophytes in mitigating the environmental stresses on plants, including heat, drought, nutrient limitations, and exposure to pollutants (Ryan *et al.* 2008; Bulgarelli *et al.* 2013; Chang *et al.* 2014; Wani *et al.* 2015; Santoyo *et al.* 2016). These previous studies collectively show positive endophyte effects on improving plant fitness and survival under the environmental stress conditions, supporting the hypothesis that the effects of endophytes on plant stress mitigation may be ubiquitous among different plant taxa and stressors. However, a systematic comparative synthesis is needed to test this hypothesis and determine the ubiquity or specificity if it exists: Stress mitigation conferred

by endophytes may be host-specific or effective only under particular experimental conditions. To draw overall conclusions about the benefits of endophytes for plant stress mitigation, it is imperative to identify the experimental conditions and host-endophyte combinations that yield the most effective stress mitigation.

A meta-analysis aims to synthesize information through an explicit statistical protocol of data aggregation and analyses from a number of individual experimental studies (Arnqvist & Wooster 1995). It is especially effective to answer research questions with broader applicability and uncover emergent properties across individual studies that may not be apparent otherwise. The power of a meta-analysis can be realized when the effects of individual studies are inconsistent in different experimental settings. Therefore, we employed a meta-analysis to amass this information by evaluating the effectiveness of endophyte inoculations in plant stress mitigation and its host specificity. To our knowledge, only a few studies have attempted to apply statistical approaches to measure the overall endophyte effects on the host plants' physiology to date (Newsham 2011; Omacini *et al.* 2012; Mayerhofer *et al.* 2013). Moreover, there is no meta-analysis that has addressed the endophyte effects on host plants under stressful conditions.

In this study, we hypothesized that: 1) plant stress mitigation conferred by endophytes is not host species specific, and 2) plant stress mitigation by endophytes is ubiquitous across plant taxa regardless of stressor types or experimental conditions. To test our postulates, we extracted and collected raw data from 209 articles and performed a meta-analysis. In addition, to investigate the host-specificity, we classified plant species into different functional groups (i.e. woody vs. herbaceous; crop vs. non-crop; eudicots vs. monocots; and $C_3$ vs. $C_4$), and then individually scored the effect sizes of each group to compare the endophyte effects on different types of host plants.

## Materials and Methods

### *Data Collection Process*

A total of 209 journal articles were retrieved through a database search using the SCOPUS database (www.scopus.com) as of October 2016. We considered bacterial, fungal, and yeast endophyte studies that focused on three stress factors: salinity, nitrogen deficiency, and drought. The keywords used in the search were 'endophyte', 'bacteria', 'fungi', 'yeast', and 'Plant

Growth Promoting Endophytes (PGPE)'. 'Salt', 'nitrogen', 'water', and 'drought' were also added as independent variables in the keywords for each stress factor. We used 'biomass' as the keyword to identify articles that included a common response variable to focus this meta-analysis. If articles reported shoot and root biomass separately, the variables were summed to analyze the effect size on total biomass. When only one variable – either shoot or root dry weight – was reported, it was considered total biomass in the analysis.

Of the 209 articles found, 108 articles met our selection criteria: 1) experiments were performed in controlled environments – a lab, growth chamber, or greenhouse environment, and 2) the design of the experiment included control and endophyte inoculated groups grown under stress conditions for proper comparisons. In addition, research articles that did not report the standard deviations (SD) or standard errors (SE) of the means were filtered out, as those values were required to calculate the effect sizes in the meta-analysis process. After this selection step, 84 articles proceeded to the analysis (Table 1). Total biomass of plants and germination rates were considered proxies of plant performance in response to the stress factors. Each combination of an endophyte strain and a plant species in one article was regarded as one data set to be analyzed, and then summed. Values in tables of the articles were collected and arranged in an MS Excel spreadsheet. Graphical data in figures were digitized using ImageJ v.1.48 (Schneider *et al.* 2012) with the 'Figure Calibration' plugin package, and then also organized in the spreadsheet.

A total of 326 datasets were imported and compiled in R version 3.2.2 (R Core team 2016). The summary statistics of the selected articles and breakdown of datasets were provided in Table 1.

***Estimation of Summary Effect Sizes***

Inoculation of endophytes was counted as a fixed effect in different environmental and experimental conditions; thus, a fixed-effect model for meta-analysis was implemented to analyze the extracted data. The obtained means, SDs, SEs, and number of replications (i.e., sample size) were further processed to be imported to the R platform to conduct the statistical analysis. Total biomass or germination rate of host plants was set to a response variable. The following is the formula to calculate Hedges' $d$ – non biased and scaled differences addressing sample sizes of datasets (Crawley 2013):

$$d = \frac{\bar{X}^T - \bar{X}^C}{S} J \qquad \text{Eq. 1}$$

$$J = 1 - \frac{3}{4(n^T + n^C - 2) - 1)} \qquad \text{Eq. 2}$$

where $\bar{X}^T$ and $\bar{X}^C$ are the means of responses from the treatment (inoculated) and the control groups (Eq. 1). *S* is the pooled within-study SD and *J* is a correction factor for small sample sizes (Eq. 2). $n^T$ and $n^C$ in the equation stand for the number of samples of the treatment and the control groups.

The variance of *d* (*Vd*) was calculated by plugging $n^T$ and $n^C$ with *d* into the following Eq. 3.

$$Vd = \frac{n^T + n^C}{n^T \times n^C} + \frac{d^2}{2(n^T + n^C)} \qquad \text{Eq. 3}$$

The bias-corrected versions of Hedge's mean differences and their variances – *g* and *Vg* – were calculated by simply multiplying *J* and $J^2$ to *d* and *Vd*. Calculated *Vg* was used in the computation of 95% confidence intervals (CI) of each *g*. These weighted measures correct the bias that could affect the effect size estimates derived from the different sample sizes in individual studies.

$$95\%\ CI = 1.96 \times \sqrt{Vg} \qquad \text{Eq. 4}$$

The individual statistics (*g*, *Vg*, and CI) were used to score the endophyte effects in an individual data set indicated with the color scale provided in Fig. 4.

The reciprocals of *Vd*s used as the weights (*W*) for determining the summary fixed effects. The sum of the products of the weights and the effects ($WY = W \times g$) was divided by the sum of the weights to finally determine the summary effect (*M*) as follows:

$$M = \frac{\sum WY}{\sum W} \qquad \text{Eq. 5}$$

The variance of the summary effect (*VM*) above is just the reciprocal of $\sum W$.

The SE of *M* (*SEM*) is,

$$SEM = \sqrt{\frac{1}{\sum W}} \qquad \text{Eq. 6}$$

Finally, the sum of *W* was used to calculate SE of the mean summary effects to further compute the *z*-test statistic ($z = M/SEM$). In the cases where the effect size was found to be significant at $\alpha = 0.05$, we calculated the fail-safe number ($n_{fs}$) in order to estimate publication bias using 'metafor' package in R (Viechtbauer 2010). If $n_{fs}$ is over $5n + 10$, it is considered to be safe to ignore publication bias as described in Rosenberg (Rosenberg 2005), where *n* is the number of studies used in the analysis.

The overall summary effects of each stress factor were split up into the effects under the following sub-categories. Group 1 compared the effects on herbaceous to woody species, while Group 2 did those on crops to non-crop species. Eudicot vs. monocot and $C_3$ vs. $C_4$ comparisons were conducted in Group 3 and 4, respectively. The effect sizes without stress and with stress were also compared using a paired *t*-test procedure in the R platform.

Combined measures of all three stress factors for *d* were represented in a heat map (Fig. 4). To investigate the endophyte effects on commercially important major plant species, we selected the five most studied plants: corn (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L) for staple crops, pepper (*Capsicum annuum* L.) as a horticultural crop, and poplar (*Populus* spp. L.) as a woody plant used for environmental services and bioenergy.

## Results

### *Synthesis of General Information*

The publication trend categorized by the stress factors indicates overall steady increases in published research about all three factors (Fig 1a). The drought stress papers gradually increased over the past 16 years from 1998, while the nitrogen stress papers rapidly increased after 2011. The salinity stress papers also increased rapidly in the past few years starting in 2009 (Fig. 1a).

Categorized by the type of endophytes, an increasing volume of articles was published on plant stress mitigation conferred by bacterial (53%), fungal (41%), and yeast (6%) endophytes over the last two decades (Figs. 1b and 2a). Yeast endophyte research is relatively new compared to the other two types; the first yeast endophyte research was published in 2012. These studies analyzed were all done in controlled experimental conditions: Greenhouse (72%), chamber (22%), and lab (6%) environments (Fig. 2b).

Methods of inoculation varied widely within a total of 108 articles (Fig. 2c). There are two main ways to deliver endophyte inocula: Seed inoculation (54%) and soil inoculation (21%). Most of the fungal endophytes were inherently infected by vertical transmission (17%). Spraying of endophyte inocula on the leaf surface (1%) and dipping plant cuttings in endophyte cultures (2%) were effective inoculation methods.

Most of (85%) the studies used single strain inocula compared to multiple strain consortia. These consortia studies make up 15% of the total data sets and they all used either bacterial mixtures or bacteria and yeast combined mixtures. None of the consortia studies we found included filamentous fungi in the consortia (Fig. 2d).

Our analysis included studies performed from a total of 29 countries, among which the United States was the leading country with 32 original research articles published (Fig. 2e).

The concentration of endophyte inocula used in the experiments varied by colony forming unit (CFU) = $2.0 \times 10^5$ to $1.0 \times 10^9$ (Table 1). Regardless of the density of the endophytes, their effects on plant physiology under the stressful conditions were found to be statistically significant in most of the articles (Figs. 3 and 4).

A substantial number of studies we examined were incompletely designed with no negative control groups to compare for stress effects. In these studies, there were comparisons between control and inoculated plants only under the stress treatment. Arranging a complete experimental design with control groups for both endophyte inoculation and stress treatment is necessary to show possible interaction effects and to test the true impacts of endophytes on plant physiology. Thirty out of 108 searched articles had an incomplete experimental design (data not shown). For those completely designed studies, a paired *t*-test to compare non-stressed and stressed treatments was used (Fig. 3).

The genus of endophytes that was used the most in our analysis was *Neotyphodium* – with a total of 7 studies – followed by *Epichloe* and *Pseudomonas* with 6 studies found for each (Table 1). In most of the research studies, herbaceous crop species were used as the host and only 22 data sets of the 326 data sets investigated the effects on woody plants (Table 1 and Fig. 3).

***Cumulative Effect Sizes on Different Functional Groups***

Overall, our results supported the hypothesis that various endophyte strains provide environmental stress tolerance to a wide range of plant hosts. Seventy-nine endophyte strains analyzed in the present study helped 41 host plant species maintain fitness under various drought, nitrogen, and salt stress conditions. There was no publication bias in the cumulative endophyte effects under all three stress factors. The fail-safety numbers of the drought/nitrogen/salinity stress cases were 989/9,805/88,586, which were all greater than the criteria (255/630/770). Even under non-stressed conditions from the same studies analyzed, the numbers were higher than these criteria (708/1,730/3,928).

Despite the smaller sample sizes, eudicot species ($n$ = 4) in the category Group 3 and $C_4$ species ($n$ = 7) in Group 4 under drought conditions showed superior performance when inoculated with endophytes according to their cumulative effect sizes ($d$ = 4.697 and 5.091, respectively; Group 3 and 4-left panels in Fig. 3). Likewise, $C_4$ plants under salt stress conditions showed a greater effect size ($d$ = 2.271; Group 4-right panel in Fig. 3) than $C_3$ plants.

There was only one study focused on woody host-microbe interactions under drought stress conditions (Group 1-left panel in Fig. 3). Fifteen and six data sets from woody plants' responses under nitrogen and salinity stresses, respectively, were used in the analysis; even so, compared to

herbaceous hosts' data sets, the size of the samples was too small to draw a conclusion about endophytes aiding shrubs and trees (all Group 1 panels in Fig. 3). Furthermore, endophyte inoculation to woody species under salt stress conditions did not produce significant effects (lower 95% CI = − 0.397 < 0, Group 1-right panel in Fig. 3).

Overall, the effects of endophyte inoculation on biomass of both non-stressed and stressed plants was statistically significant in all three stress factor studies ($P < 0.001$, Overall panels in Fig. 3). The summed effect sizes were 0.553/0.505/0.324 in drought/nitrogen/salinity stress studies for non-stressed plants and 0.563/0.717/0.986 for stressed plants. All these numbers were statistically greather than 0 (no effects). However, the effect size of endophyte inoculations did not differ between non-stressed and stressed hosts in drought and nitrogen studies. In the salinity stress studies, there was a significantly higher endophyte effect on plants' biomass gain under the stress than non-stressed controls.

***Endophyte Effects on Five Major Host Plants***

The selected five major plant groups all positively responded to the endophyte inoculations as shown in Fig. 4. The summed effect sizes (the sum of the color scale values) was greatest in pepper, followed by corn, wheat, rice and poplar. The maximum score was recorded in *Zhihengliuela* on pepper under salinity stress conditions ($d = 26.34$). The minimum effect size was found in the combination between *Pseudomonas* and *Zea mays* ($d = 0.229$). Interestingly, there was no study in this analysis that observed increases in plant stress tolerance with the most commonly used endophytes – *Neophodium* and *Epichloe* (counts: 7 and 6) – on these five crops. As shown in the Supplementary Material (endo_host_heatmap.html), 128 combinations between endophyte strains and host plant species were used in plotting their inter-relationships. The number of all possible combinations was 3,948 (endophyte strain × plant species = 94 × 42), indicating only 2.3% of the total combinations has been reported by the literature. The maximum effect was found in the *Penicillium* spp. and cucumber (*Cucumis sativuas*) combination ($d = 26.89$) under salinity stress whereas the minimum effect was found in the *Neotyphodium* spp. and *Lolium perenne* combination ($d = -0.83$, harmful effect) in drought stress. Seven combinations showed negative endophyte effects on biomass of hosts under stress conditions.

## Discussion

Published studies on plant stress mitigation by endophytes have been increasing considerably in recent years. Our meta-analysis provides a synthesis of valuable findings from a large number of experimental studies that were conducted in a diverse mix of host-endophyte combinations, treatments, and environmental conditions found in the literature to date.

### *Trends in Publication Show Growing Interests in Topic*

General statistics about the publications clearly shows the increasing attention to this research topic (Fig. 1). This trend is likely to continue, given the soaring demand for plant stress research especially in response to environmental stresses associated with a rapidly changing climate and the need for finding adaptive solutions to the climate impacts in crops.

Drought stress mitigation by fungal endophytes in several $C_3$ grass species has been reviewed by Rodriguez et al. (Rodriguez *et al.* 2009) with an emphasis on the ecological impacts of the *Neotyphodium* and *Epichloe* genera since 1995 (Fig. 1). Further work is being published more focusing on employment of the technique (Berg 2009; Porras-Alfaro & Bayman 2011) and elucidation of the mechanisms of molecular communication between the hosts and the endophytes (Kusari *et al.* 2012, 2014).

Compared to fungal strains, bacterial endophytes or Plant Growth Promoting Bacteria (PGPB) research appeared to have a slower start in the early 2000s, but has gained more attention in recent years focusing on their ability for biological nitrogen fixation and phytohormone production. For example, various strains of diazotrophic bacterial and yeast endophytes were isolated from poplars in their native habitats (Doty *et al.* 2009a) and have been successfully inoculated into a range of other host species (Khan *et al.* 2012d, 2016; Knoth *et al.* 2013a; Kandel *et al.* 2015). These bacterial strains have been found to alleviate nutrient deficiency of plants. The number of articles reporting endophyte effects under nitrogen-limited conditions has been rising rapidly since 2010; this trend is likely to reflect a renewed interest in non-nodulating diazotrophs that are endophytic or rhizospheric PGPBs (Omacini *et al.* 2012). Unlike fungal and bacteria strains, only a few studies (6%, Fig. 2a) examined yeast endophytes for their ability to confer stress tolerance (Khan *et al.* 2012d, 2016).

There was no standard protocol for inoculation throughout the literature, though similar procedures were followed in different experiments from the same research groups (Fig. 2c). The two most frequently used techniques were seed and soil inoculation techniques, which attributed to 54% and 21% of the methods, respectively, we analyzed. Seed inoculation refers to a method where experimenters co-cultivate prepared liquid inocula and introduce the inocula to host plants when they are still in the seed or seedling stage, mostly in petri-dishes or small containers. In comparison, soil inoculation is usually performed directly into root media or pots where host plants are grown.

A notable observation is that multiple studies have used a mixture of assorted endophyte strains hypothesizing that the mixture (or often called a consortium) would be more representative of the original microbiome consisting of multiple strains providing unique and synergistic benefits than single strains (da Silva *et al.* 2014) (Fig. 2d).

***Endophytes Mitigate Plant Stress in a Wide Range of Species***

We found positive endophyte effects on biomass accumulation of host plants, which is in accordance with previous meta-analytic reports (Newsham 2011; Omacini *et al.* 2012; Mayerhofer *et al.* 2013). To be specific, our results showed these positive impacts of endophytes on hosts' growth under drought, nitrogen deficiency, and salinity stress conditions (Fig. 3). While the intensity of the imposed stresses was variable, the results corroborate the effectiveness of endophyte inoculation to mitigate plant stress with little host specificity. An exception to this general pattern may be found in the $C_3$ vs. $C_4$ comparison (Fig. 3). That is, $C_4$ plants benefited more by having endo-symbionts under drought and salinity stress conditions than $C_3$ plants did. $C_4$ species inherently have higher water use efficiency (WUE) than $C_3$ species through the specialized photosynthetic pathway (Lambers 2008). Endophytes may help boost this trait by increasing the increment of biomass gain, leading to further increase WUE under water-deficit conditions. This result is opposed to the effect sizes of arbuscular mycorrhizae on $C_3$ vs. $C_4$ plants gaining biomass under drought conditions reported in Worchel et al. (Worchel *et al.* 2013). This may be due to their different symbiotic styles; arbuscular mycorrhizae help host plants survive mainly by increasing water and nutrient acquisition from the rhizosphere (Kivlin *et al.* 2013), whereas endophytes do rather by providing phytohormones and inducing the defense related secondary metabolisms while residing in the plants (Wani *et al.* 2015).

Underlying mechanisms for the difference found in endophyte effects between $C_3$ and $C_4$ plants are unknown and call for additional attention in future studies.

Mycorrhizas are another type of mutualistic associates with plants that has been studied over many decades. There are meta-analytic research articles about these symbionts that summed the effect sizes on gaining host biomass under drought and salt stress conditions (Worchel *et al.* 2013; Chandrasekaran *et al.* 2014). The effect sizes of endophytic symbiosis on gaining plant biomass we analyzed were greater than those of mycorrhizal symbiotic interactions. For example, the summed endophyte effect sizes under drought/salinity stress conditions were 0.563/0.986 out of 49/152 data sets. These are higher than 0.160/0.429 out of 57/93 from mycorrhizas. This suggests that endophytic association may offer more benefits overall, although species' preference in forming a specific type of symbiosis should be considered in the context of the application.

***Considerations on Cumulative Effect Sizes – Differences in the Effects Found at Various Life Stages of Plants and Some Negative Effects in Specific Cases***

Some of the articles argued (e.g., (Saikkonen *et al.* 1998; Schulz & Boyle 2005; Porras-Alfaro & Bayman 2011)) that the benefits of endophytes were conditional, and they questioned the effects over long periods of time or under certain circumstances. Indeed, 23.4% of the analyzed data sets were from the experiments conducted within three months when the plant materials were not fully grown to their final harvesting stages. However, some studies did perform experiments to the last phases of host plants' growth and development, discussing the endophyte effects on biomass over time (Usuki & Narisawa 2007; Upadhyay *et al.* 2011; Nia *et al.* 2012; Knoth *et al.* 2013a; Patel & Saraf 2013). As Newsham (Newsham 2011) stated, long-term effects need to be investigated to confirm the results found in the literature.

The summarized effect sizes on the increase in plant biomass under the three environmental stresses were all significantly positive without a publication bias, but noteworthy is that negative effects were also found in a few articles. Contrary to the mostly positive responses to inoculation, seven data sets in our analysis were found to be negative as either no changes or decreases in the hosts' biomass were observed in those studies (Fig. S1, (Faeth *et al.* 2004; Marks & Clay 2007; Ren & Clay 2009a; Patel & Saraf 2013; Oberhofer *et al.* 2014; Yin *et al.*

2014; Song *et al.* 2015)). Similarly, Nadeem et al. (Nadeem *et al.* 2014) presented PGPB's harmful effects on plant physiology possibly derived from the production of cyanide, the over-production of auxin, or some metabolites the endophytes produced.

### *Impacts on Biochemical Processes of Plants by Endophytes Help Explain Underlying Mechanisms*

Recent studies using molecular and '-omics' technologies have begun to address the underlying mechanisms of host-microbe interactions under environmental stresses. One of the most plausible explanations uncovered to date is that selected endophytes' characteristics relieve reactive oxygen species (ROS) activity by enhancing anti-oxidative enzyme systems in host plants (Zhang & Nan 2007; Rodriguez *et al.* 2008; Redman *et al.* 2011; Bu *et al.* 2012; Alikhani *et al.* 2013; Gond *et al.* 2015). ROS as a stress response agent results in cell death in plants while anti-oxidative enzymes counteract to scavenge ROS. Yet, communication between microbes and hosts must be closely investigated to examine how endophytic micro-organisms send signals and trigger the scavenging reactions, and how they produce antioxidant scavengers by themselves. Another conceivable mechanism regards the ability to create phytohormones or to modulate phytohormone biosynthesis of host plants. Empirical data have supported the idea that auxin, gibberellic acid, abscisic acid, salicylic acid, and ethylene biosynthesis processes are likely related to the delay of stress responses in hosts (Siddikee *et al.* 2011; Cheng *et al.* 2012; Alikhani *et al.* 2013; Straub *et al.* 2013b; Chang *et al.* 2014; Khan *et al.* 2015a; Yaish *et al.* 2015). Using molecular tools, such as knocking-out specific functional genes involved in phytohormone or anti-oxidant production by the endophytes, would open more opportunities to explore the mechanisms of the interactions and the crosstalk between the host and symbionts.

### *Suggestions for Future Studies*

First, from our literature review, the effects of endophytic inoculation under the stress conditions were found to be significant, despite differences in delivering methods. However, from an industrial perspective, consistent guidelines would allow more efficient and reliable application of the technology. Minimizing the number of microbial strains used in treatment media while delivering the maximum effects will be one of the most applicable aspects, together with finding a new inoculation medium, such as a dried powder or coating on seeds, to decrease the cost and efforts of application.

Second, varying research methods of stress implementation and levels of stress treatment made the analysis less powerful than we originally expected. Different stress regimes were even used within a single research article, making it difficult to explicitly evaluate the effects. Referring to current opinions on methods of imposing stress to plant materials in future studies would allow for more robust statistical analysis and therefore more accurate interpretation of data. Though it is difficult to enforce standardized stress intensities, it in fact would facilitate developing an influential tool to gauge a threshold for the hosts' inhabiting endophytes under stress conditions – in other words, a metabolic cost-benefit analysis.

Third, there is much room for improvement in determining the most ideal combinations between endophyte strains and host plant species, considering that the most suitable plant-microbe combination may vary depending on the soil type (Johnston-Monje *et al.* 2014). All three – plant, microbes, and soils – should be factored in the equation for better application. In addition, focus should be placed on the extent of the endophyte host range, including a diversity of plant types to explore many application uses of this biological mitigation of environmental stress impacts– not only for commercial importance, but for restoring endangered species in native habitats as well (Gonzalo-Turpin *et al.* 2010; Emery & Rudgers 2013). To meet this demand, developing an efficient screening tool for endophyte impacts on plants (Jia *et al.* 2008) would be required.

Fourth, the timing of harvesting during plants' growth and developmental stages was crucial to investigate the dynamic interactions between the microbes and the hosts. Knoth et al. (Knoth *et al.* 2013a) reported significant growth promotion of sweet corn grown in nitrogen limited condition by bacterial and yeast endophytes at 25 days after inoculation. Eventually, however, the control reached the statistically same biomass of the inoculated plants at 90 days after inoculation. In contrast to this study, Kandel et al. (Kandel *et al.* 2015) showed an initial negative effect of endophyte inoculation on above-ground plant growth 1 month after planting rice. But, in the long run, the inoculated plants had greater height, tillers, and biomass 3 months after planting. Empirical results over time must be done to support this idea, eventually leading to finding a key to maximize the endophyte effects in application of the knowledge in the field.

Finally, there were limitations of the meta-analysis due to technical difficulties in controlling environmental factors and evaluating the endophyte effects without other potential symbionts in

experiments, so the data sets were only collected from controlled greenhouse or chamber environments in the present study. This will hinder researchers from estimating the precise impacts of endophytes in real agricultural or outdoor ecosystems. Emerging interests in the topic are promising, but the studies did not provide robust data for the entire plant science community. There are redundant articles that appear to be readily comparable to each other. The next generation must utilize the mechanistic approach to determine how to maximize the benefits from the knowledge we have gained by providing high quality of experimentation.

In conclusion, our study demonstrated improvements in plant growth by endophyte inoculation under three different environmental stress conditions. This benefit does not involve host-specificity, so we can call it interspecific functionality. As there is an increasing attention to this microbial stress mitigation tool for sustainable agriculture, it is time to fill the gap between whole-plant level physiological responses and understanding of biochemical mechanisms. By doing so, research communities will be able to find a key to utilize its full potential with wider applications in the field.

## Supplemental Information

The interactive version of Fig. 4 with the full information about the meta-analysis is available (Fig. S1, 'endo_host_heatmap.html') online at:

## Author Contributions

Conceived the idea: HR SHK. Designed the analysis and collected the data: HR MH SLK JC. Performed the analysis: HR. Provided materials and resources: SLD SHK. Wrote the paper: HR MH SLK SLD SHK.

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List of the papers processed in the analysis.

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## Tables & Figures

**Table 1** General statistical information from the articles found on the SCOPUS (www.scopus.com) database about the endophyte effects on plant fitness under drought, nitrogen, and salt stress conditions (as of October 2016). The differences of biomass or germination rates of the host plants with and without the stress factors were evaluated as a response variable of the statistics. The number of datasets per article is arranged in the Data column. A summary of the meta-analysis statistics from the literature selected is provided in the supplementary materials.

| Category | Overall Statistics | Individual Statistics (drought, nitrogen, salt) |
|---|---|---|
| The number of articles found in the search results | 209 | 53, 121, 35 |
| The number of articles that met the selection criteria | 108 | 30, 44, 34 |
| The number of articles actually used in the analysis | 84 | 23, 37, 24 |
| The number of datasets analyzed | 326 | 49, 125, 152 |
| The endophyte genus that conferred maximum benefits to the host | *Penicillium* (on *Cucumis sativas*, $d$ = 26.89) | |
| The endophyte genus that conferred minimum benefits (or harmful effects) to the host | *Neotyphodium* (on *Lolium perenne*, $d$ = -0.830) | |
| The range of inoculum density used in the studies | $2.0 \times 10^5$ – $1.0 \times 10^9$ CFU/mL inocula | |
| The strain used most in the analysis | *Neotyphodium* sp. (count: 7) | |

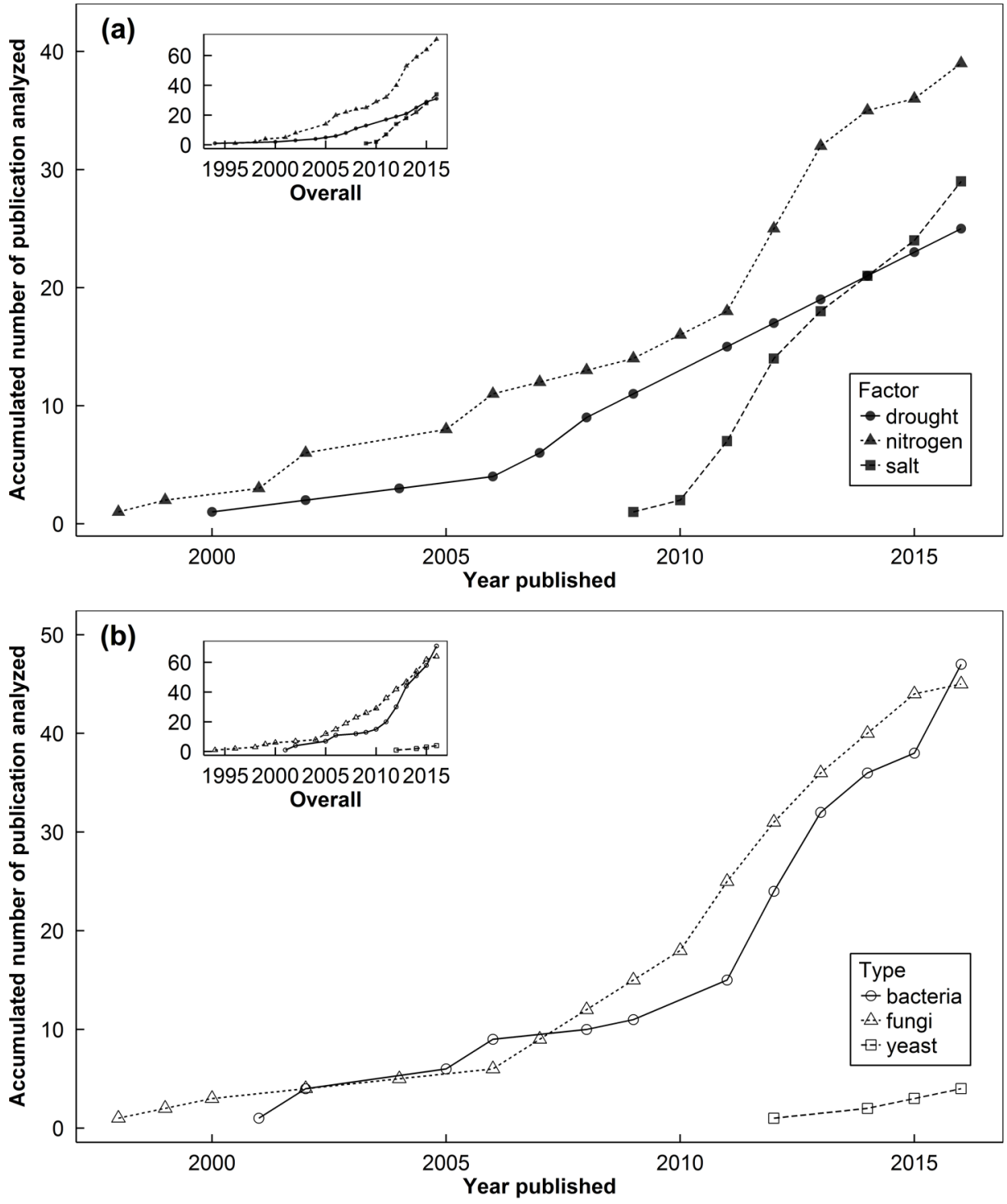


**Fig. 1** The accumulated number of publications about endophyte effects on plant stress physiology posted in the SCOPUS database in the last two decades. The data are sorted by **(a)** stress factor and **(b)** type of endophyte. The inlets in the main plots show the overall publications ($n$ = 136) found in the search, while the main plots present the number of articles ($n$ = 84) used in the analysis.


**Fig. 2** General statistical information of the studies ($n$ = 108) used in the analysis, separated by **(a)** type of endophytes used, **(b)** environmental control of experiments conducted, **(c)** method of inoculation (including 5% NA indicated as white), **(d)** single vs. multiple strains in inocula, and **(e)** location where the studies were carried out.

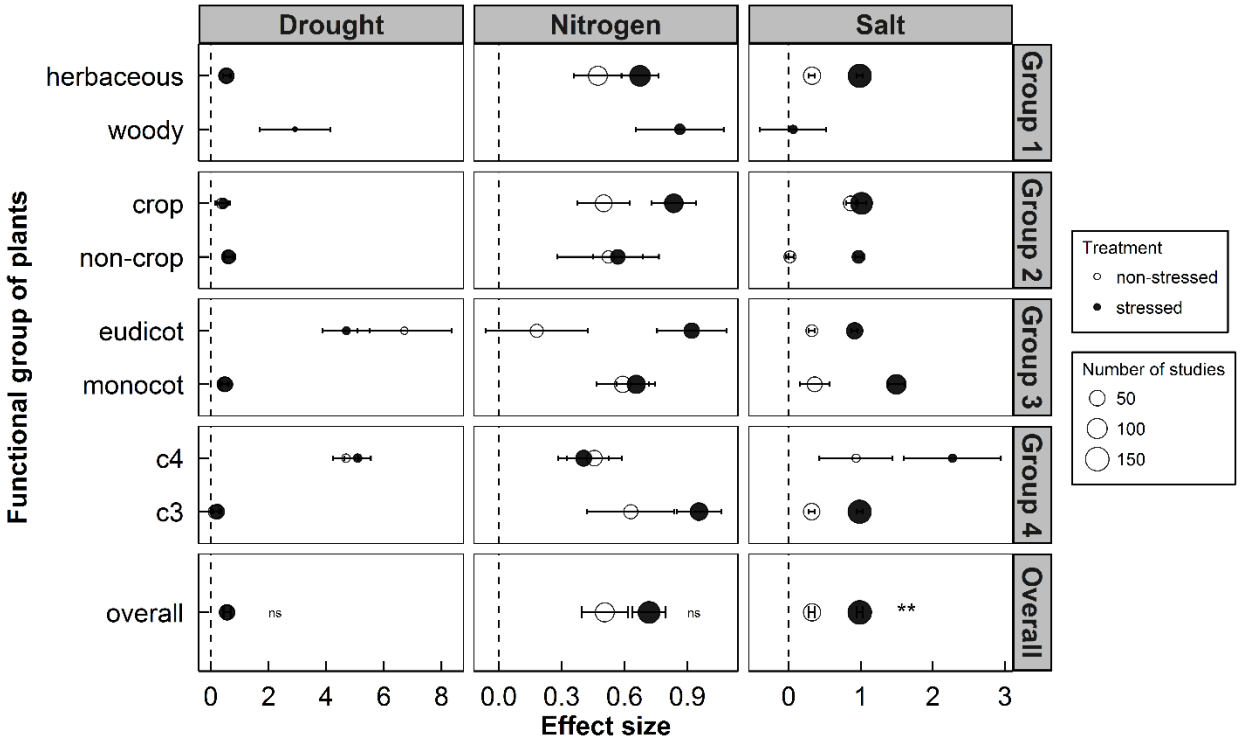


**Fig. 3** Cumulative endophyte inoculation effect sizes on host plants' gaining biomass under drought, nitrogen, and salt stress conditions arranged by functional group of host plant species in the vertical subplots. The size of the symbols indicates the number of the studies combined to calculate the measures. The open and closed symbols present the effect sizes of endophytes on non-stressed plants and stressed plants, respectively. The horizontal error bars stand for ± 95% confidence intervals (CI). If the CIs do not cross the vertical dashed lines ($d = 0$), the effect size for a combination of a certain functional group under the stress factor is significant at $P < 0.05$. The overall summary effect sizes are presented in the bottom panels without (non-stressed) and with (stressed) drought ($n = 42$ and 49), nitrogen ($n = 88$ and 124), and salt ($n = 66$ and 152) stresses. "ns (not significant)" and "** (significant at $P < 0.01$)" in the overall effect size panels show paired *t*-test results of endophytes effects under non-stressed vs. stressed conditions.

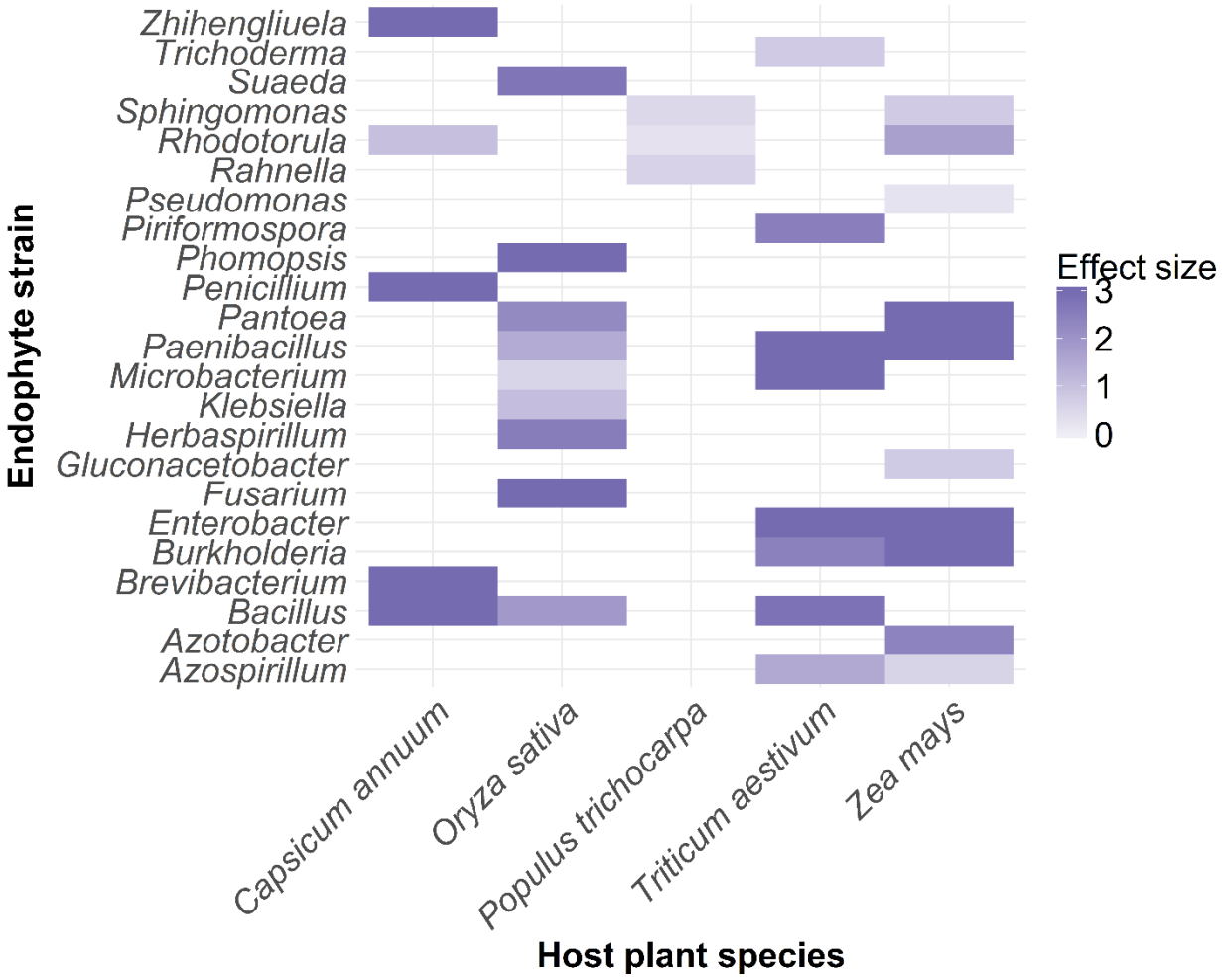


**Fig. 4** Endophyte effect sizes on gaining plant biomass under all stressed conditions (drought, nitrogen, salt combined) in each endophyte genus and host plant species combination. A total of 35 combinations from 23 endophyte genera and 5 major host species were plotted. The full interactive version of this heatmap with a total of 128 combinations is provided in the supplementary materials (Fig. S1, 'endo_host_heatmap.html'). The numbers of the endophyte strains and the host species examined were 94 and 42, respectively.

# CHAPTER 1B

## Endophyte Effects on Photosynthesis and Water Use of Plant Hosts: A Meta-analysis

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## Abstract

Endophytes – bacteria, fungi, and yeast living inside plants – can provide multiple benefits such as growth promotion and stress tolerance for their host to be successful in diverse environments. In this chapter, we review current literature to provide a meta-analysis on how the benefits conferred by endophytes are modulated through photosynthesis and water relations of the host plants. A total of 30 articles collectively demonstrated that endophyte inoculations led to photosynthetic improvements characterized by an increase in chlorophyll content, photochemical efficiency, or net $CO_2$ assimilation rate. An increase in leaf N content by either biological N fixation or N assimilation and translocation facilitated by endophytes, in part, accounted for these improvements. In addition, water use efficiency (WUE) of the host plants was enhanced by endophytes but this response was highly variable among studies conducted under different environmental conditions. An efficient osmoregulation and stomatal control modulated by endophytes are among the potential mechanisms that explain the improvements in WUE. We suggest that future studies need to consider the cost-benefit perspective of the plant-endophyte associations in terms of carbon, water, and nitrogen balances.

**Keywords:** endophytes, meta-analysis, photosynthesis, water use efficiency, nitrogen fixation, osmoregulation, stomata functions, symbiosis

## Introduction

Like animals do, plants host microbiomes. These include endophytes, beneficial bacteria, fungi, and yeast forming microbial communities *in planta* (Dobereiner 1992). They are found to enhance growth and fitness of host plants sustaining in harsh environmental conditions by offering them various benefits. These constructive effects include, but are not limited to, biological fixation of the atmospheric di-nitrogen (Knoth *et al.* 2014; Doty *et al.* 2016), production/promotion/regulation of plant growth hormones (Xin *et al.* 2009a; Waqas *et al.* 2012; Barnawal *et al.* 2016), facilitation of nutrient acquisition (Costa & Loper 1994; Wakelin *et al.* 2004), and provision of tolerances to abiotic/biotic environmental stressors (Rodriguez *et al.* 2008; Redman *et al.* 2011; Khan *et al.* 2016). All of these impacts promote growth and increase fitness of plant hosts and are well documented and summarized throughout the literature (Ryan *et al.* 2008; Rodriguez & Freeman 2009; Bulgarelli *et al.* 2013).

Most of the literature on endophytes has emphasized either the symbiotic characteristics/functions of the symbionts *in vitro* systems at the lab scale or the influences on overall biomass gain at the field

scale. Therefore, understanding endophyte effects on plant functional traits has become important to shape our knowledge about symbiotic interactions and to highlight the importance of plant microbiomes (Friesen *et al.* 2011). Many studies have uncovered some plant physiological mechanisms affected by endophytes, and the authors provide some mechanistic explanations of endosymbiosis in different contexts. However, the studies are often too species-specific or environmental condition specific to provide a consensus of their impacts on functional traits.

The scope of this chapter is centered on photosynthesis and water relations among the plant functional trait differences gained by endophytic symbiosis (Fig. 1). Friesen *et al.* (2011) argued that the impacts of endophytes on photosynthetic pathways is implausible (Table 1).

However, Straub *et al.* (2013) demonstrated the expression of the genes related to the photosynthetic light reaction of *Miscanthus sinensis* was up-regulated upon a bacterial endophyte inoculation. Ghabooli *et al.* (2013) also showed that photosynthetic enzymes, e.g. Rubisco large/small chain, chlorophyll a-b binding proteins, were up-regulated by a fungal endophyte inoculation under drought stress conditions. Indeed, despite some controversial reports, there exists a body of evidence indicating the effects on photosynthetic properties and water relations of plants affected by endophytic symbionts. This chapter reviews these effects of bacterial, fungal, and yeast endophytes on broad photosynthetic characteristics and water relations of plants under a variety of stress-imposing environmental conditions.

## Definitions of Terms and Methods of Analysis

### *Endophytes*

Endophytes can be classified mainly under three categories: Bacterial, fungal, and yeast endophytes. They are different domains of microorganisms, but share a common characteristic of living inside plants providing multiple benefits to the host. Most commonly investigated endophytic bacterial genera are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Streptomyces*, and *Staphylococcus* (Friesen *et al.*, 2011). Fungal species include *Neotyphodium* and *Epichloe* (Saikkonen *et al.* 1998) which are the two most studied endophyte strains amongst all. A study shows a yeast species – *Rhodotorula* – can form an endophytic mutualism with various plant hosts (Khan *et al.* 2012c). One noteworthy approach is blending multiple strains of bacterial or yeast endophytes together in hopes of stacking the functional benefits of the endophytes when mixed (Khan *et al.* 2012d, 2015b, 2016, Knoth *et al.* 2013a, 2014; Kandel *et al.* 2015). The mixture is called a consortium and it is considered in our meta-analysis. The articles referred in this chapter contain all these categories of endophytes (Table 4.1).

***Photosynthetic Efficiency***

Photosynthesis can be defined broadly as $CO_2$ uptake or $O_2$ production or specifically as $CO_2$ assimilation rate, photosynthetic light harvesting efficiency, chlorophyll content, or biochemical properties of the Calvin cycle (Rubisco content/activity). The specific terms are used to represent photosynthetic capacity and responses of leaf physiology to environmental cues. Sometimes photosynthesis as a net assimilation rate is expressed by certain biomass terms (Hunt *et al.* 2002), but we restrain our discussion here to leaf scale photosynthetic capacity. In relation to this, there are two key processes of photosynthesis in leaf physiology: The light reactions and the dark reactions or carbon reactions.

The light reactions are related to the light harvesting process which takes place in the lamellae sides of chloroplasts in mesophyll cells. Chlorophyll concentration and its efficiency are important factors in determining the efficiency of the first light harvesting aspect of photosynthesis. The most frequently used parameters are chlorophyll content (Chl) and photochemical efficiency (Flr). Chlorophyll content can represent the efficiency of the light harvesting process, while Flr can represent the efficiency of electron transport during the following photochemical reactions in between photosystem II (PSII) and photosystem I (PSI). Chlorophyll can be quantified with various experimental methods, from *in vitro* quantification (Lichtenthaler 1987) to *in vivo* estimation (Cerovic *et al.* 2012). These have been developed and widely used in plant sciences. Both methods were considered as a representative for Chl in our analysis. Photochemical efficiency refers to the rate of the light energy that is processed to the available form of biochemical energy – ATP and NADPH – in further photosynthetic $CO_2$ assimilation processes. It can be measured by a chlorophyll fluorescence technique which is currently broadly implemented in plant stress physiology (Baker 2008). Especially, $F_v/F_m$, defined as maximum quantum efficiency of PSII photochemistry, was our focus of our meta-analysis to evaluate photochemical efficiency.

The carbon reactions, also known as dark reactions, on the other hand, are associated with the $CO_2$ assimilation process on the stroma sides of chloroplasts in mesophyll cells/bundle sheath cells in $C_3/C_4$ plants (Taiz & Zeiger 2010). It begins with the uptake of atmospheric $CO_2$ from the leaf surfaces via stomata. By diffusion through the intercellular structures of the leaf, $CO_2$ molecules encounter a series of resistances that limit the supply of $CO_2$ to the Calvin-Benson cycle at the site of carboxylation. Once $CO_2$ is captured, it is staged to the carboxylation process where Rubisco catalyzes C fixation. All the enzymes related to operating the Calvin-Benson cycle work to incorporate $CO_2$ molecules to the 3 C skeletons. The efficiency of the carboxylation process can be

defined by how fast this $CO_2$ assimilation process occurs by the activities of the associated enzymes. The rate of net $CO_2$ assimilation ($A$) now can be measured easily *in vivo* by a commercial gas exchange system equipped with infrared gas analyzers (IRGAs) (Long & Bernacchi 2003).

***Stomatal Conductance and Transpiration***

Stomata – the interface of gas exchange on the leaf surfaces – play a crucial role in both photosynthetic $CO_2$ uptake and coupled $H_2O$ loss of plants (Woodward *et al.* 2002). As stomata are the location where $CO_2$ and $H_2O$ are exchanged mostly during the daytime, it is important to evaluate stomatal reactions to connect photosynthesis with water relations of plants.

Stomata open and close in response to many environmental cues, such as vapor pressure deficit (VPD) (Bunce 2006), atmospheric $CO_2$ concentration (Hetherington & Woodward 2003), water availability from the rhizosphere (Tardieu & Davies 1992), and pathogenic attacks (Melotto *et al.* 2006)c. The opening/closure – the aperture – of stomata can be determined by either microscopic observations (Milla *et al.* 2013) or *in vivo* gas exchange activity measurements (Farquhar & Sharkey 1982). To represent the actual amount of water release and $CO_2$ uptake during the stomatal actions, instantaneous stomatal conductance ($g_s$) is used as a unit of stomatal activity. Stomatal conductance has become the most commonly used leaf physiology parameter. It also finds significance in linking photosynthesis to water relations in various model approaches (Damour *et al.* 2010) and is a reciprocal of the $CO_2$ diffusional resistance by the movement of guard cells. It can be expressed in both conductance of water vapor or carbon dioxide. Stomatal conductance of water vapor can be determined by measuring the rate or the velocity of water vapor diffusion *in vivo*.

Transpiration ($E$) represents how much water is lost throughout stomata in response to VPD between the atmospheric air and the leaf surfaces. It is a parameter that expresses gas exchanges and water relations of plants. Along with $g_s$, temperature and relative humidity (RH) in the ambient air, which in turn influence VPD, come into play in determining $E$. Many plant species lose water through stomata and this is thought to occur only in the daytime as the transpiration demand increases when the air temperature is elevated while RH decreases. However, Snyder *et al.* (2003) reported some species had positive $g_s$ and $E$ at night in an arid environment. This suggests some plants actively transpire under water-limited conditions as a survival strategy. It highlights the importance of $g_s$ and $E$ in water management of plants.

In many cases, $g_s$ and $E$ are estimated or measured by a commercial IRGA system together with other leaf photosynthetic and gas exchange parameters, for instance, $A$ and $F_v/F_m$.

### *Water Use Efficiency*

As water becomes scarce in irrigated agricultural crop lands, now it is well-recognized as one of the key resources for crop yield (Rosegrant *et al.* 2009). Recent efforts in the current challenging climate conditions focus on how to increase the productivity of crops with limited water (Bernacchi & VanLoocke 2015). As a consequence, water use efficiency (WUE) stands great importance in agriculture.

Plant WUE is cataloged into three measures: Intrinsic WUE, extrinsic WUE, and WUE of productivity. The former two are leaf physiological parameters, whereas the latter one is a whole-plant physiological parameter. To understand these parameters, we revisit aforementioned leaf gas exchange parameters: $A$, $g_s$, and $E$. The basic idea of all these WUE is to express how much $CO_2$ is incorporated into plant biomass with a given amount of water consumed during the process (Lambers *et al.* 2008b).

Intrinsic WUE refers to the WUE determined by inherent leaf physiological processes. It is calculated by "$A/g_s$". Extrinsic WUE refers to the WUE externally determined by external surrounding factors, i.e. air temperature, RH, VPD that would affect $E$ of plants. It is calculated by "$A/E$". These are short-term measures of WUE. A gas exchange machine can compute these WUE instantaneously on the leaf being measured.

In contrast, WUE of productivity is a long-term measure of WUE. It is calculated by "total biomass gain/water consumption" over time. WUE of productivity could be an accumulated result of intrinsic/extrinsic WUE. One can quantify WUE of productivity by measuring the differences in biomass at between initial and harvest stages, together with the records of water consumption.

### *Meta-Analysis*

Several studies reviewed plant-endophyte interactions with respect to plant growth and stress (Ryan *et al.* 2008; Berg 2009; Bulgarelli *et al.* 2013; Wani *et al.* 2015). However, few of these review articles addressed ecophysiological processes involving photosynthesis and water relations of the host plants. There is also limited analytic review for generalized photosynthetic and water use responses of endophyte symbiotic plants with few exceptions (Newsham 2011; Omacini *et al.* 2012).

Meta-analysis is a means to quantitatively evaluate an overall effect of a certain treatment on different subjects reported in various studies (Hedges *et al.* 1999). In general, it is capable of addressing the heterogeneity of studies used in the analysis. Compared to conventional literature

reviews, it has an advantage of enabling researchers to make statistical conclusions on research questions from different studies. One can design a meta-analysis starting with refining keywords to search articles that meet the investigators' criteria. Collected data are processed to estimate "effect size" which represents the effectiveness of a treatment on response variable(s). If the variances of the effect sizes, usually presented by 95% confidence intervals (CI), do not overlap the zero effects on the scale, then one can conclude the effects are statistically significant on increasing/decreasing the reported response variable(s).

In the present meta-analysis, research questions we posed were: 1) Do endophytes improve photosynthetic and water use efficiency of host plants? and 2) Do the endophyte effects differ when the hosts are under stress in comparison to non-stress conditions? "Endophyte inoculation" is the treatment to be estimated for its effect size and the leaf physiology parameters mentioned above (i.e., Chl, Flr, *A*, $g_s$, *E*, and WUE) are the response variables to be analyzed. We gathered a total of 30 articles from the SCOPUS (https://www.scopus.com/) database and statistically meta-analyzed 93 data sets extracted from the papers. Details about the articles used in this chapter are provided in Table 1.

## Synthesis of Information

A total of 37 articles were gathered, but only 30 were used due to lack of necessary information for a meta-analysis in the other seven articles. Since some articles reported results from multiple strains or experiments, we procured 86 data sets from the selected articles. Only three studies did not conduct endophyte experiments under abiotic/biotic stress conditions. Most other studies tested the endophyte effects under stress conditions compared to non-stressed controls. Most of the plant species used in the studies were important agricultural crops, e.g. rice, corn, wheat, tomatoes, and soybeans. This suggests that there is a consensus of attempting to use endophyte symbioses as growth promotion and stress mitigation means for crops particularly in light of climate change, sustainability, and resource use efficiencies in agriculture (Berg 2009).

We analyzed 11/18/3 for bacterial/fungal/yeast endophytes used as a single strain or a consortium in the analysis. These different types of endophyte inoculants were introduced to experimental plants in various ways: Coating seed surfaces with inocula or soaking seeds with inocula before sowing (Ghimire & Craven 2011; Patel & Saraf 2013; Gond *et al.* 2015), applying inocula to seedlings grown in aseptic conditions (Rojas-Tapias *et al.* 2012), soaking stem cuttings in inocula (Rogers *et al.* 2012), or directly drenching inocula to the soils where the plant materials were prepared (Khan *et*

*al.* 2016). In some cases, plants were infected by vertically transmitted endophytes from a parental generation while non-infected plants served as controls (Morse *et al.* 2002; Anzhi *et al.* 2006), which is only found in fungal endophyte cases (Classes 1 and 2 fungal endophytes as discussed in Rodriguez *et al.*, 2009). Horizontally transmitted endophytes from neighboring plants were also found in one study (Ren & Clay 2009b).

Most of the studies were carried out in well-controlled environments. Except four studies, all were lab, chamber, or greenhouse experiments. This likely becomes a potential drawback of the endophyte research which hinders us from making strong conclusions about their effects at the field scales.

***Improvement of Photosynthetic Efficiency by Endophytes: Both the Light and the Dark Reactions***

Endophyte inoculation effects under different experimental conditions were found highly significant on increasing photosynthetic efficiency represented by Chl, Flr, and *A* (Figs. 2, S1, S2, and S3). In addition, the effects on the increases were more pronounced under stressed conditions than under non-stressed conditions except Chl of plants. The results suggest that both photosynthetic light reactions and carbon reactions were effectively enhanced by symbiotic associations.

The increase in photosynthetic light reaction activities characterized by Chl and Flr was one of the most common leaf physiological responses from the studies investigated. Especially, due to the simplicity of the assessment, Chl measured by a handheld chlorophyll meter was the most frequently used leaf physiology parameter ($n$ = 72). The observed results were likely to be derived from better nitrogen (N) availability by either symbiotic N fixation (Knoth *et al.* 2013a, 2014) or improved N nutrient uptake and assimilation (Yang *et al.* 2014b). Chl is a N rich molecule that consists of up to 75% of leaf N (Poorter *et al.* 1990). Chl has a strong positive correlation with leaf N level in a variety of plant species (Evans 1989). Scaling down to a molecular level, the up-regulation of associated gene expression supported this hypothesis (Woodward *et al.* 2012; Straub *et al.* 2013b). Chl has a positive correlation with Flr (Netto *et al.* 2005). The increases in a Chl content often leads to increases in Flr.

Separately from the light reactions, the dark reactions are also affected by endophyte symbiosis in a positive direction. There are multiple factors that determine the rate of gas exchange, including stomatal, mesophyll, or biochemical limitations (Grassi & Magnani 2005). Amongst those, considering the integral part of the $CO_2$ assimilation process, alleviation of biochemical limitation, represented by up-regulation of Rubisco engaged genes, is crucial evidence for the improvement (Bae *et al.* 2009; Ghabooli *et al.* 2013). This is also related to the available N level in leaves as

approximately 50% and 20% of N is allocated to Rubisco biosynthesis in $C_3$ and $C_4$ plants, respectively (Parry *et al.* 2003). Therefore, as Friesen *et al.* (2011) discussed, N allocation and metabolism are the core parameters resulting in these beneficial effects on photosynthetic efficiency.

However, in some cases, no apparent changes to photosynthetic components of the symbiotic plants were observed (Rogers *et al.* 2012; Knoth *et al.* 2013a). Several reports found different effects on photosynthesis at various stages of growth (Morse *et al.* 2002; Knoth *et al.* 2013a). This explains the complexity of the endophyte study and increases the uncertainty of implications. Costa Pinto *et al.* (2000) found impairment of the light reactions due to fungal endophyte infection in banana and maize plants in their early stage of growth (30-45 days after seeding). Similarly, Belesky *et al.* (1987) observed significantly lower *A* in endophyte infected tall fescue. Interesting findings are both Belesky *et al.* (1987) and Rogers *et al.* (2012) reported increases in total biomass accumulation by endophytes though photosynthetic capacity was decreased and not changed. The former paper found the explanation from the increases in tiller numbers and the latter paper did from the increases in leaf area of the plants. It seems that the endophytes-infected plants opted to invest carbon to producing more source organs rather to boosting productivity of sink tissues. Still, the mechanisms underlying these growth dynamics are largely unknown, requiring more research to fully benefit from the symbiotic interactions and to maximize the potential use of this biological adaptation strategy.

### *Importance of Leaf N Level in Photosynthetic Efficiency and Contribution of Biological N Fixation*

Nitrogen is the most important element in plant nutrition and metabolism other than C, H, and O. Approximately 1.5% of the total biomass is composed of N. It is the most abundant macronutrient obtained from the soil (Taiz & Zeiger 2010). Plants actively absorb N mostly from the root systems in the form of $NO_3^-$ or $NH_4^+$. Plants then transport N to shoots or leaves where N assimilation occurs to convert them into bioavailable forms (amino acids) by a series of enzymatic responses. These available N sources are then delivered to the photosynthetically active leaf tissues – newer leaves rather than older leaves (Xu *et al.* 2012b). Plants invest N into photosynthetic machineries in response to the environmental and other nutrient conditions (Xu *et al.* 2012a). Either the light harvesting process, the electron transport process in PSII, or the $CO_2$ assimilation process can be a target of the investment. Note that each process relates to Chl, Flr, or *A* in the analysis. Evans & Terashima (1987) demonstrated that these three parameters (shown as Chl, PSII activity, and Rubisco activity in the original article) were most strongly correlated to leaf N content than any other processes of photosynthetic machineries.

Nitrogen, therefore, alters leaf related plant functional traits in a significant way through this allocation adjustment between photosynthetic functions (Friesen *et al.* 2011). It is straightforward to connect plant N to photosynthetic efficiency as it consists of a substantial portion of growth N. It is not surprising that some studies postulated that the improvement of photosynthesis and further overall biomass by endophyte inoculation was due to increased N availability from biological N fixation (BNF) by the symbionts (Knoth *et al.* 2014).

As a consequence, to capitalize on these photosynthetic benefits, it is imperative to assess BNF activities in different strains of endophytic microbes; both *in vitro* and *in vivo* on a smaller scale. Doty *et al.* (2016) demonstrated the variable capacity of $^{15}N$ incorporation in cuttings of wild poplar plants, and suggested that specific strains may be required for the high N-fixation activity. Identification of these key strains from the symbiotic microorganism pools is required to develop better combinations of endophyte consortia to augment the extent of the benefits. On a larger scale, it will be required to evaluate to what extent endophytic BNF gives benefits through improving nitrogen use efficiency (NUE) in host plants. Endophyte and other associative bacteria engaged BNF is estimated to be 0.5, <4, or <14 Tg/year in sugar cane, non-legume crop, and grazing croplands respectively (Herridge *et al.* 2008). This contributes to 25-35% of total BNF of 50-70 Tg/year in agricultural croplands. Alternation of N uptake efficiency (NUpE), N utilization efficiency (NUtE), or photosynthetic N use efficiency (pNUE) by endophyte symbioses should be examined to compare this sustainable method with other recent approaches to enhance photosynthetic capacity (Zhu *et al.* 2010; Evans 2013; Ort *et al.* 2015; Xin *et al.* 2015). In the case of legume-rhizobium symbiosis, this benefit of BNF will be even more substantial in the future climate conditions with elevated atmospheric $CO_2$ concentrations (Rogers *et al.* 2009).

***On a Cost-Benefit Approach of Photosynthates***

Despite convincing evidence of the photosynthetic improvements, one should also consider the cost of endophyte symbiosis. That is, the additional carbohydrates fixed in the host plants through an increase in photosynthetic capacity or in leaf area facilitated by the endophytes might not be allocated directly to sink tissues where new biomass synthesis occurs but drained to feed and harbor endophytes. Mutualists, like pathogenic microorganisms, traffic the sugar transport through the phloem stream. Likewise, it is a well-established finding that symbionts demand the cost for their benefits to the hosts in the form of sucrose or inorganic acids (Lemoine *et al.* 2013).

To date, carbohydrate costs of endosymbiosis has not been actively discussed compared to the other types of plant-microbe symbioses such as legume-rhizobia and plant-mycorrhizal associations. Researchers have used cost-benefit analysis to quantify carbon cost of these other symbiotic microbes. For instance, 20% of photoassimilates was used by mycorrhizae (Jones *et al.* 2009), but it varied from 4-20% specifically in arbuscular mycorrhizal symbiosis (Lambers *et al.* 2002). The majority (83%) of this photosynthate use was the respiratory loss by the fungi themselves and/or the affected increases in plant root respiration (Baas *et al.* 1989). Likewise, symbiotic $N_2$ fixing rhizobacteria cost the plants about 14-25% of photosynthates (Lambers *et al.* 2008b; Kaschuk *et al.* 2010). In certain cases, microbial strains may cheat the hosts to drain more carbon based food sources when the plants have multiple symbiotic partners including those cheaters (Kiers *et al.* 2003, 2011).

Therefore, the increases in *A* should not be directly interpreted as the increases in overall biomass production or yield of plants as *A* is a proxy of instantaneous $CO_2$ assimilation determined by simultaneous gas exchanges on the leaf surfaces. The symbionts may lead to an increased production of photosynthates as a means to mine their own investments towards the hosts. Figure 4.2 shows there were 50-100% (corresponding to 0.5-1.0 effect size on the scale) increases in *A* upon endophyte associations; however, a significant part of these increases could be reduced by the C drain from the symbionts. Assuming they serve as active C sinks in plants, it would be interesting to test how strong their sink strength is. Providing classical cost-benefit analysis results would also be informative for pinpointing the actual C gain over loss of the plants.

***Varying endophyte effects on water relations – stomatal control in different contexts***

Compared to significant improvements of photosynthetic efficiency, the effects on water relations and use efficiency were more variable throughout the literature we analyzed (Figs. 4.2, A4, A5, and A6). Stomatal conductance was increased by endophyte inoculation under both non-stressed and stressed conditions. Transpiration rate and WUE were not changed under non-stressed conditions while they were increased by endophyte inoculation only under stressed conditions.

Overall, $g_s$ increased with endophytes and this stomatal opening could explain the increases in *A* by allowing more atmospheric $CO_2$ to diffuse into the leaf inside. This helps plants produce more carbohydrates at the risk of losing $H_2O$ on the leaf surfaces. For example, Shukla *et al.* (2012) conducted a study with rice and 5 strains of fungal endophytes under drought stress conditions in

which study endophyte inoculation delayed the onset of drought and induced reductions in photosynthesis and other gas exchange parameters – $g_s$ together with *A* and Chl.

On the contrary, Malinowski & Belesky (2000) reported several cases of the opposing endophyte effects on $g_s$. Fungal endophyte infection decreased $g_s$ of grasses that were subjected to drought stress conditions. These strains include *Neotyphodium*, *Phialophora*, and *Acremonium* families (Turner 1986; Belesky *et al.* 1987; Richardson *et al.* 1993a; Elmi & West 1995; Barker *et al.* 1997; Buck *et al.* 1997). They hypothesized that endophyte inoculation could induce stress metabolism of hosts, leading to preconditioning the hosts to any other biotic stresses. These earlier observations in the fungal endophyte research suggested abscisic acids as a possible hormonal signal to mediate $g_s$ responses, but few experimental approaches have been made to test the hypothesis. Also, increased secondary metabolites (Redman *et al.* 2011; Tiwari *et al.* 2013) in symbiotic plants without stressors support this hypothesis. A recent bacterial and yeast endophyte study (Khan *et al.* 2016) showed similar mechanistic arrays of evidence; the rapid stomatal closure to drought stress promoted the tolerance of the host plants. This seemed to be related to the hormonal status of inoculated plants as the strains used in the study were known to produce stress stimulated jasmonic acids, salicylic acids, and abscisic acids. Also, the inoculated hybrid poplar plants showed a reduced production of reactive oxygen species, which is a strong indicator of enhanced stress responses.

This adaptive strategy with endophytes contrasts to other symbiotic styles. A good example is mycorrhizal symbiosis. Mycorrhizal fungi stimulate stomatal opening in such a way to increase photosynthetic responses of the hosts under stressful conditions (Augé *et al.* 2014). They tend to provide more $H_2O$ to the hosts by increasing surface areas of root systems rather than to conserve more $H_2O$ by regulating stomatal opening (Worchel *et al.* 2013). Even the directions of symbiotic mechanisms on the same functional traits are opposite to each other, both mutualisms provide the hosts with a better chance to tolerate water deficits.

Some researchers discovered osmotic adjustment as the reason for the improved drought stress tolerance with regard to efficient water use (Richardson *et al.* 1993a). This mode of action is different from the stomatal closure theory. Rather, endophyte-infected plants show higher $g_s$ than uninfected plants and this causes the plants to increase the photosynthetic gas exchange. In turn, this increases carbohydrates available for osmotic adjustment as a conditioning tool to defend against the threat of drought. Decreases in complex sugars (e.g., fructans) and subsequent increases in simple sugars suggest this mechanistic adaptation.

### *Alternative Views on Varying Endophyte Effects on WUE*

The promotion of rapid stomatal responses in reaction to the environmental changes likely leads to the decreases in water loss by transpiration, and in turn to the increases in WUE over time. This interpretation is in line with Franks' *et al.* (2015) genetic engineering approach to increase WUE of plants. They manipulated the genes involved in stomatal development to decrease stomatal density, specifically by knocking out *epf2* genes and subsequent drops in $g_s$ over the course of drought stresses. They concluded that the reduction of water loss while maintaining photosynthetic activity was the key to improve WUE.

Interestingly, a number of articles analyzed in the present study reported no changes to water relations with certain strains of endophytes inoculated to some plants (Rogers *et al.* 2012; Shukla *et al.* 2012a; Knoth *et al.* 2013a, 2014). Furthermore, there are contrasting reports about endophyte effects on WUE found in the literature. For example, lower WUE was measured in endophyte-infected perennial ryegrass in response to drought (Eerens 1998) as opposed to higher WUE reported in salinity stress (Bu *et al.* 2012).

Changes in WUE induced by endophytes also seem to be affected by water availability of the soils. Morse *et al.* (2002) observed decreased WUE in endophyte-infected *Festuca arizonica* under well-watered conditions while contrasting increased WUE under water-limiting conditions. The authors suggested stomatal closure might help plants withhold water in leaves, leading to conserve soil moisture when drought began. With less use of water, the symbiotic plants could conserve soil moisture during the drought. Subsequently, as the drought became severe, the conserved water allowed the plants to maintain photosynthetic gas exchanges – $A$ and $g_s$ – that probably increased WUE over a long-term period. Bae *et al.* (2009) also demonstrated similar changes in the gas exchange properties of *Theobroma cacao* plants in inoculated with *Trichoderma* spp. under drought conditions.

Nonetheless, it is noteworthy that WUE used in this chapter is a measure of instant responses of gas exchanges. It does not necessarily lead to increases in long-term, biomass based WUE. There are other factors to be considered in a long-term measure; for example, biomass production is caused not only by the amount of photoassimilates, but also by hormonal impacts. Auxin is one of the major phytohormones that is known to stimulate growth responses of plants. Many of the endophyte strains reported were found to produce microbial IAA (indole-3-acetic acids, a naturally occurring auxin) or

thought to promote the production of endogenous auxins (Nassar *et al.* 2005; Xin *et al.* 2009b; Khan *et al.* 2012d; Barnawal *et al.* 2016).

***Other Considerations on Endophyte Effects and Dynamics of the Associations***

Some articles demonstrate there were not significant gains in physiological parameters in response to endophyte inoculations. Rather, they pointed out the changes in plant fitness component (i.e. biomass) by alteration of endogenous hormonal balance (Rogers *et al.* 2012).

The same endophyte does not necessarily produce the same treatment effects over time. For instance, Knoth *et al.* (2013) showed different endophyte effects on plant physiology at different life stages of corn plants suggesting that seasonal variation should also be considered.

The influence of environmental stresses is another consideration. The overall effect sizes of endophytes on all the parameters analyzed here were larger under stressed conditions despite different types of stress factors with a varying range of the intensity of the stressors (Table 1). As an example, Bu *et al.* (2012) investigated effects of fungal endophytes, *Suaeda salsa*, on photosynthetic ability of *Oryza sativa* under five $Na_2CO_3$ stress conditions (0, 5, 10, 15, and 20 mM $Na_2CO_3$). They found that *A* and *E* increased in inoculated plants compared to non-inoculated controls under all $Na_2CO_3$ stress levels. However, WUE, $g_s$, Flr in inoculated plants showed stress intensity dependent responses; they were increased under high $Na_2CO_3$, while there was no significant difference under low $Na_2CO_3$. These results suggested that the fungal endophyte effects on improvement of plant performance represented by the photosynthetic parameters are dependent on $Na_2CO_3$ stress level.

Surrounding environmental conditions also affect the benefits of symbiosis in different ways. Davitt *et al.* (2011) demonstrated the context-dependency of symbiosis by differing light intensity. Endophyte effects have plasticity and plants are also flexible in their response to symbiotic stimulation of physiological traits. From an experimenter's point of view, this could bring a challenge. Assessment of a certain strain/consortium can be difficult and reproducibility can be problematic. On top of that, endophyte effects can be species-specific. Interaction effects should also be considered when endophytes are used as a factor of the experiments that have multiple treatment factors employed. Morse *et al.* (2002) showed the interaction effects of fungal endophyte inoculation and drought stress treatment on *A*, $g_s$, *E*, and WUE over time. Saikkonen *et al.* (1998) reviewed variable endophyte-plant interactions and pointed out the interactions could range from antagonistic to mutualistic. The direction of the relationships could be determined by mode of transmission,

pattern of infections and life span of the host plants. This shows dynamic responses of the host plants to endosymbionts and poses challenges to endophyte research.

## Conclusions

Regardless of types or strains, overall, endophytes were shown to enhance photosynthetic capacities of various host plants. They also improved water relations of host plants through different physiological pathways. A minority of reports pointed to negative endophyte effects on both aspects of plant physiology. Indeed, how to explicitly explain these varying effects remains challenging.

Increased N availability through direction provision or indirect stimulation by endophytes seems to be the main reason for the photosynthetic improvement in most of the studies. Tighter stomatal control or osmotic regulation, and further water management induced by endophytes seems to be the main reason for the better water use efficiency.

However, based on the contexts of experimental settings or treatments, mechanistic explanations could differ. Future endophyte physiology studies may focus on detailed biochemical and molecular level examinations to support lower-level changes in the signaling cascades that shape plant functional traits. To facilitate this process, constructing endophyte mutants lacking genes encoding some functional *in vitro* characteristics, plant mutants lacking genes involved in some functional traits, or a combination of them will be required to conduct functional trait studies.

The benefits of endophytes in plant physiological functions are clear, but the costs are still uncharacterized. What are the C costs of the endosymbiosis? How do plants govern these interactions? What environmental cue/condition can encourage endophyte-plant associations and benefit the two the most? All these questions need to be answered for better understanding of the ecophyphysiology of plant-endophyte interactions.

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List of papers used in the meta-analysis. Find details in Table 1.

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## Tables & Figures

**Table 1** General information about the studies meta-analyzed in this chapter.

| Reference | Type of endophytes | Genus of endophytes | Species of host plants | Leaf physiology parameters | Number of data sets | Stressor | Experimental environment |
|---|---|---|---|---|---|---|---|
| (Ali *et al.* 2014) | Bacteria | *Pseudomonas* | *Solanum lycopersicum* | Chl, Flr | 8 | Salinity | Greenhouse |
| (Barnawal *et al.* 2016) | Bacteria | *Brachybacterium* | *Chlorophytum* | Chl | 1 | Salinity | Greenhouse |
| (Bu *et al.* 2012) | Fungi | *Suaeda* | *Oryza sativa* | Chl, *A*, $g_s$, *E*, WUE | 4 | Salinity | Chamber |
| (Gagné-Bourque *et al.* 2016) | Bacteria | *Bacillus* | *Phleum pretense* | *A*, $g_s$ | 2 | Drought | Chamber |
| (Ghimire & Craven 2011) | Fungi | *Sebacina* | *Panicum virgatum* | Chl, Flr | 3 | Drought | Greenhouse |
| (Gond *et al.* 2015) | Bacteria | *Pantoea* | *Zea mays* | Flr | 1 | Salinity | Lab |
| (Khan *et al.* 2012c) | Fungi | *Exophiala* | *Cucumis sativus* | Chl, Flr | 1 | Heat | Chamber |
| (Khan & Lee 2013) | Fungi | *Penicilium* | *Glycine max* | Chl, Flr | 1 | Metal | Chamber |
| (Khan *et al.* 2012a) | Fungi | *Metarhizium* | *Glycine max* | Chl, Flr, *A*, $g_s$, *E*, WUE | 2 | Salinity | Chamber |
| (Khan *et al.* 2011b) | Fungi | *Penicilium* | *Glycine max* | Chl, *A*, $g_s$, *E*, WUE | 2 | Salinity | Chamber |
| (Khan *et al.* 2016) | Bacteria and yeast | Consortia of *Acinetobacter, Burkholderia, Curtobacterium, Pseudomonas, Rahnella, Rhizobuim, Rhototorula, and Sphingomonas* | *Populus deltoides x P. nigra* | Chl, Flr, $g_s$ | 1 | Drought | Greenhouse |
| (Knoth *et al.* 2013b) | Bacteria | Consortia of *Acinetobacter, Burkholderia, Herbaspirillum, Pseudomonas, Rahnella, Rhizobuim, Rhototorula, and Sphingomonas* | *Zea mays* | Chl, Flr, *A*, $g_s$, *E*, WUE | 6 | Nutrient | Field |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (Knoth *et al.* 2014) | Bacteria and yeast | Consortia of *Acinetobacter, Burkholderia, Enterobacter, Herbaspirillum, Pseudomonas, Rahnella, Rhizobuim, Rhototorula, and Sphingomonas* | *Populus trichocarpa clone nisqually-1* | Chl, Flr, *A*, $g_s$, *E*, WUE | 3 | Nutrient | Field |
| (Li *et al.* 2012c) | Fungi | *Neotyphodium* | *Achnatherum sibiricum* | Flr | 4 | Nutrient | Chamber |
| (Li *et al.* 2012a) | Fungi | *Suaeda* | *Oryza sativa* | Chl, *A*, $g_s$, *E*, WUE | 3 | Metal | Chamber |
| (Madhaiyan *et al.* 2013) | Bacteria | *Enterobacter* | *Jatropha curas* | Chl | 1 | Nutrient | Greenhouse |
| (Morse *et al.* 2002) | Fungi | *Neotyphodium* | *Festuca arizonica* | Flr, *A*, $g_s$, WUE | 1 | Drought | Field |
| (Naveed *et al.* 2014) | Bacteria | *Burkholderia* | *Zea mays* | Chl, *A*, $g_s$, *E*, WUE | 4 | Drought | Greenhouse |
| (Newman *et al.* 2003) | Fungi | *Neotyphodium* | *Festuca arundinacea* | Flr, *A* | 1 | Nutrient | Field |
| (Patel & Saraf 2013) | Bacteria | *Enterobacter* | *Jatropha curas* | Chl, Flr | 3 | Salinity | Lab |
| (Rogers *et al.* 2012) | Bacteria | *Enterobacter* | *Populus deltoides x P. nigra* | *A*, $g_s$, WUE | 1 | - | Field |
| (Rojas-Tapias *et al.* 2012) | Bacteria | *Azotobacter* | *Zea mays* | Chl, Flr | 4 | Salinity | Chamber |
| (Shahabivand *et al.* 2012) | Fungi | *Piriformospora* | *Triticum aestivum* | Chl, Flr, *A* | 3 | Metal | Chamber |
| (Shukla *et al.* 2012b) | Fungi | *Trichoderma* | *Oryza sativa* | Chl, *A*, $g_s$, WUE | 5 | Drought | Lab |
| (Vahabi *et al.* 2016) | Fungi | *Piriformospora* | *Arabidopsis thaliana* | Flr | 2 | Shading | Chamber |
| (Waqas *et al.* 2012) | Fungi | *Penicilium* | *Oryza sativa* | Chl, Flr | 8 | - | Chamber |
| (Woodward *et al.* 2012) | Fungi | *Fusarium* | *Oryza sativa* | *A* | 2 | - | Lab |
| (Yang *et al.* 2014a) | Fungi | *Phomopsis* | *Oryza sativa* | Chl | 2 | Nutrient | Chamber |
| (Zarea *et al.* 2012) | Fungi | *Piriformospora* | *Triticum aestivum* | Chl | 6 | Salinity | Greenhouse |
| (Zhang & Nan 2007) | Fungi | *Neotyphodium* | *Elymus dahuricus* | Chl | 1 | Drought | Chamber |

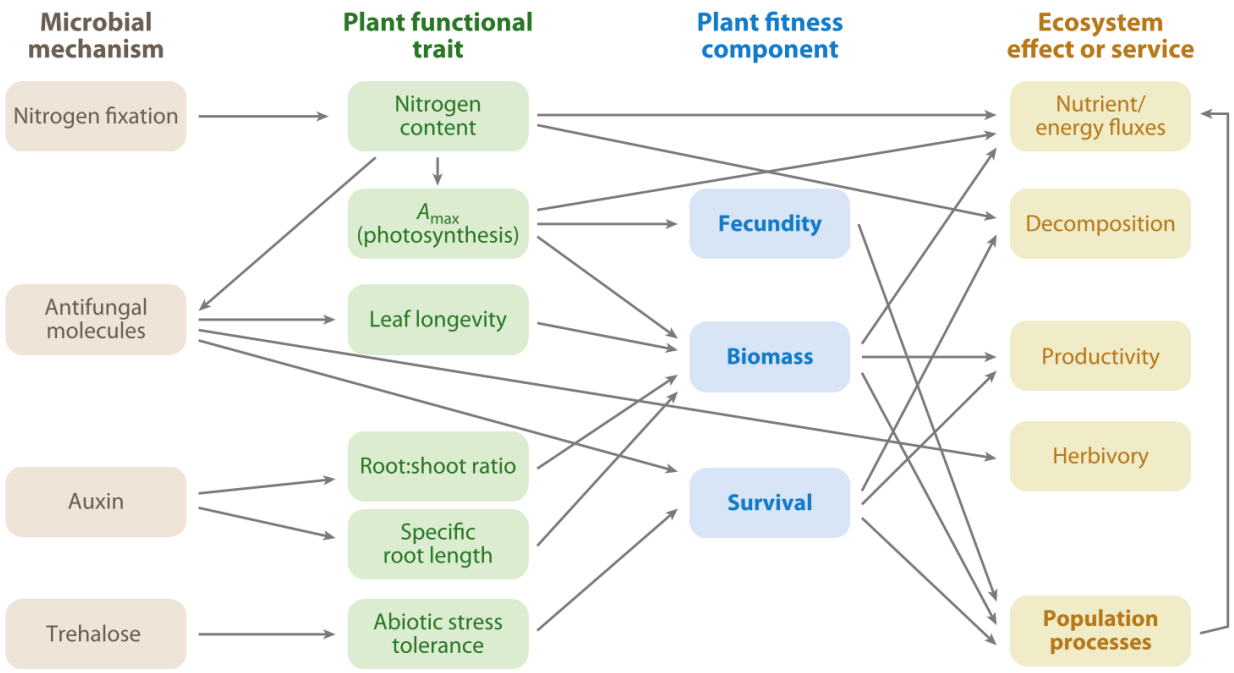


**Fig. 1** Exemplary interactions between host plants and endophytic microorganisms and their relationships in metabolic and physiological components. Not all connections are described in the diagram. In this chapter, the focus falls on plant functional trait with emphasis on photosynthetic and water use efficiency. Adapted from Friesen *et al.* (2011).

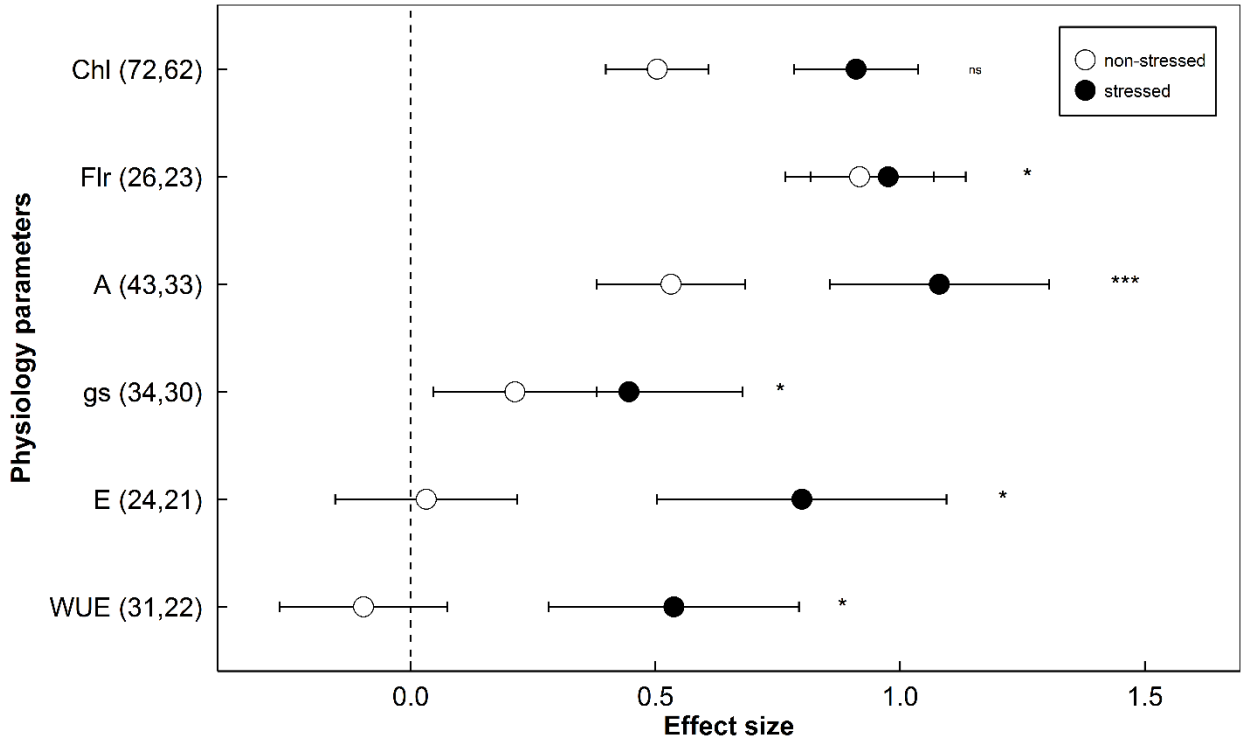


**Fig. 2** Overall effect sizes of various endophytes on host plants' photosynthetic and water relations parameters. A total of 84 studies are used in the meta-analysis. Chl, Flr, A, gs, E, and WUE refer to chlorophyll content, photochemical efficiency, net $CO_2$ assimilation rate, stomatal conductance to water, transpiration rate, and intrinsic water use efficiency defined by A/gs. The numbers in parentheses mean the number of data sets used in the meta-analysis for each parameter (under non-stressed, stressed conditions). Open and closed circles show the mean overall effect sizes of endophyte inoculation on the physiological parameters under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. Significance codes on the right present paired *t*-test results of the differences of the effect sizes between non-stressed and stressed host plants (ns, non-significant; * and *** significant at $P < 0.05$ and 0.001 levels). Detailed breakdowns of the effect sizes of individual studies on each physiological parameter are provided in supplementary figures (**Figs S1-6**).

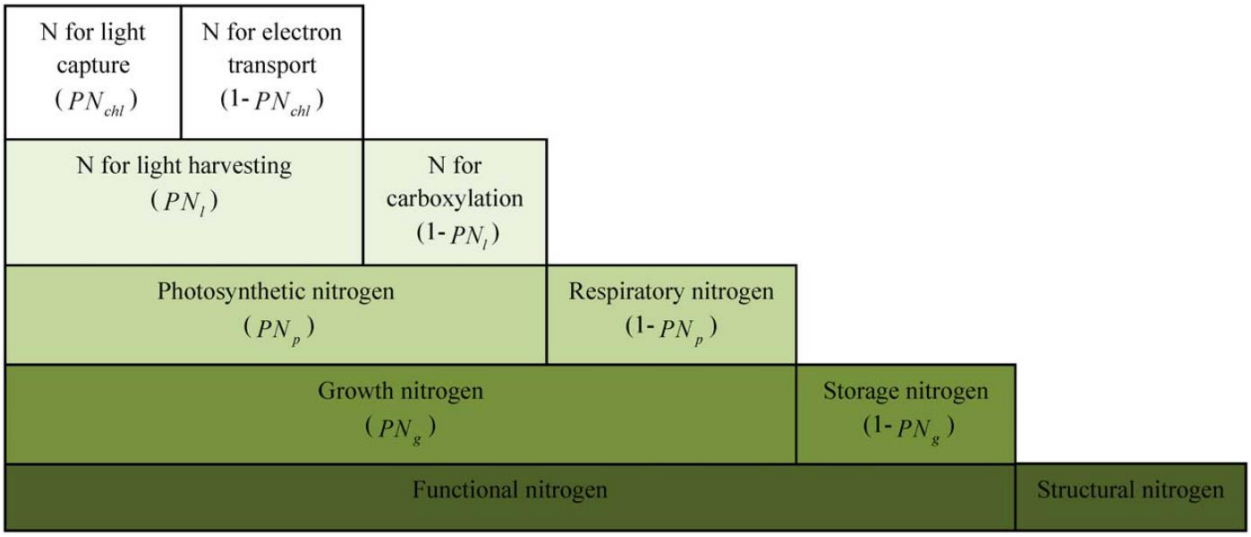


**Fig. 3** Nitrogen is an essential nutrient in plant metabolisms. Up to 40% of growth nitrogen is incorporated to photosynthetic machinery components enclosing light harvesting enzymes and $CO_2$ assimilation associated enzymes, e.g. Rubisco, in the Calvin-Benson cycle. This determines photosynthetic efficiency under different nitrogen availability. Adapted from Xu *et al.* (2012)

## Supplementary Material

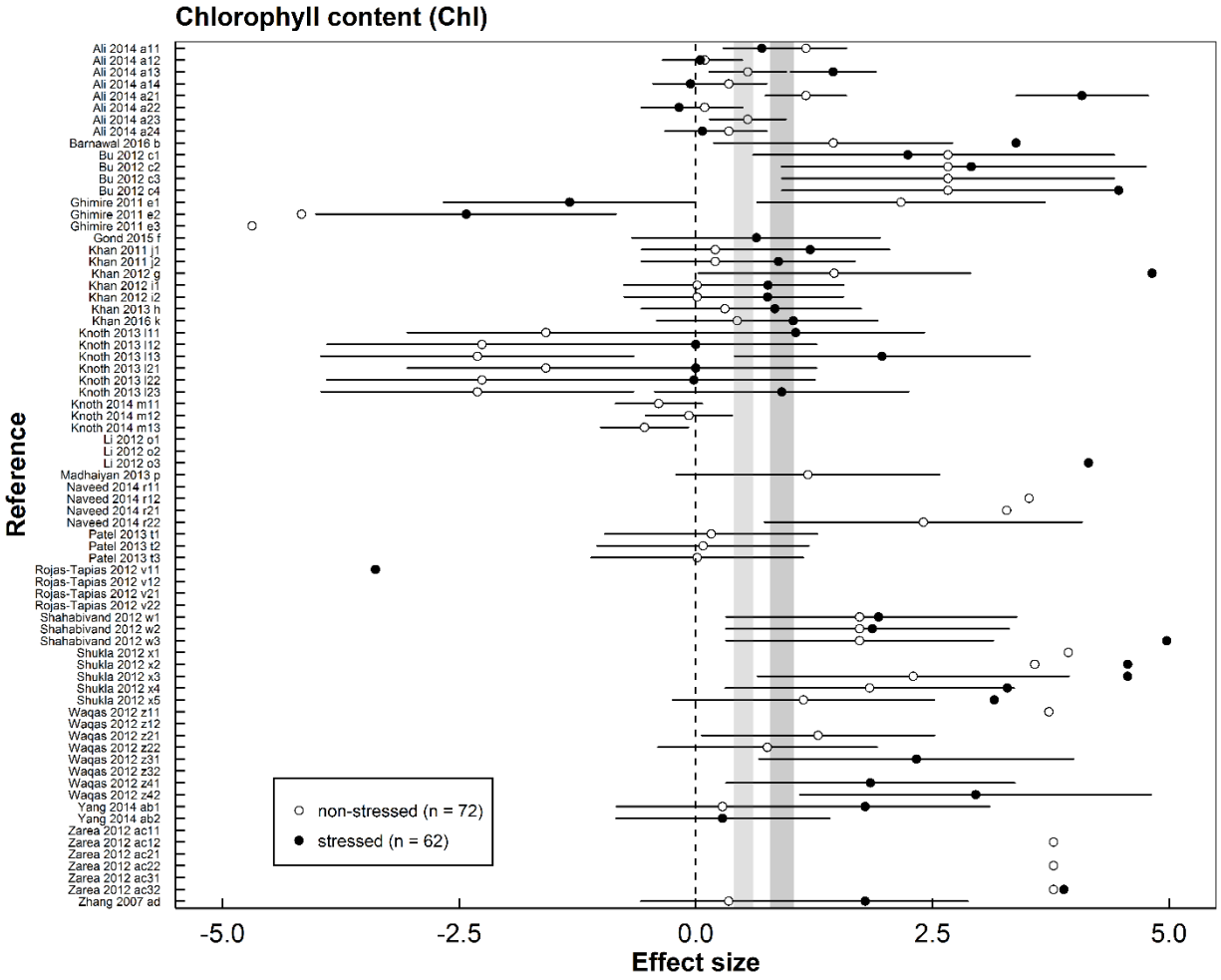


**Fig. S1** Effect sizes of various endophytes on host plants' chlorophyll content (Chl). Open and closed circles indicate the mean endophyte effect sizes on Chl under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 72 and 62 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

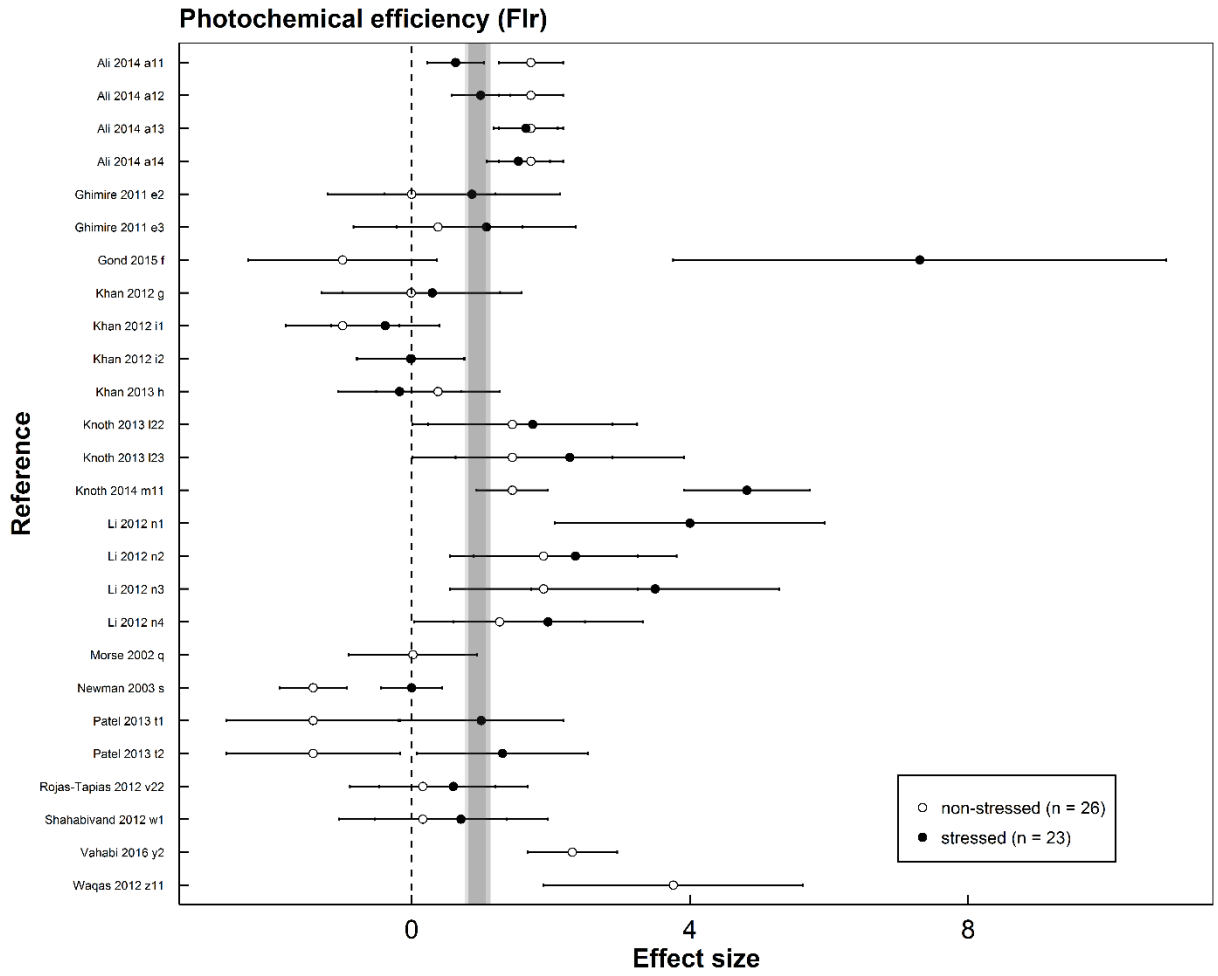


**Fig. S2** Effect sizes of various endophytes on host plants' photochemical efficiency (Flr). Open and closed circles indicate the mean endophyte effect sizes on Flr under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 26 and 23 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

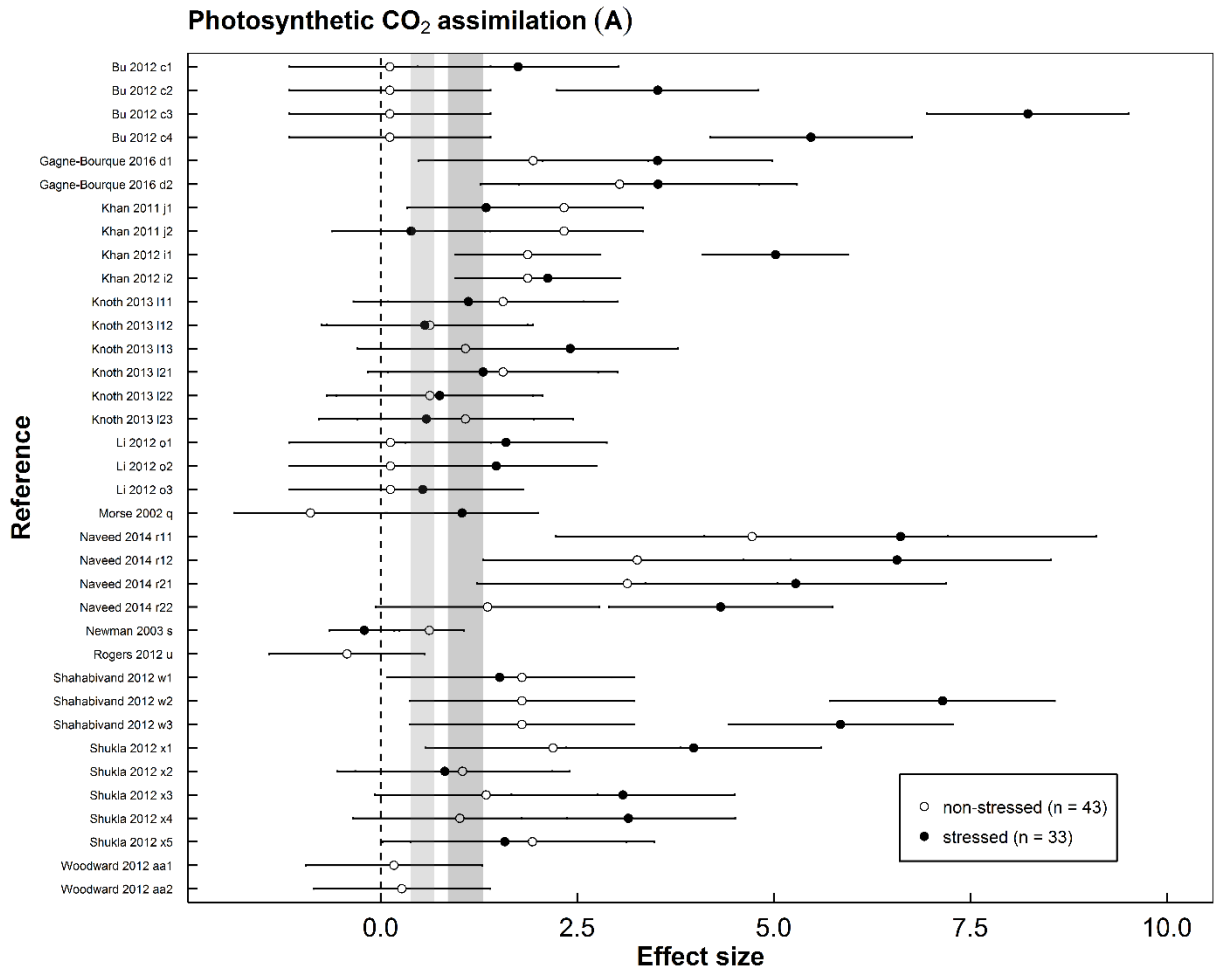


**Fig. S3** Effect sizes of various endophytes on host plants' net $CO_2$ assimilation rate (A). Open and closed circles indicate the mean endophyte effect sizes on A under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 43 and 33 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

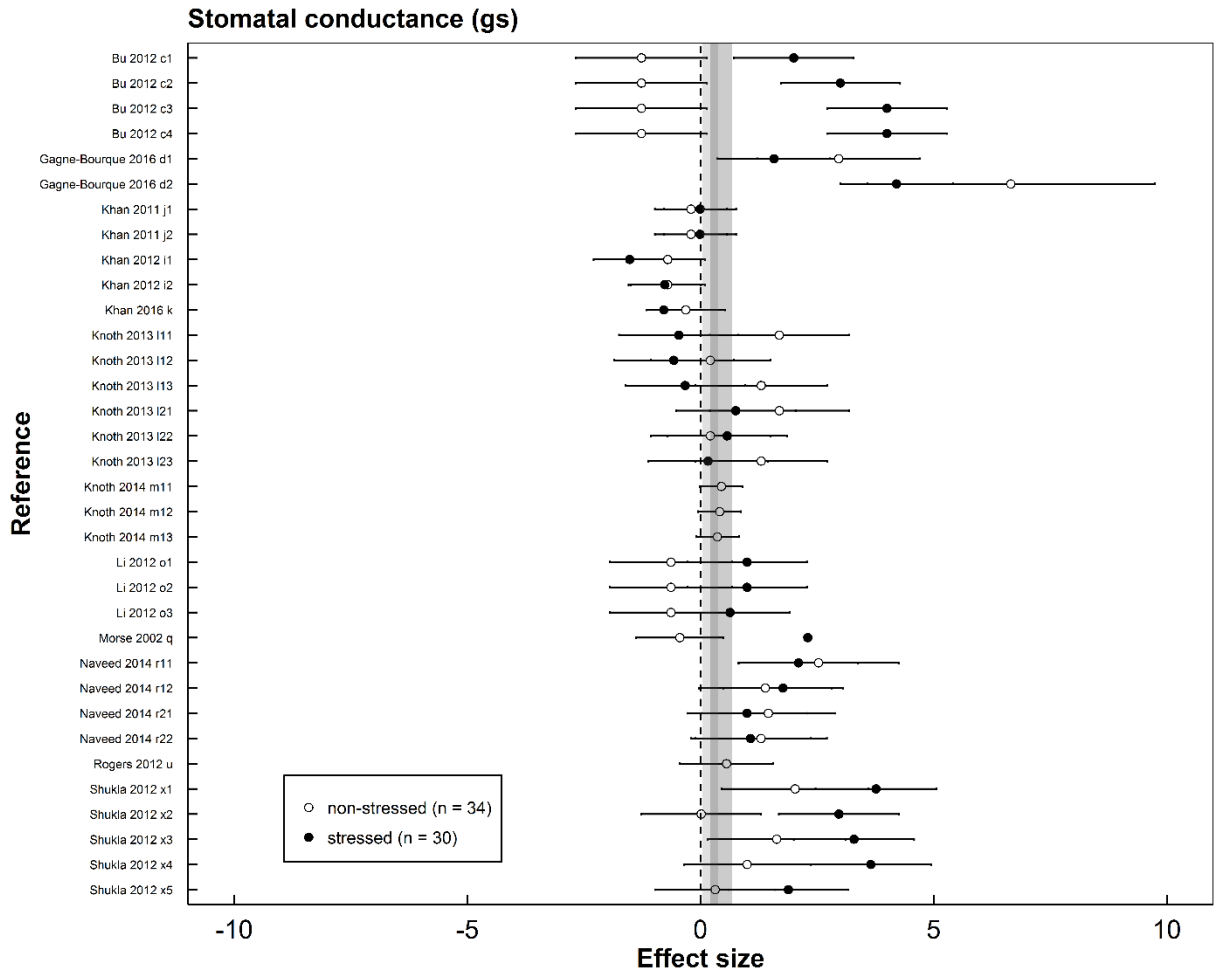


**Fig. S4** Effect sizes of various endophytes on host plants' stomatal conductance (gs). Open and closed circles indicate the mean endophyte effect sizes on gs under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 34 and 30 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

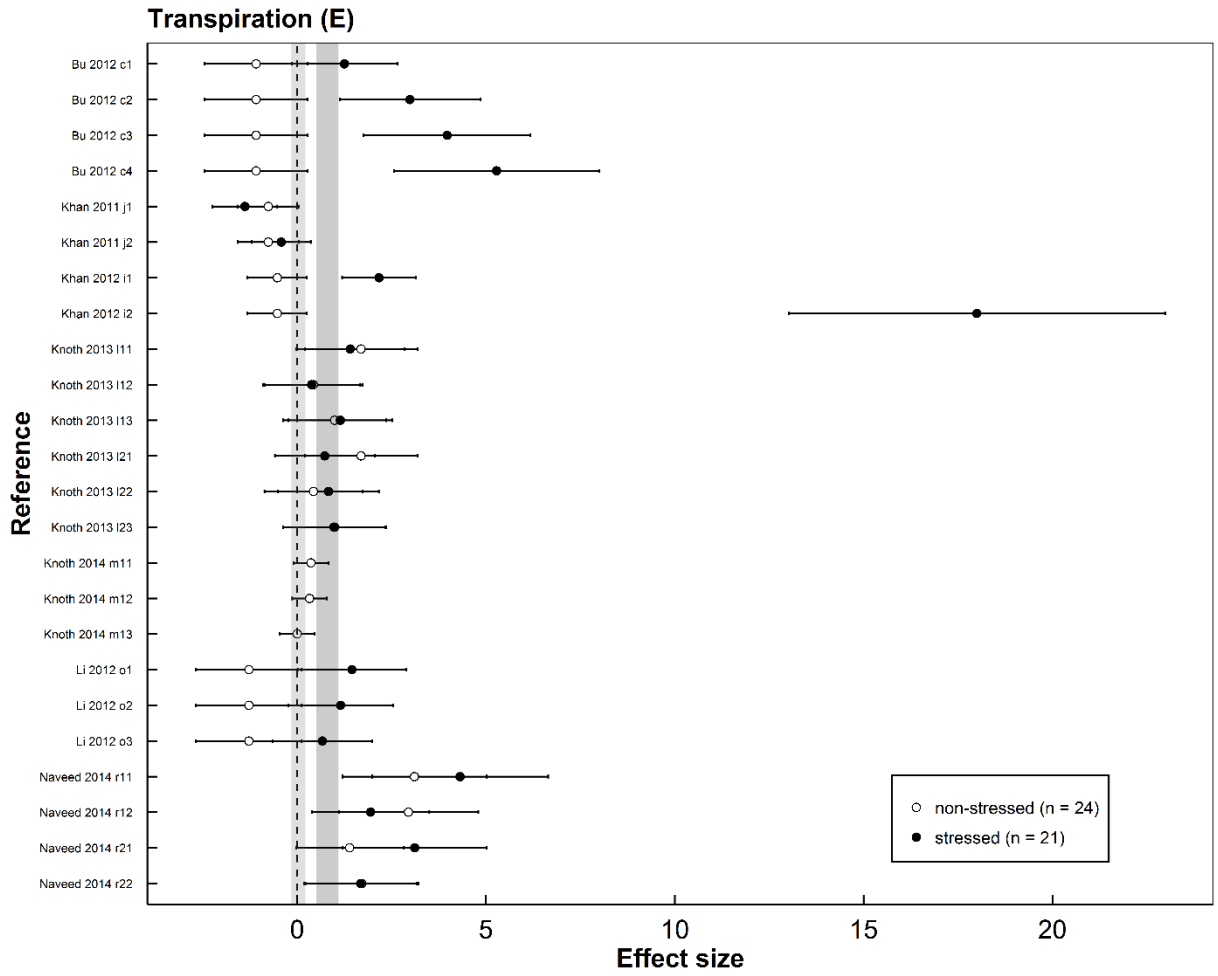


**Fig. S5** Effect sizes of various endophytes on host plants' transpiration rate (E). Open and closed circles indicate the mean endophyte effect sizes on E under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 24 and 21 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

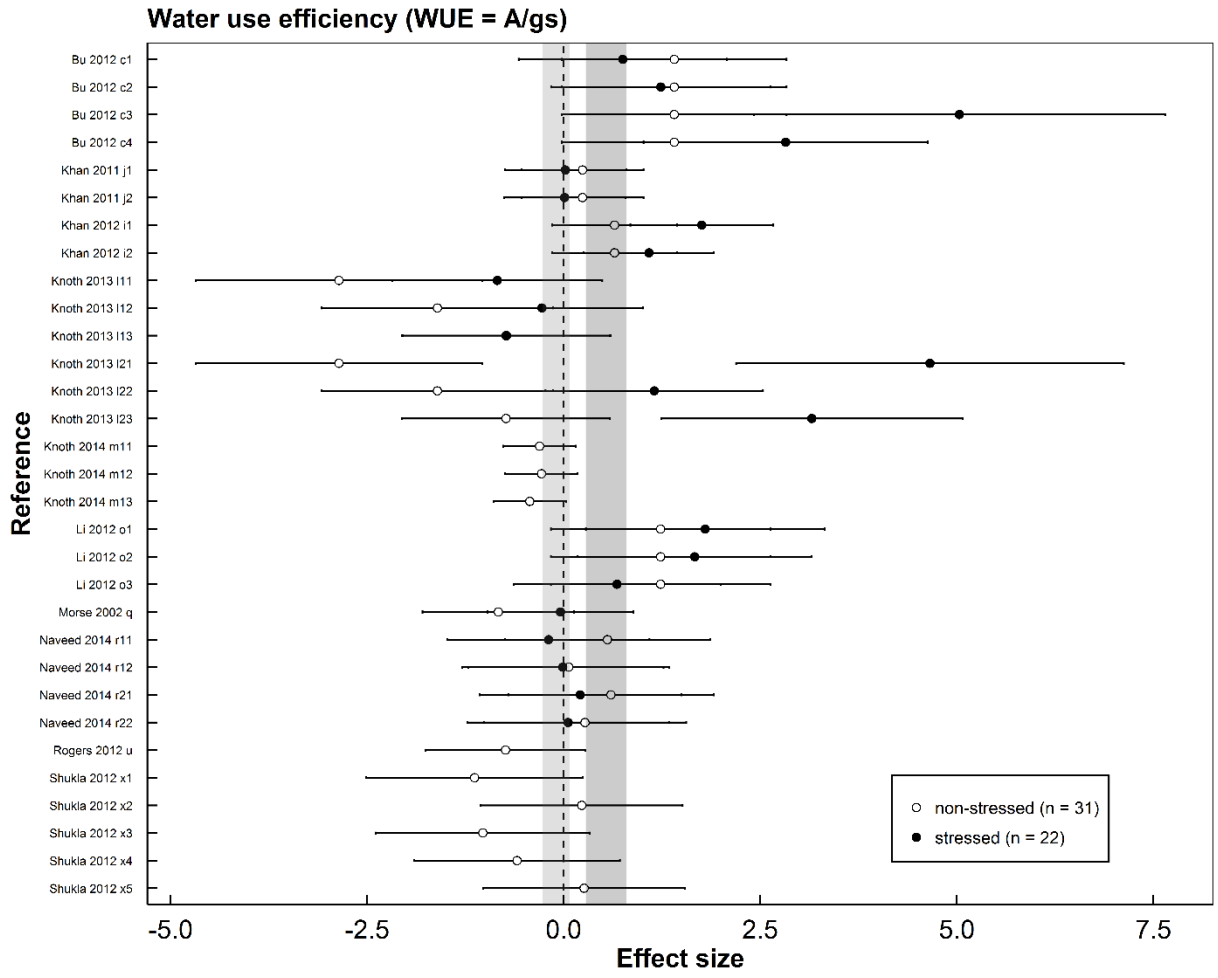


**Fig. S6** Effect sizes of various endophytes on host plants' intrinsic water use efficiency (WUE). Open and closed circles indicate the mean endophyte effect sizes on WUE under non-stressed and stressed conditions. Error bars indicate ± 95% confidence intervals of the means calculated by the Hedge's method (Hedges *et al.* 1999). If the error bars overlap the dotted line (effect size = 0.0), the effect is considered non-significant. The light and dark grey shades represent the overall endophyte effect sizes (the aggregates of 31 and 22 studies, corresponding to one in **Fig. 2**) under non-stressed and stressed conditions.

# CHAPTER 2

## Salicaceae Endophytes Modulate Stomatal Behavior and Increase Water Use Efficiency in Rice

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## Abstract

Bacterial and yeast endophytes isolated from the Salicaceae family have been shown to promote growth and alleviate stress in plants from different taxa. To determine the physiological pathways through which endophytes affect plant water relations, we investigated leaf water potential, whole-plant water use, and stomatal responses of rice plants to Salicaceae endophyte inoculation under $CO_2$ enrichment and water deficit. Daytime stomatal conductance and stomatal density were lower in inoculated plants compared to controls. Leaf ABA concentrations increased with endophyte inoculation. As a result, transpirational water use decreased significantly with endophyte inoculation while biomass did not change or slightly increased. This response led to a significant increase in cumulative water use efficiency at harvest. Different endophyte strains produced the same results in host plant water relations and stomatal responses. These stomatal responses were also observed under elevated $CO_2$ conditions, and the increase in water use efficiency was more pronounced under water deficit conditions. The effect on water use efficiency was positively correlated with daily light integrals across different experiments. Our results provide insights on the physiological mechanisms of plant-endophyte interactions involving plant water relations and stomatal functions.

**Keywords:** endophytes[1], rice[2], stomatal conductance[3], water potential[4], water relations[5], water use efficiency[6], water deficits[7], ABA[8]

## Introduction

Climate change has become a great challenge in agriculture by reducing potential yield of crops as environmental stresses on crops increase (Cai *et al.* 2015). Growing human population will reach 9.6 billion by 2050 (United Nations Department of Economic and Social Affairs 2015), which ominously implies the demand for crop production will concurrently increase substantially. As a result, two central themes to food security must be addressed: 1) how to increase crop plant resiliency with dynamically changing environmental conditions, and 2) how to improve crop yield with more sustainable methods that possibly lessen the burden of chemical and irrigation inputs used for both fertilizers and environment management (Flexas 2016).

Amongst all of the resources in the agricultural industry, field irrigation water usage draws the most attention of scientists and the public alike. Currently, 53% of cereal production is met by irrigation. If this trend continues, agriculture will remain as the biggest player in draining freshwater globally by 2050 (Rosegrant *et al.* 2009). Moreover, considering the fact that climate change brings unstable precipitation, more frequent runoffs, and weather extremes such as the 2012-2014 drought in

California USA (Griffin & Anchukaitis 2014), it will necessitate more efficient, innovative approaches to water use.

Recent efforts in achieving higher crop sustainability involve increasing plant water use efficiency (WUE), including but not limited to, genetic manipulation of plants to form less stomata (Franks *et al.* 2015), selection of drought tolerant genotypes through fast screening methods (Condon *et al.* 2004), and alteration of canopy structure to maximize light acceptance (Drewry *et al.* 2014). Nevertheless, these techniques are not readily available in the field, still under testing, or maybe unrealistic for larger scale applications.

Endophytes are microorganisms living in the intercellular spaces of plants, providing several benefits to hosts, in turn, receiving carbohydrate-based nutrients for their growth (Dobereiner 1992). These are mutualistic symbionts that can enhance plant fitness and performance in terms of increasing host biomass under stressful conditions (Goh et al. 2013). For example, endophytes that confer water stress tolerance are well studied (Rho *et al.* 2017) and a number of research articles show their efficacy in plant fitness and plasticity under low water availability (Khan *et al.* 2015a, 2016; Gagné-Bourque *et al.* 2016). Thus, a realistic aim is to supplant other current agriculture methods with endophytic symbioses to increase crop WUE and yields.

Doty et al. (2009) isolated diazotrophic – di-nitrogen fixing – endophytic bacteria and yeast from the Salicaceae family of plants – native poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) growing in primary substrate of natural riparian zones. In this environment the nutrient supply to the plants was severely limited due to frequent flooding. The isolates were characterized in the paper and subsequent publications clearly demonstrated their potential symbiotic traits in other host species across taxa including grass species such as rice (Khan *et al.* 2012e; Knoth *et al.* 2013a, 2014). A major goal of our past and current studies has been to test the effectiveness of the endophytes from Salicaceae hosts on improving the growth and mitigating stress of host plants from other taxa, especially crops. Since then, we have been using these isolates to find out their potential benefits in other agricultural crops and to explore their mechanistic level impacts. In line with these efforts, our recent publication, Kandel et al. (2015), demonstrated that this endophyte consortium significantly increased biomass of the same rice variety, leading to the increases in yield potential. However, Kandel et al. (2015) did not provide information on the physiological benefits and their underlying mechanisms. In this context, the present study delivers a mechanistic view of having endophyte symbiosis focusing on water relations of rice as a host.

Previous results have provided evidence for prospective impacts of the select endophytes on plant functional traits. The select endophytes were shown to have multiple potentially symbiotic traits including phytohormone production (indole-3-acetic acid in Xin et al. 2009), biological nitrogen fixation (Knoth *et al.* 2014; Doty *et al.* 2016), and cross-host biological nitrogen fixation capacity (Knoth *et al.* 2013a; Kandel *et al.* 2015). In a recent study (Khan *et al.* 2016), it was demonstrated *in vitro* with liquid chromatography that select endophytic bacterial and yeast strains produce plant hormones, including abscisic acid (ABA) – a key player in stomatal control and development. In the article, the authors inoculated poplar cuttings to test stomatal response and photochemical efficiency under water deficit conditions, examining daytime stomatal conductance ($g_s$) and chlorophyll fluorescence ($F_v/F_m$). The results showed significant decreases in $g_s$ over time and higher $F_v/F_m$ in inoculated cuttings compared to controls. Multiple possible mechanisms based on microbial assays and genomic analysis were provided. Based on the findings of endophytic ABA production, we postulate that these symbionts can trigger stomatal closure and affect stomatal development of host plants, as potential mechanisms for the observed drought tolerance.

Rice (*Oryza sativa*) is currently the second most important staple crop worldwide following maize (FAOSTAT 2015). Since rice is suggested to be an isohydric species (Parent *et al.* 2010), it is likely to be sensitive to daytime water demand as increasing transpiration rate aligns with rising air temperature and light intensity – both leading to an increase in vapor pressure deficits (VPD) between the air and leaf surface – during the daytime. The control of losing and blocking water vapor exchange on the surface of leaves is important for managing the resource as well as operating photosynthetic machinery by absorbing enough amount of $CO_2$ from the ambient air. Consequently, isohydric species have developed tighter governing of stomatal controls together with stable water potential adjustment to endure losing water during daytime, when gas exchanges of leaves occur most actively, and to survive in unfavorable water conditions (Tardieu & Davies 1992).

Nonetheless, there are contradictory results about endophyte effects on stomatal control over numerous host plant species (Malinowski & Belesky 2000). Some reported they facilitated the stomatal closure to conserve water inside, while others reported the opposite results; they assisted the hosts with opening stomata to absorb more $CO_2$ from the atmosphere to photosynthesize and eventually leading to more biomass gain. However, since the experiments were conducted in different contexts with unquestionably various combinations between the hosts and symbionts, clear conclusions cannot be drawn.

To date, multiple studies examined the impacts of endophytes on whole plant physiology related to water relations and leaf gas exchange (Richardson *et al.* 1993b; Elmi & West 1995; Morse *et al.* 2002; Rogers *et al.* 2012), but their complete mechanisms matching with inoculated endophytes' characteristics have not yet been demonstrated. To provide a comprehensive understanding of endophyte effects on host plant water relations, we conducted a series of greenhouse experiments to examine endophyte effects on physiological attributes of rice water relations: Stomatal responses, water potential, water use and biomass gain of rice plants upon endophyte inoculation. In this study, we tested hypotheses: 1) the ABA-producing endophytes reduce diurnal $g_s$ leading to reduced total water use and improved WUE, 2) the inoculation effects on plant water relations differ between different endophyte strains, and 3) the effect size of endophyte inoculation varies with environmental conditions such as water deficits, elevated $CO_2$, and light levels.

## Materials and Methods

Preparation of microbial materials for the four independent experiments shared the same protocols. All four experiments were conducted in a greenhouse in the Douglas Research Conservatory at the University of Washington (47°39′27″N, 122°17′21″W; 10 m elevation), Seattle, WA, USA. Details about the experimental settings are provided in Table 2.

### *Origins of endophytes and preparation for inoculation*

Nine different strains of diazotrophic endophytic bacteria and yeast were used in this study whose *in vitro* characteristics were identified and reported previously by Doty et al. (2009). WP1, WP5, WP9, WP19, WPB, WW5, WW6, and WW7C were isolated from wild black cottonwood (*P. trichocarpa*) and wild willow trees (*S. sitchensis*) at their native habitat, the Snoqualmie River, Western Washington, whereas PTD1 was isolated from hybrid poplar (*P. trichocarpa* × *P. deltoides)* (Doty *et al.* 2005). Details about the endophyte strains are provided in Table 1. All of these endophyte strains have been shown to be potentially diazotrophic by having positive amplification of *nifH* marker gene for nitrogenase reductase (Doty *et al.* 2009a; Knoth *et al.* 2013a). Their positive effects on biomass increase of different host plants were also demonstrated by several articles in consequent studies (Khan *et al.* 2012e, 2015b, 2016; Knoth *et al.* 2014; Kandel *et al.* 2015). In addition, their colonization efficiency on host plants was established and reported in our previous work (Knoth *et al.* 2013a; Kandel *et al.* 2015, 2017a). We used the same inoculation method that was developed from the prior studies.

The selected endophytes were grown on N-limited combined carbon medium (NL-CCM, Rennie 1981) for growth of endophytes to maintain their nitrogen fixation ability. All of them grew well on the media, visually identified after 48-hour growth, and then cell suspension cultures were started in flasks containing NL-CCM broth. Three to five days later, optical density of the bacterial culture was measured using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, USA). The final concentration of the bacterial solution for inoculation was adjusted to $OD_{600} = 0.1$ (equivalent to approximately $1 \times 10^7$ cells) using sterile deionized water and N-free liquid media (Doty *et al.* 2009a). A mock-inoculum for the control group was prepared just with the N-free liquid media. All microbiological tasks were done in a sterile condition using proper aseptic sterilization techniques.

***Experiment 1: A greenhouse study using multiple endophyte strains***

*Preparation of plant and microbial materials*

Three bacteria strains were used in this greenhouse study. WP5, WPB, and PTD1 were selected to compare their effects on water relations of the host plants with emphasis on stomatal behaviors and leaf water potential components. Experiment 1 was conducted from October 15$^{th}$ 2014 to March 28$^{th}$ 2015.

We used a very early to early maturing, semi-dwarf, Japonica variety M-206 rice (*O. sativa*) that was identified as the best responding rice variety to the endophyte inoculation in a previous study (Kandel *et al.* 2015). Rice seeds were surface-sterilized by imbibing them with 3% NaOCl for 4 hours to remove any debris and contaminants on the seed coat. The seeds were rinsed with sterilized deionized water for four times to wash out remaining NaOCl. Although this surface sterilization technique does not guarantee the removal of all microbes in the seeds due to possible internal/inherent microbiome inside (Nelson 2017), it is a widely used procedure to eliminate at least the ones on the surface. Thus, any differences in responses from the plants can be statistically interpreted as the effects of endophyte inoculation treatments.

The surface sterilized seeds were planted in 1-gal pots placed into plastic buckets. Horticultural root media (Sunshine Mix #2, Sun Gro Horticulture, Agawam, MA, USA) were used to grow the plants. There were four treatments groups, three with single strain inoculations (WP5/WPB/PTD1) and a mock-inoculated control (CTRL) with ten replicates in each treatment group. As such, the total number of pots was forty, and eight plants were planted in a pot. The subjects were placed in a greenhouse bench space where the air temperature and relative humidity (RH) are automatically controlled. To account for the potential environmental gradient along a greenhouse bench, we used a

randomized complete block design with blocks placed across the bench. To further minimize location effects of the greenhouse, the position of pots within a block was reset every week. The average air temperature of the greenhouse environment during the experiment was 22/19°C, 16/8 hrs day/night supplemented with high pressure sodium light (400 W single phase bulbs, Phillips Electronics North America Corp., Andover, MA, USA) to compensate for the lack of sufficient sunlight during the winter-time. The average daily light integral (DLI) was 9.5 mol $m^{-2}$ $d^{-1}$ of photosynthetically active radiation (PAR). The air temperature and light intensity were recorded in pendant type data loggers (UA-002-08, Onset Computer Corporation, Bourne, MA, USA) at 30-min intervals during the experiment period. Relative humidity was 48/54% day/night in average (Table 2).

After inoculation, plants were given 200 mL of a N-free liquid nutrient solution adjusted at the quarter-strength modified Hoagland solution (Hoagland & Arnon 1950). Deionized water was fully given until the water filled up to a 10-cm mark of the buckets to simulate the field growing conditions of paddy soil growing rice. The amount of water and fertilizer supplied was recorded weekly.

*Gas exchange measurements*

We refer to Moldenhauer et al. (2013)'s classification to specify the growth and development stages of rice plants hereafter.

Gas exchange measurements were taken three times during the experiment on day 58 (V3-4), 118 (V5), and 153 (R1-2) using portable photosynthesis systems equipped with IRGAs (LI6400XT, Li-Cor, Inc., Lincoln, NE, USA). The measurements were taken between 12 pm and 5 pm each time. 2-$cm^2$ leaf chamber fluorometers (6400-40, Li-Cor, Inc.) were set to measure photosynthetic assimilation rate ($A$), $g_s$, and real-time intrinsic WUE ($A/g_s$). Settings of the sensor heads were 1500 μmol $m^{-2}$ $s^{-1}$ PFD for saturating light intensity, 400 ppm of $CO_2$ concentration in the reference cell of the instruments, 25°C block temperature, 300 μmol $s^{-1}$ flow rate, and 40-70% RH to optimize the microclimate for photosynthesis during the measurements. Before the measurements, a minimum of 3 minutes was given for the response time of the leaf samples.

*Stomatal conductance measurements*

163 days after germination, at about R3-4 stage of their growth, the diurnal changes of $g_s$ 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 first measurements taken at 9 am.

*Leaf water potential measurements*

One plant per pot was randomly selected to measure destructive leaf water potential to analyze water relations of the hosts that may be influenced by the microorganisms. A pressure bomb (Model-1000, PMS, Albany, OR, USA) was used to measure midday (12 pm to 1 pm) leaf water potential of the samples. The second or third youngest leaf from the top was chosen and immediately after the readings, the rest of the plants were harvested for taking measurements of solute potential of plant extracts. This approach was used to parse out osmotic potentials for the entire leaf, assuming homogenized whole plant solute potential is the same as the leaf solute potential. The harvested leaves were transported to a −80°C deep freezer for breaking the cell walls to mix apoplastic and symplastic solutions. The frozen samples were loaded and sapped in a hydraulic plant sap press (Plant Sap Press #2720, Spectrum Technologies, Inc., Aurora, IL, USA). 400 μL of the extracted plant solution was collected in sample cups of a thermocouple psychrometer (SC-10, Decagon Devices, Inc.) to measure osmotic potential of the solution. Soluble sugar content (SSC) of the remainders was assessed by a handheld refractometer (RHB-10/ATC, Horiba, Japan).

*Calculation of stomatal density*

Specimens for evaluating influences of the bacteria on stomatal development of the hosts were collected using a common stomatal imprint technique (Gay & Hurd 1975). The sampling was done two days after $g_s$ was measured on day 165. The imprints were collected from the abaxial side of the leaves using nail polish. We counted stomata to calculate stomata density of the specimen. Three field of views were observed and the variables were counted for each sample. The triplicated observed variables were averaged to calculate the parameters. ImageJ program (Schneider *et al.* 2012) was used with an add-on package to count the numbers of stomata and epidermal cells of the specimen with 40X magnification from a standard compound microscope.

*Verification of colonization*

General colonization characteristics of the endophytes and efficacy of the inoculation method used in the study are fully documented in Kandel (2016). Also, an extended review can be found in Kandel et al. 2017.

One rice plant per pot was harvested 99 days after germination to verify colonization of the endophytes. Leaf, stem, and root tissues were separately harvested and the surfaces of the tissues were sterilized by submerging them in 3% NaOCl for 3 minutes for leaf and stem tissues and 8 minutes for root tissues, followed by rinsing with sterilized deionized water four times to remove any

remnants of NaOCl. The final rinsing water from each sample was collected to confirm the efficacy of the surface sterilization process. Approximately 100 mg of the sterilized tissues were transferred into 1.5-mL microtubes containing 400 μL NL-CCM solution and then they were ground with sterilized microtube pestles. The extracts were diluted into $10^{-4}$ using an aseptic serial dilution. The diluted solution was plated onto NL-CCM containing petri-dishes. After 48 hours of incubation at room temperature, photos of the plates were taken on a photo stand. The photos were downloaded to further process colony forming unit (CFU) count. CFUs were counted with ImageJ program to compare the bacterial counts in the colonized tissues among the treatment groups. Total CFU count from the leaf, stem, root tissues combined was marginally higher for the inoculated plants compared to the control plants ($P = 0.085$).

***Experiment 2: A $CO_2$ enrichment study using a single endophyte strain***

A $CO_2$ enrichment study was designed and conducted to test stomatal responses affected by endophytes under two different atmospheric $CO_2$ concentration.

*Preparation of plant materials*

Experiment 2 was conducted from March 21st 2014 to September 2nd 2014. For each pot, surface sterilized four M-206 rice seeds were sown in a 3-gal plastic pot containing the same horticultural root media (Sunshine Mix #2, Sun Gro Horticulture) that was fully irrigated with tap water after seeding. Before placement in closed top chambers, the pots were nested in 4-gal plastic pails for easier measurements of fertilizer and water to be supplied. The watering and fertilizing were done through the gaps between the pots and the pails using a plastic funnel. The amount of water and fertilizer supplied was recorded weekly. A total of thirty-two pots were prepared; eight pots per chamber randomly assigned to receive control (E-) or endophyte (E+) treatments.

*$CO_2$ treatment and inoculation*

The experiment was a 2 × 2 factorial with eight replications; two levels of atmospheric $CO_2$ concentration – ambient $CO_2$ (AMB, approximately targeted to 400 ppm) and elevated $CO_2$ (ELE, app. 800 ppm) – and two levels of inoculation status – mock-inoculated control with surface sterilized seeds (E-) and diazotroph endophyte-inoculated treatment group (E+). Due to the nature of the $CO_2$ treatment with a chamber, a split plot design was applied to deploy the pots in two sets of chambers. For the detailed environmental controls and specifications of the chambers, refer to Kinmonth-Schultz and Kim (2011) and Nackley et al. (2016).

A total of four chambers were used in the experiment. Two were AMB chambers with ambient air supplied through air ducts from outside of the greenhouse and the other two were set to ELE chambers with pure $CO_2$ supplied through air tubing, of which concentration was controlled by flowmeters. Accordingly, a single chamber accommodated both four E- and four E+ plant pots; eight pots, plus a pot without plant samples for monitoring the amount of weekly evaporation from the soil surface by the airflow of the chambers.

The air temperature, RH, light intensity, and $CO_2$ concentration were monitored and recorded in a data logger (CR1000, Campbell Scientific, Logan, UT, USA). The average temperature during the experiment was 23/19°C and RH of the air in the chambers was 60/66% day/night. The light regime of the greenhouse was set to 16/8 hrs day/night (7 am – 9 pm as a photoperiod) and the average DLI from the sunshine and the supplementary lighting of the facility was 9.1 mol $m^{-2}$ $d^{-1}$ of PAR. The average atmospheric $CO_2$ concentrations of the two AMB chambers and the other two ELE chambers were 437/886 ppm, respectively. See Table 2 for more details about the environmental settings.

In this experiment, one reference strain, WP5, was used. Seven days after sowing when 95% of the seeds were germinated, 2 mL of the prepared WP5 inoculum were added to each seedling. We pipetted the inoculum to the crown of the rice seedlings for the roots to easily absorb the solution. The other half of the plants were mock-inoculated with the same volume of the mock-inoculum for setting the control group.

As in Experiment 1, 200 mL of a quarter strength nitrogen with the nitrogen free medium was supplied and the plants were fully irrigated through the plastic pail to prevent any stress responses from water deficit.

*Stomatal conductance measurements*

At 128 days after germination around R3-4 stage of their growth, $g_s$ of the youngest fully expanded leaves was measured at 3-hour intervals to examine diurnal changes of the parameter using steady state leaf porometers (SC-1, Decagon Devices, Inc.). We applied the same measurement procedure described in Experiment 1.

***Experiment 3: A water deficit study using endophyte consortia***

The third experiment was designed to test the endophyte effects on long-term WUE under both well-watered and water deficit conditions. The trial was conducted from July 14$^{th}$ to October 6$^{th}$ in 2015. The same plant material, M-206 rice, was prepared and used in this trial. The differences from the

previous experiments were the endophyte treatment and the fertilization and irrigation conditions outlined below. Measured metrics were weekly pot-based transpiration and biomass allocation at harvest.

*Preparation of plant materials and inoculation/water deficit treatments*

The average air temperature recorded in data loggers (UA-002-08, Onset Computer Corporation) was 29/20°C in day/night. The average DLI was 35.8 mol $m^{-2}$ $d^{-1}$ of PAR with a 16/8 hrs day/night photoperiod of supplemental lighting. The air temperature and light intensity were recorded every 15 minutes. RH was 57/71% day/night in average (Table 2).

After the seed surface sterilization, four rice seeds were placed into 1-gal pots containing horticultural root media (Sunshine Mix #4, Sun Gro Horticulture). A total of thirty-two pots were prepared and placed into the same number of plastic buckets. Seven days after the germination, we inoculated the half of the samples with the prepared consortium of endophytes (Table 1). Targeted $OD_{600}$ per strain was approximately 0.011, and so $OD_{600}$ of the consortium of the selected nine strains was 0.1. Each plant received 2-mL of the consortium inoculum (MIX) using the inoculation technique outlined above. The other half was given the same volume of the mock-inoculum consisted of a N-free solution (CTRL).

Like Experiment 1, a randomized complete block design was applied to assign the pots on a bench in the greenhouse. Six pots without plants were located in the middle of the experimental design to collect weekly evaporation rate of the soil, and then the rate was used to calculate weekly transpiration during the experimental period. The total volumes of the water in the pots with plants were subtracted by the averaged volume of the water in the pots without plants every week to calculate weekly transpiration. Every week, 200 mL of the aforementioned fertilizer adjusted at a full strength nitrogen level of Hoagland was supplied and the pots were fully irrigated to 15-cm marks on the side of the buckets.

Six weeks after the germination, half of the pots were subjected to water deficit conditions. We stopped the weekly irrigation for these half (water deficit stressed, S), whereas continued full irrigation for the others (non-stressed, NS). From this point on, there were four treatment groups in the design: non-stressed control (NS_CTRL), non-stressed inoculated (NS_MIX), stressed control (S_CTRL), and stressed inoculated (S_MIX) with eight replications per each. To monitor the soil water status after the water deficit treatment, the soil water potential was measured by using a thermocouple psychrometer (SC-10, Decagon Devices, Inc.).

*Water use efficiency calculation*

Weekly transpiration was recorded throughout the experimental period until the plants harvested after 4 weeks of induced water deficits. Total transpiration was calculated by adding up the weekly transpiration at harvest. Measured dry weights were divided by total transpiration after harvesting and 72-hr drying at 70°C. The pot-based total transpiration and the measured dry weights were used to calculate long-term WUE.

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

***Experiment 4: A growth chamber study using endophyte consortia***

To provide support for a mechanistic understanding of the stomatal conductance responses, we repeated Experiment 2 in the previously used growth chamber of the same greenhouse facility from March 23$^{rd}$ 2017 to July 20$^{th}$ 2017. The objective of this experiment was to test the hypothesis that ABA produced endophyte consortia would increase *in vivo* ABA concentrations of the host plants.

*Preparation of plant materials and inoculation*

For preparing the plant samples, the protocols from Experiment 2 were used. For preparing the microbial samples and inoculation process, the protocols from Experiment 3 were used. In short, a total of 32 rice plants were grown in 3-gal pots in four sunlit growing chambers. The half of the surface sterilized plants were inoculated by the *nifH* endophyte consortium seven days after germination. They were grown under well-watered and nitrogen limited conditions until measurements and sampling taken over 104 days. The average air temperature recorded in a data logger (CR1000, Campbell Scientific) was 21/17°C in day/night. The average DLI was 10.8 mol $m^{-2}$ $d^{-1}$ of PAR with a 16/8 hrs day/night photoperiod of supplemental lighting. The air temperature and light intensity were recorded every 15 minutes. RH was 57/59% day/night in average. Further experimental details can be found in Table 2.

*Stomatal conductance measurements*

At 66 days after germination around V6-7 stage of their growth, $g_s$ of the youngest fully expanded leaves was measured at 12 and 6 pm to examine diurnal changes of the parameter using steady state leaf porometers (SC-1, Decagon Devices, Inc.). We applied the same measurement procedure described in Experiments 1 and 2.

in vivo *ABA assay*

Diurnal *in vivo* ABA content was determined biochemically using the Phytodetek enzyme-linked immunosorbent assay (ELISA) kit (PDK 09347/0096, Agdia, Elkhardt, IN, USA). At 96 days after germination around R3-4 stage, the rice leaf samples were harvested at 12 pm and 6 pm. The fully expanded youngest leaves were immediately submerged into liquid nitrogen in centrifuge tubes. They were frozen and stored at −80$^{o}$C until further analysis. The samples were ground into fine powder and approximately 100 mg of the powder of each was transferred into a microtube. ABA was extracted using 1 mL of 80% methanol at 4$^{o}$C overnight. On the following day, the mixture was centrifuged at 10,000 rpm for 5 mins. The supernatant was collected in a new microtube. The pellet was resuspended and 1 mL of fresh 80% methanol was used to repeat the extraction process at 4$^{o}$C overnight. Again, by centrifuging the mixture at 10,000 rpm for 5 mins and the supernatant was combined with the extracts from the previous day. The pooled supernatant was dried down using a vacuum concentrator until approximately 50 µL of liquid remained. Then, TBS buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 3 mM $NaN_3$) was added up to a final volume of 500 µL of the extract. The buffered extract was then diluted 10-fold in TBS buffer. The diluted sample was used to further detect ABA, following the Phytodetek ELISA assay kit manual. The ABA concentrations were measured using a multichannel spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). Each sample (CTRL/MIX) at each time point was replicated eight times.

***Statistical analysis***

All physiology parameters measured in Experiments 1 through 4 were analyzed with R version 3.2.2 (R Development Core Team, 2014). For Experiment 1, the measures were subjected to contrast matrix to compare control vs. three single strain inoculated plants with blocking effect included in the model. For Experiment 2, the variables recorded were tested for the effects of a split-plotted $CO_2$ treatment with a proper statistical analysis. The chamber effect on variation was found not to be significant $\alpha = 0.05$ level. Consequently, two-way ANOVA was implemented to the variables corresponding to a two-way factorial design. Experiment 3 was designed with a 2 × 2 factorial structure with blocking effects on the experimental plot. Two-way ANOVA was applied to analyze the response variables. The data from Experiment 4 were analyzed using a simple *t*-test procedure at each time point to see significant differences between control vs. inoculated plants. The numbers of replication for Experiments 1, 2, 3, and 4 were eight, ten, eight, and eight, respectively.

## Results

### *Experiment 1: Greenhouse study using multiple endophyte strains*

Stomatal conductance ($g_s$) decreased during the daytime in E+ plants with multiple strains of the bacteria (PTD1/WP5/WPB). An average 27% decrease in $g_s$ by multiple single strain endophytes was found with $P = 0.124$, 0.005, and < 0.001 at 12, 3, and 6 pm from Experiment 1 (Fig. 1).

Similar to $g_s$ response, stomatal density also decreased by 12% ($P = 0.012$) in response to endophyte inoculation (Fig. 2). That is, compared to 492 stomata/mm$^2$ in the control (CTRL), the average stomatal counts were 433 stomata/mm$^2$ in the inoculated plants (PTD1/WP5/WPB).

Intrinsic WUE, a proxy of short-term WUE, was not significantly changed in E+ plants ($P = 0.106$, Fig. 3). Overall biomass and total transpiration over time did not respond to endophyte inoculation, suggesting little differences in long-term WUE in Experiment 1 (data not shown).

Leaf water potential decreased (21% more negative) in E+ plants regardless of the endophyte strains used ($P = 0.025$, Fig. 4A). Osmotic potential, on the other hand, was increased (18% less negative) by the endophyte inoculation ($P < 0.001$, Fig. 4B). This increase in osmotic potential was compensated by the 27% decrease in turgor pressure in the inoculated samples shown in Fig. 4C ($P < 0.001$). No significant difference was found in soluble sugar content for PTD1/WP5/WPB (Fig. 4D).

### *Experiment 2: $CO_2$ enrichment study using a single endophyte strain*

Similar to Experiment 1, endophyte inoculation decreased $g_s$ significantly during the daytime. An average 18% decrease in $g_s$ by a single strain endophyte was observed with $P = 0.037$, 0.013, and 0.081 at 12, 3, and 6 pm from Experiment 2 (Fig. 5). No statistical differences were found in the values taken at other time points of the day between AMB and ELE conditions. There were no differences found in measurements made at 9 am between E- and E+ plants ($P = 0.195$ in Fig. 5). During the peak time of photosynthetic gas exchange activities (12-3 pm), the differences in $g_s$ became more pronounced, showing 20 to 21% decreases in E+ plants. High $CO_2$ lowered $g_s$ by 29% across E- and E+ treatments (Fig. 5).

### *Experiment 3: Water deficit study using endophyte consortia*

We found increases in biomass along with decreases in total transpiration over time and subsequent increases in WUE of productivity of rice plants under both non-stress (NS) and water deficit stress (S) conditions (Fig. 6).

The water deficit treatment affected all three measures significantly: a decrease in biomass by average 62% ($P < 0.001$, Fig. 6A), a decrease in total transpiration by an average of 85% ($P < 0.001$, Fig. 6B), and an increase in WUE of productivity by 221% on average ($P = 0.002$, Fig. 6C) of the all plants (CTRL and MIX pooled). E+ plants showed an increase in biomass by 16% over E- plants across water deficit treatments ($P = 0.039$, MIX in Fig. 6A). The endophyte effects on reducing total transpiration were greater under S than NS treatment ($P = 0.009$, the interaction effect – INT – in Fig. 6B). The magnitudes of the decreases were 30 and 22% in S and NS treatment, respectively ($P = 0.096$ and $< 0.001$).

The endophyte effects on WUE of the NS and S plants combined were significant (84% increase, $P = 0.047$, Fig. 6C), and this was presumably derived from the decreases in total transpiration (26% decrease, $P < 0.001$, Fig. 6B) rather than from the increases in biomass (16% increase, $P = 0.039$, Fig. 6A). The endophyte treatment was more effective in S as the WUE increases were more than two-fold compared to NS (116 vs. 52% in Fig. 6C).

Under NS conditions, the remaining water in MIX was greater than one in CTRL while there was no significant difference in the soil water potential between CTRL and MIX (NS panels in Fig. S1). The water deficit treatment completely dried the remaining water in the buckets and the pots at harvest (S panels in Fig. S1).

***Daily transpiration over daily light integrals***

As expected, daily transpiration increased with DLI over the growing periods in both E+ and E- plants (Fig. 7). The interactions between DLI and endophyte inoculation on daily transpiration were highly significant ($P = 0.006$). This result suggests that reduction in whole-plant transpiration due to endophyte inoculation is more pronounced in high light conditions.

***Experiment 4: in vivo ABA content affected by endophyte consortia***

There were no significant differences found in both $g_s$ and *in vivo* ABA content between E- and E+ rice leaves at noon (Fig. 8). In the afternoon at 6 pm, the decrease in $g_s$ by the endophytes was found significant ($P = 0.043$, in Fig. 8A). Also, the increase in *in vivo* ABA concentrations in E+ rice leaves was found at 6 pm ($P = 0.006$, in Fig. 8B). The endophyte inoculation caused almost a three-fold increase in *in vivo* ABA concentrations in rice leaves.

## Discussion

In the present study, we evaluated the effects of endophyte inoculation on water relations of a rice host. We tested the hypothesis that endophytes alter the host physiology to reduce transpiration of rice through stomatal regulations. A series of experiments conducted in this study illustrated that several changes in water relations took place when a rice plant was inoculated with diazotrophic endophytes. These physiological changes included significantly higher ABA concentrations, lower stomatal conductance, lower stomatal density, lower leaf water potential likely through a reduction in turgor pressure, and eventually an increase in WUE of the whole-plant as these responses accumulate over time and space.

Malinowski and Belesky (2000) reviewed plant physiological mechanisms of drought and mineral stress tolerance offered by cool-season grass endophytes, focusing on the impacts of fungal endophytes. The authors pointed out that some fungal species, even in the same fungal endophyte class, had opposite modes of actions on stomatal reactions and the following plants' water stress tolerance mechanisms. Similarly, some articles reported increases in $g_s$ (Bae *et al.* 2009; Shukla *et al.* 2012a; Gagné-Bourque *et al.* 2016), while others reported decreases in $g_s$ (Turner 1986; Richardson *et al.* 1993b; Elmi & West 1995), yet highlighting that either action both resulted in the hosts withstanding water deficit conditions.

### *Decreases in stomatal conductance during daytime by endophytes*

In Experiments 1, 2, and 4, we observed clear patterns of stomatal behavior with decreases in $g_s$ in the afternoon (Figs. 1, 5, and 8A). It is evident that stomatal movement of the host plants colonized by the endophytes is heavily dependent on time of day. These experiments performed under different environmental conditions revealed similar patterns of a decrease in $g_s$ in the afternoon (Figs. 1, 5, and 8A).

Two potential mechanisms may explain this afternoon reduction of stomatal conductance: 1) hormonal influences from the endophytes – endogenous ABA production, or 2) recycling of microbial respiratory $CO_2$ in the Calvin cycle of the plants.

The first possibility of altered diurnal stomatal movement is the effects of ABA-production by endophytes. ABA is a key hormone of stomatal control and its diurnal fluctuation is well described by the dual source model (Tallman 2004). The inoculated plants may have had a higher ABA level due to additional ABA provided by the endophytes, reaching the threshold faster to close stomata. Another related possibility is that endophytes might induce faster circadian clock responses to

environmental cues fluctuating in a 24-hr cycle (Seo & Mas 2015). The two-fold increase in WUE by endophytes in Experiment 3 under water deficit conditions would be in line with this respect (Fig. 6C). This implies the endophytes can cause the host to respond to the environmental changes more sensitively, enabling more efficient use of water as resources.

Stomatal control determines the efficiency of water relations over any other mechanisms, which is governed by ABA concentration (Tardieu & Davies 1992). All the endophyte strains used in this study produced ABA ranging from 0.404 – 0.831 μg $mL^{-1}$.in an *in vitro* assay from our earlier work (see Table 1 in Khan et al. 2016). Also, we directly showed the increases in *in vivo* ABA concentration along with the decreases in $g_s$ in the afternoon in Experiment 4 (Fig. 8). This confirms that the impacts of endophytes on ABA production and resulting decreases in $g_s$ in rice were significant.

Another piece of evidence supporting ABA production by endophytes is the decreases in stomatal density of the inoculated plants. Together with the stomatal closure during the daytime, it is possible that endophyte-producing ABA affected stomatal development. ABA is also known to involve stomatal development under water deficit conditions (Chater *et al.* 2014). *In vitro* ABA production capacity of the select nine strains in Experiment 3 was assayed in our previous study, Khan et al. 2016, where the drought tolerance of the inoculated poplar trees was significantly enhanced. This decrease in stomatal density allowed the plants not only to conserve water during the daytime in response to the environmental change, but also may save the metabolic cost to build up guard cells rather than photosynthetic cells. Franks et al. (2015) demonstrated the genetically engineered *Arabidopsis* having less stomata, and therefore so lowered stomatal density, had an advantage over the wild type regarding WUE. Also, Gray et al. (2015) showed that the stomatal density lowered mutant consumed less water over time, displaying higher soil water content than the wild type. Both studies presented the importance of stomatal density as a key control parameter for WUE. Endophyte inoculation can be a novel approach to modify stomatal density as a way to increase WUE.

Our second explanation is the microbial respiration and recycling of $CO_2$ molecules by the plants. As $g_s$ decreased during the afternoon (Figs. 1, 5, and 8A), $CO_2$ supply from the atmosphere would drop in E+ plants. However, the increases in WUE (Fig. 6C) suggest that somehow E+ plants could maintain the rate of photosynthetic $CO_2$ assimilation with less $CO_2$ through stomata. This implies that other source of $CO_2$ supply to the site of carboxylation may possibly contribute to the assimilation process. When carbohydrates are given to endophytes respiring in the intercellular spaces of the

leaves, perhaps, this respired $CO_2$ is readily available for the Calvin cycle. This endophytic respired carbon would not have to travel through the diffusional pathway of $CO_2$, having advantage of the shorter travel distance. Indeed, Bloemen et al. (2013) reported that even $CO_2$ respired by root tissues was re-assimilated by the aboveground tissues up to 10-20% in poplar trees. Busch et al. (2013) also demonstrated that up to 24-38% of photorespired and respired $CO_2$ were re-assimilated, resulting in an increase of photosynthesis by 8-11% in rice and wheat. These two examples signify how important respired $CO_2$ sources from other parts of plants can be in the $CO_2$ assimilation process. Although challenging to experimentally prove, our hypothesis – the recycling endophytic respiratory $CO_2$ by the hosts' photosynthesis – holds a possibility.

As opposed to our results with bacterial and yeast endophytes, a meta-analysis from Augé et al. (2014) revealed an average 24% increase in $g_s$ by mycorrhizae. They conducted the statistical analysis on 400+ individual studies including plants grown under water stressed conditions. Mechanistic differences exist between the two distinguished symbiotic styles. Mycorrhizae aid host plants in absorbing water from the rhizosphere, conferring drought tolerance likely by increasing relative water content of the plants. Conversely, endophytes – from this study – seem to assist host plants in conserving existing water mainly by decreasing $g_s$.

***Decreases in leaf water potential by endophytes***

Stomatal control comes first in importance before osmotic regulation of water potential in rice plants (Parent *et al.* 2010). We therefore examined leaf water potential components. The hypothesis behind this was that endophytes would reduce sugar content as they drain different forms of carbohydrates and organic acids from the hosts, leading to decrease in the ability to regulate osmolytes of the plants.

The endophyte inoculation resulted in a reduction in leaf water potential (Fig. 4A). Interestingly, this reduction was mostly due to a reduction in turgor pressure whereas the osmotic potential increased with inoculation (Fig. 4B and 4C). As the endophytes consume carbohydrates produced from the mesophyll cells and are involved in trafficking the transportable form of carbohydrates to the apoplast– mostly sucrose as a basic form (Lemoine *et al.* 2013) –, osmotic potential of the host cells was likely to be increased (i.e., less negative) (Fig. 4B). The consumption of other osmolytes (e.g. organic acids) by endophytes can be another possibility of the increase in osmotic potential. As a consequence, the turgor pressure of the cells might have dropped further in part because lower sucrose and organic acid levels in the symplast as they are concurrently consumed and recycled by

endophytes. Collectively, with lower $g_s$ and water potential, the inoculated plants tend to have lower operational cost of water than the control plants.

***Increases in water use efficiency of hosts***

The alterations of stomatal development and diurnal behaviors accompanied with plasticity of cell water relations would offer host plants an advantage of saving water during the daytime, especially under high light and warmer conditions when evapotranspiration demand is high. Although stomata were closed and the $CO_2$ supply from the atmosphere to the intercellular, photosynthetic $CO_2$ assimilation was not affected by the endophyte inoculation. This advantage is likely to be cumulative over the entire growing period, implying that if there are more sunny days than cloudy and overcast days, the influences will be greater. The differences in our environmental conditions among Experiments 1 through 3 corroborate this point (Table 2). We did not find significant increases in biomass and following increases in WUE of productivity in Experiments 1 and 2 (data not shown). Experiment 1 was conducted during mostly winter and early spring featuring the lack of accumulated light intensity and lower air temperature even in the controlled greenhouse environment. Experiment 2 was conducted in the naturally-lit growth chambers where the frames of the chambers shaded the plants grown inside and lowered the actual air temperature of the chamber space. On the other hand, the plants grown in the summer season in Experiment 3 showed increases in biomass and long-term WUE (Fig. 6). Experiment 3 was conducted under full sunlight condition – the average air temperature was approximately 8°C higher and the average DLI was around 4-fold higher than the previous two experiments. The reduction in daily water use by endophytes also upholds this point (Fig. 7). The magnitude of the reduction is positively correlated to DLI.

Another possibility is that a consortium of endophytes would benefit the plants more than a single strain of endophytes. This has to do with the collection of microbiota simulating the natural habitat would help the hosts more by interacting with each other (Bulgarelli *et al.* 2013). Knoth et al. (2014) also reported a consortium of multi-strain endophytes was more effective on increasing gain of final biomass of poplar clones in a greenhouse experiment. We used single strain endophytes in Experiments 1 and 2 where the decreases in $g_s$ were observed, but no changes in WUE (Fig. 3). In Experiment 3, however, where we used a consortium of nine endophyte strains, we found significant increases in long-term WUE (Fig. 6C).

The decreases in the cumulative total transpiration of the inoculated plants were more significant under the water deficit conditions (Fig. 6B). Endophytes helping plants under stress is reported in

copious articles. Their beneficial effects on the host are thought to be augmented under various stress conditions because the endophytes inside plants signal stress response pathways before the stress is imposed and these microorganisms appear to turn on defense mechanisms of the plants (Pandey *et al.* 2012).

## Conclusions

We showed that select bacteria and yeast endophytes decreased $g_s$ suggesting that this stomatal response was the main reason for increases in WUE of the rice plants. The rice plants inoculated with multiple strains of the endophytes all showed the decrease in $g_s$ and stomatal density. The decrease in $g_s$ was also observed under a $CO_2$ enrichment condition. This stomatal response resulted in reduction of total transpiration of plants over growing periods. The effect size of the reduction was greater when DLI were higher and water supply to plants was limited under water deficit conditions. The reduction in total transpiration was the main reason for increases in long-term WUE in the rice plants. We suggest that the increases in ABA production by endophytes can be a possible mechanism for these stomatal reactions and the resulting whole plant physiological benefits.

## Author Contributions

Conceived idea and designed experiments: HR, SK. Conducted experiments: HR, NW, VVE. Analyzed data: HR, SK. Provided materials and resources: SD, SK. Wrote the article: HR, VVE, SD, SK.

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## Tables & Figures

**Table 1** List of the endophyte strains used in this study. The rRNA gene of each strain was compared with the BLAST NCBI database and identified as the closest match (Doty *et al.* 2009a).

| Endophyte | Closest rRNA Match | Source | Reference | Experiment |
|---|---|---|---|---|
| PTD1 | *Rhizobium* sp. | Hybrid poplar (*Populus trichocarpa* × *P. deltoids)* | Doty et al. (2005) | 1, 3, and 4 |
| WPB | *Burkholderia* sp. | Wild poplar (*P. trichocarpa*) | Doty et al. (2009) | 1, 3, and 4 |
| WP1 | *Rhodotorula* sp. | Wild poplar (*P. trichocarpa*) | Khan et al. (2012) | 3 and 4 |
| WP5 | *Rahnella* sp. | Wild poplar (*P. trichocarpa*) | Doty et al. (2009) | 1, 2, 3, and 4 |
| WP9 | *Burkholderia* sp. | Wild poplar (*P. trichocarpa*) | Doty et al. (2009) | 3 and 4 |
| WP19 | *Acinetobacter* sp. | Wild poplar (*P. trichocarpa*) | Doty et al. (2009) | 3 and 4 |
| WW5 | *Sphingomonas* sp. | Wild willow (*Sitka sitchensis*) | Doty et al. (2009) | 3 and 4 |
| WW6 | *Pseudomonas* sp. | Wild willow (*S. sitchensis*) | Doty et al. (2009) | 3 and 4 |
| WW7C | *Curtobacterium* sp. | Wild willow (*S. sitchensis*) | Doty et al. (2009) | 3 and 4 |

**Table 2** Experiment designs in this study.

| Category | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
|---|---|---|---|---|
| Growing season | Autumn to Spring (10/15/2014 – 3/28/2015) | Spring to Summer (3/21/2014 – 9/2/2014) | Summer to Autumn (7/14/2015 – 10/6/2015) | Spring to Summer (3/23/2017 – 7/20/2017) |
| Growing duration | 158 days | 165 days | 84 days | 119 days |
| Endophyte strains used | PTD1/WP5/WPB | WP5 | *nifH* MIX (see Table 1 for details) | *nifH* MIX (see Table 1 for details) |
| Experimental settings | 4 (CTRL/PTD1/WP5/WPB) | 2 x 2 (CO2 x INOC) | 2 x 2 (DRT x INOC) | 2 (INOC) |
| Experimental environments | Greenhouse benches | Sunlit growing chambers | Greenhouse benches | Sunlit growing chambers |
| Nitrogen fertilization | ¼X N | ¼X N | 1X N | ¼X N |
| Average RH (day/night) | 48/54% | 60/66% | 57/71% | 57/59% |
| Average air temperature (day/night) | 22/19°C | 23/19°C | 29/20°C | 21/17°C |
| Average instantaneous light intensity | 174.0 µmol $m^{-2}$ $s^{-1}$ | 176.3 µmol $m^{-2}$ $s^{-1}$ | 313.7 µmol $m^{-2}$ $s^{-1}$ | 201.7 µmol $m^{-2}$ $s^{-1}$ |
| Average daily light integral | 9.5 mol $m^{-2}$ $d^{-1}$ | 9.1 mol $m^{-2}$ $d^{-1}$ | 35.8 mol $m^{-2}$ $d^{-1}$ | 10.8 mol $m^{-2}$ $d^{-1}$ |

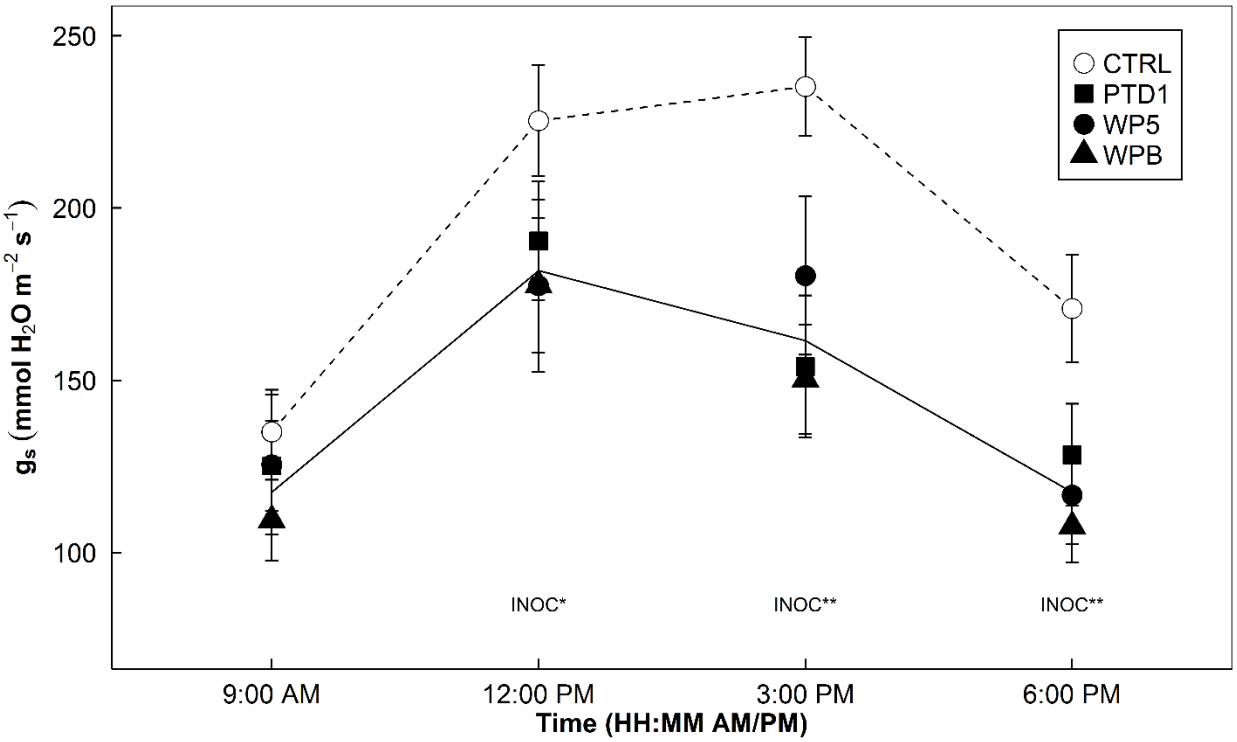


**Fig. 1** Diurnal patterns of stomatal conductance ($g_s$) of rice leaves on 163 days after germination in a greenhouse bench experiment (Experiment 1). Open symbols indicate mean $g_s$ of control groups, whereas closed symbols indicate mean $g_s$ of single strain-inoculated groups (square/circle/triangle = 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.

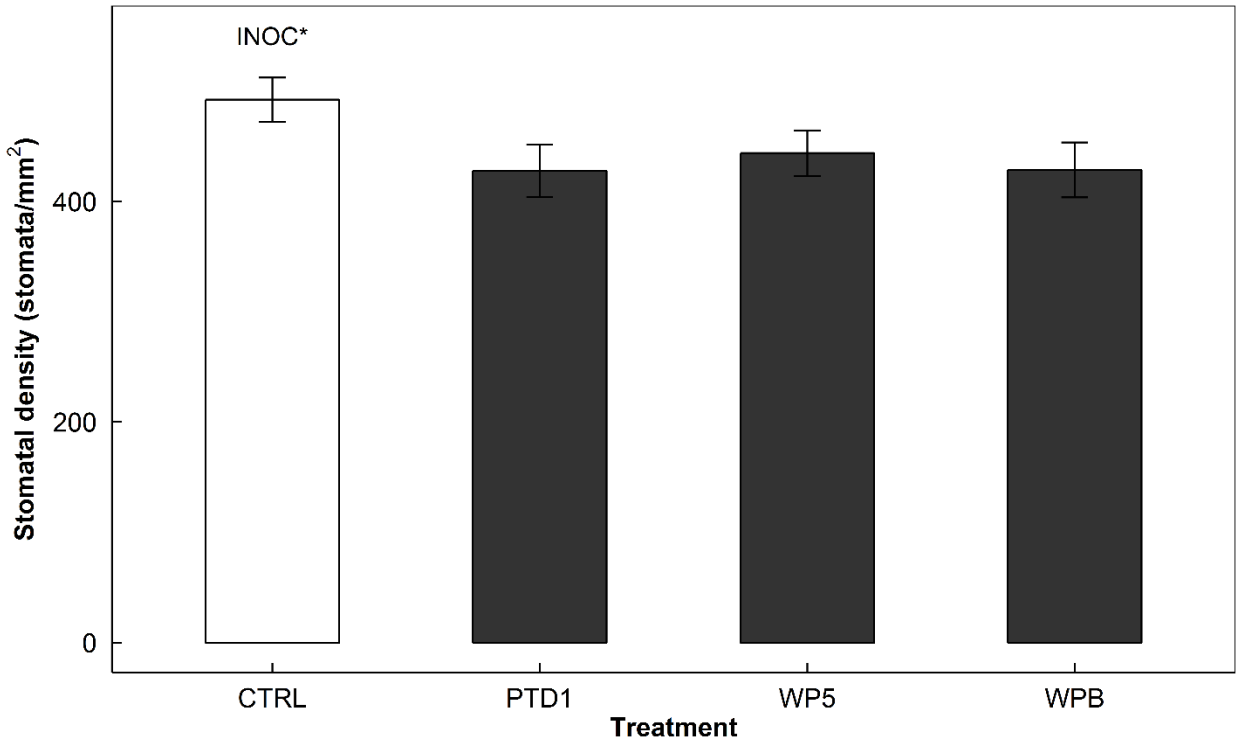


**Fig. 2** Stomatal density of adaxial sides of rice leaf surfaces on 165 days after germination in a greenhouse bench experiment (Experiment 1). The stomatal imprints were collected from the same leaves that were used in stomatal conductance measurements in Fig. 1. The bars present mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves. The error bars indicate ± 1 S.E. of the means ($n$ = 10). Single strain endophyte inoculation effect (INOC) is provided at $P < 0.05$ (*) level. Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

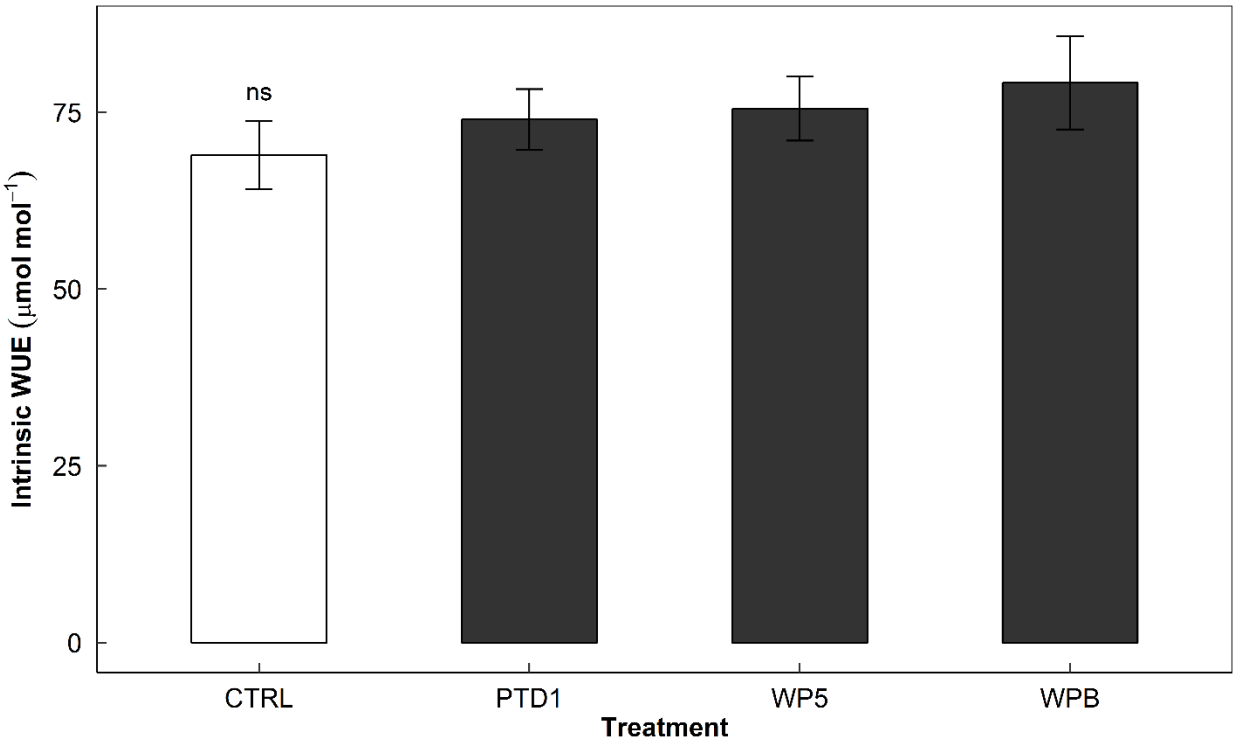


**Fig. 3** Intrinsic water use efficiency (WUE), expressed as $CO_2$ uptake/$H_2O$ loss in moles, of rice leaves measured on 58/118/153 days after germination in a greenhouse bench experiment (Experiment 1). The bars present aggregated mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves collected on the three days as the *time* effect on the measure were non-significant at $\alpha = 0.05$ level. The error bars indicate ± 1 S.E. of the means ($n = 30$). Single strain endophyte inoculation effect (INOC) was not significant (ns, $P = 0.106$). Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

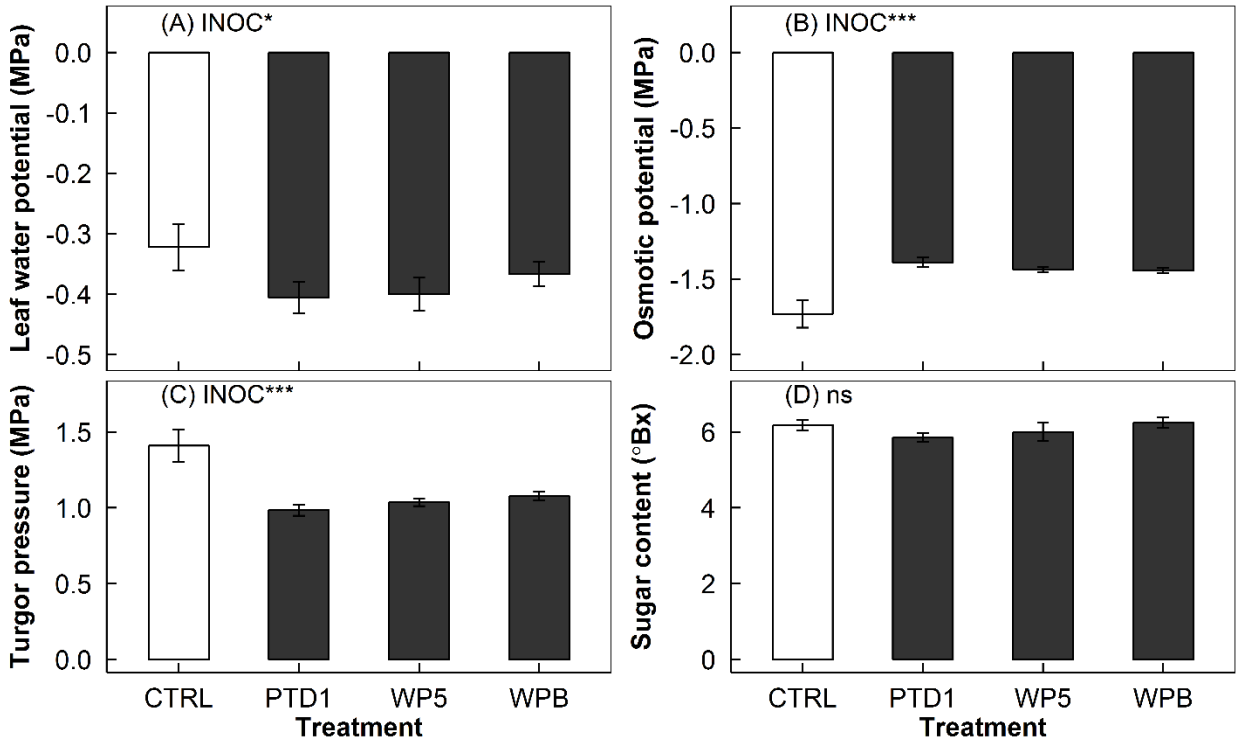


**Fig. 4** Leaf water potential **(A)**, osmotic potential **(B)**, turgor pressure **(C)**, and soluble sugar content **(D)** of rice leaves on 165 days after germination grown in a greenhouse bench experiment (Experiment 1). The bars present mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves. The error bars indicate ± 1 S.E. of the means ($n$ = 9). Single strain endophyte inoculation effect (INOC) is provided at $P < 0.05$ (*), 0.001 (***) level. No significance was found in soluble sugar content (ns). Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

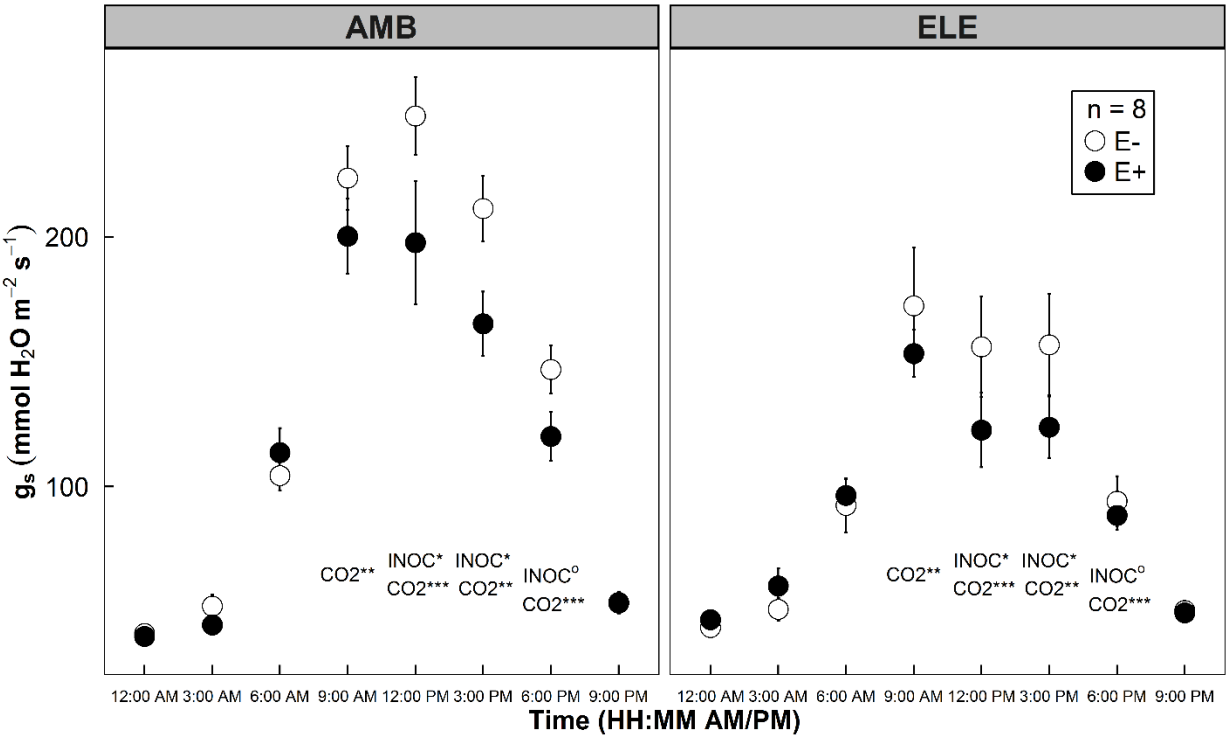


**Fig. 5** Diurnal patterns of stomatal conductance ($g_s$) of rice leaves grown under two atmospheric $CO_2$ conditions: ambient (**AMB**, app. 400 ppm on the left panel) and elevated (**ELE**, app. 800 ppm on the right panel) in a sunlit chamber experiment (Experiment 2). Open symbols indicate mean $g_s$ of control groups (E-), whereas closed symbols indicate mean $g_s$ 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. $CO_2$ treatment effect (CO2) and endophyte inoculation treatment effect (INOC) are provided at $P < 0.10$ (°), 0.05 (*), 0.01 (**), 0.001 (***) levels.

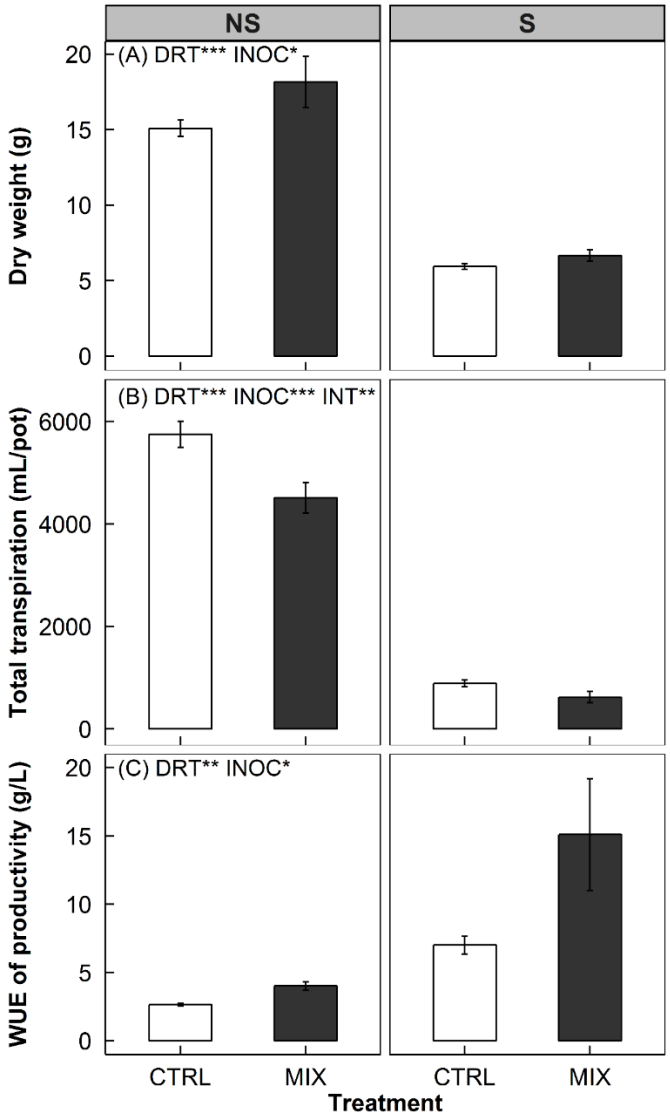


**Fig. 6** Total biomass (top panels, **A**), total transpiration over time (middle panels, **B**), and water use efficiency (WUE) of productivity (bottom panels, **C**) of rice without (left panels, **NS**) and with (right panels, **S**) water deficits at harvest in a greenhouse bench experiment (Experiment 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.

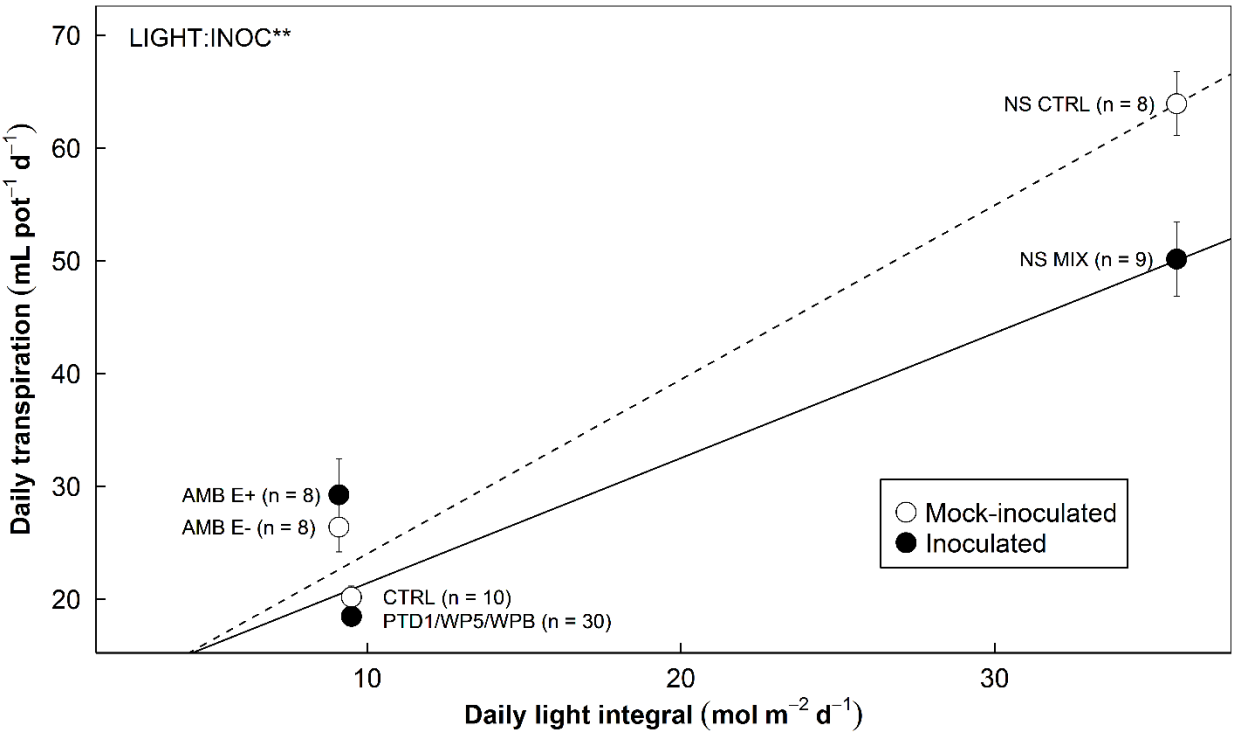


**Fig. 7** The relationship between daily transpiration by rice plants and daily light integral during growing periods affected by endophyte inoculation in Experiments 1, 2, and 3 (left, middle, and right). Different endophyte and experimental settings were used (refer to Table 2). Open symbols (CTRL and E-) stand for the mean responses of mock-inoculated control groups. Closed symbols (PTD1/WP5/WPB, E+, and MIX) stand for the mean responses of endophyte-inoculated treatment groups. Error bars indicate ± 1 S.E. of the means. The sample sizes are provided in the parentheses. Dotted/solid line show the trends of the responses of control/inoculated groups. Data from elevated $CO_2$ in Experiment 2 and water deficit treatment in Experiment 3 are not included in this figure. The relationship is significantly affected by endophyte inoculation (INOC) at $P < 0.10$ level. There is an interaction effect (LIGHT:INOC) at $P < 0.01$ level.

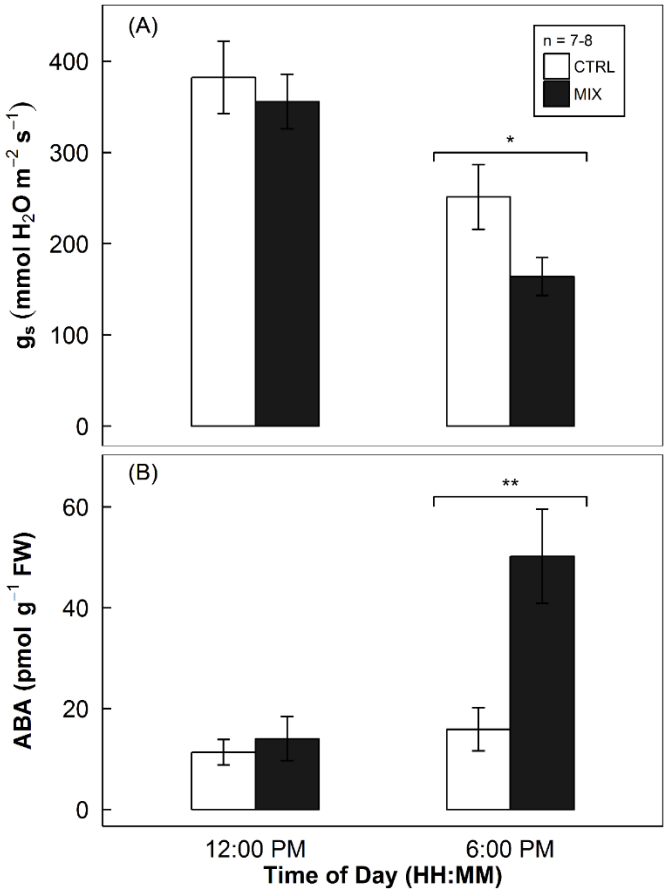


**Fig. 8** Stomatal conductance of rice leaves at round V7-8 stage (top panel, **A**) and *in vivo* ABA concentrations (bottom panel, **B**) of rice leaves harvested at around R3-R4 stage in a greenhouse sunlit chamber experiment (Experiment 4). Open and closed bars indicate mean responses of mock-inoculated 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.

## Supplementary Material

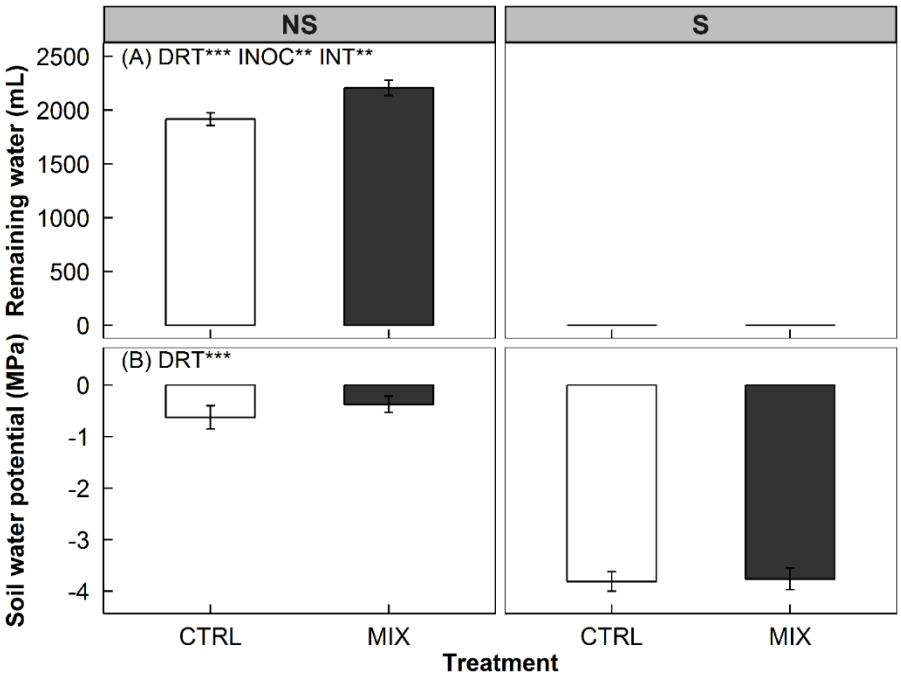


**Fig. S1** The remaining water in the buckets (top panels, **A**) and water potential of the soil in the pots (bottom panels, **B**) without (left panels, **NS**) and with (right panels, **S**) water deficits at harvest. Open and closed bars indicate means of mock-inoculated controls (CTRL) and endophyte 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 $P < 0.01$ (**) and 0.001 (***) levels.

# CHAPTER 3

## Salicaceae Endophytes Alleviate Photosynthetic Down-Regulation and Enhance Leaf Gas-Exchange Properties in Rice Grown under Elevated $CO_2$

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## Abstract

As the current atmospheric [$CO_2$] rises, this changing climate is likely to cause decreases in the $CO_2$ fertilization effects on crop yield due to down-regulation of $C_3$ photosynthesis in response to elevated [$CO_2$]. Diazotrophic microbes are N-fixing organisms, a subset of which form symbiotic associations with host plants and are known to mitigate down-regulation of photosynthesis. In this study, we tested the hypothesis that N-fixing endophytes would function similarly to eliminate the down-regulation of photosynthesis under elevated [$CO_2$]. We inoculated rice plants with diazotrophic endophytes isolated from Salicaceae hosts and examined leaf gas exchange and biochemical properties of rice leaves in response to $CO_2$ enrichment. We found that at the panicle initiation growth stage, control plants grown under elevated [$CO_2$] showed apparent down-regulation of photosynthesis compared to those grown under ambient [$CO_2$] conditions. However, endophyte-inoculated plants did not show this down-regulation. Instead, inoculated plants exhibited the higher photosynthetic electron transport rates and enhanced mesophyll conductance that collectively facilitate $CO_2$ assimilation processes under elevated [$CO_2$]. These improvements were more substantial when N supply was limited. Our results suggest that inoculation with N-fixing endophytes can be an effective means for improving plant growth in response to shifting climatic conditions by maximizing photosynthesis under elevated [$CO_2$] without apparent photosynthetic down-regulations.

**Keywords:** photosynthesis$_1$, rice$_2$, endophytes$_3$, $CO_2$ enrichment$_4$, electron transport rate$_5$, mesophyll conductance$_6$, water use efficiency$_7$, down-regulation$_8$, acclimation$_9$

## Introduction

Climate change poses a complex set of problems that one country or individual alone cannot address. We now see compelling evidence of multifarious environmental changes, just as climatologists have predicted (IPCC 2013). The main driver of global climate change is an increase in atmospheric concentrations of carbon dioxide [$CO_2$], 13.5% of which are attributed to current unsustainable agricultural practices, such as the use of excessive amounts of chemical fertilizer and resource-intensive farming techniques (Solomon *et al.* 2007). Manufacturing nitrogen (N) fertilizers through the standard Haber-Bosch process emits greenhouse gases into the atmosphere, negatively impacting environmental processes by exacerbating global warming

(Vance 2001). Moreover, excessive N fertilizer application has disrupted cropland ecological N cycles, further disturbing the nutrient balances of rivers and oceans in a process known as eutrophication, which has become a serious environmental concern (Mueller *et al.* 2014).

The main driver of climate change, an increase in [$CO_2$], is reported to positively affect performance of $C_3$ crop species – an effect that has been empirically tested through a body of literature. When this effect was first discovered and documented, it was translated into an optimistic projection that $CO_2$ enrichment would increase crop biomass (Drake *et al.* 1997). Biomass increase in response to elevated [$CO_2$] was often referred to as a $CO_2$ fertilization effect (Thornton *et al.* 2007). However, overall increases in $C_3$ plant biomass have fallen short of what was previously anticipated in theoretical models (Moore *et al.* 1999; Long *et al.* 2006). There is now consensus around the inability of $CO_2$ enrichment to solve food security issues in many regions, and it appears that a serious deficit in food production will remain as global agricultural infrastructure attempts to support an increasing human population (Tilman *et al.* 2011).

Since many experimental approaches have revealed that this down-regulation of $C_3$ photosynthesis is heavily dependent on N supplies and subsequent N metabolism (Stitt & Krapp 1999; Reich *et al.* 2006; Shimono & Bunce 2009), the impacts of elevated [$CO_2$] on crop yield then become a matter of either N acquisition from the rhizosphere or N use efficiency in plants (Ainsworth & Rogers 2007; Zhu *et al.* 2010). The mechanistic explanation for this effect is that N metabolic processes engaged during Rubisco and other Calvin-Benson cycle-associated enzyme turnovers are decelerated when excess glucose production acts as a transduction signal (Stitt & Krapp 1999).

Legumes, however, are one category of $C_3$ plants which are unlikely to be negatively affected by increasing [$CO_2$] (Ainsworth & Rogers 2007; Rogers *et al.* 2009). Previous studies have found that the mitigation effects on down-regulation in legumes under elevated [$CO_2$], in contrast to the effects on non-legume $C_3$ plants, can be attributed to their capability to reset the balance of C and N metabolic processes that stimulate biological N fixation (BNF) under nutrient-limited conditions (Ainsworth & Rogers 2007; Rogers *et al.* 2009).

Increases in N supply due to nodule-forming rhizobium bacteria in legumes are also known to help increase carbon sink strength in plants under elevated [$CO_2$] conditions, alleviating down-

regulation of photosynthesis (Ainsworth & Rogers 2007). With a sufficient N pool and plentiful carbohydrates from increased photosynthesis, plants can produce more sink tissue, which reduces carbohydrate levels in phloem. The ease of the carbohydrate signal and the subsequent increase in the rate of carbohydrate export provides positive feedback, allowing source tissue to carry out more photosynthesis (Ainsworth & Bush 2011). In addition, the symbiotic nodulating bacteria serve as an active 'biological sink' (Ainsworth *et al.* 2004), because they cost their host plants about 14-25% of photosynthates (Lambers *et al.* 2008b; Kaschuk *et al.* 2010).

Endophytes are also microbial symbionts (bacteria, fungi, or yeast), but are different from nodule-forming rhizobium bacteria in that they live inside plants, typically in the intercellular spaces or vascular tissues. While living inside plants, they provide the hosts with various benefits for growth and development under various stressful environmental conditions (Rho *et al.* 2017).

A series of beneficial bacteria and yeast strains were isolated from native Washington State Salicaceae (poplar and willow) trees by Doty *et al.* (2009), and from genomic analysis, some were characterized to contain *nifH* genes. A follow-up *in vitro* study found that the amount of N biologically-fixed by these select Salicaceae endophytes was comparable to that of the free-living $N_2$-fixing *Azotobacter* sp (Kandel *et al.* 2017b). Moreover, an *in planta* endophyte inoculation study confirmed their capacity to perform BNF in leaves (Knoth *et al.* 2014). Salicaceae diazotrophic endophytes have been found not only to impart biomass increases to various interspecific plant hosts (Khan *et al.* 2012f; Knoth *et al.* 2013b), but also to increase drought tolerance in poplar cuttings (Khan *et al.* 2016) and water use efficiency (WUE) in rice (Rho *et al.* 2018). However, their capacity to enhance photosynthesis and mitigate the down-regulation of photosynthesis in $C_3$ plant hosts under elevated [$CO_2$] has not been examined.

We hypothesized that Salicaceae endophytes, like rhizobium bacteria in legumes, would mitigate the down-regulation of photosynthesis in plants when acting as diazotrophic symbionts. To test our hypothesis, we used an interspecific host, rice (*Oryza sativa*), as a model $C_3$ crop and investigated its photosynthetic biochemistry by simultaneous measurements of leaf gas exchange and chlorophyll fluorescence properties. Thus, the present study is the first research article investigating endophyte effects on $C_3$ photosynthesis under elevated [$CO_2$], providing insight for comparison with what is known about nodulating mutualists.

## Materials and Methods

To test our hypothesis, we conducted a series of greenhouse $CO_2$ enrichment studies. The greenhouse is located in the Douglas Research Conservatory at the University of Washington Center for Urban Horticulture (47°39′27″ N, 122°17′21″ W; 10 m elevation). Our study was conducted over two growing seasons (spring to summer) in 2013 and 2014, for Experiments (Expts) 1 and 2, respectively. Expt 1 was carried out under high N conditions (HN), whereas Expt 2 occurred under low N conditions (LN). Details about experimental design and environments are outlined in the following section and summarized in Table 1.

### *$CO_2$ enrichment system and environmental conditions*

For the $CO_2$ treatment, we used four sunlit closed-top chambers. Fresh atmospheric (outdoor) air was supplied to the chambers from outside of the greenhouse using flexible dryer aluminum ducting (15.24 × 609.6 cm) with variable speed inline fans (Model FR, Fantech, Sarasota, FL, USA). Along with fresh air, $CO_2$ gas balanced with $N_2$ gas was supplied through clear Tygon tubing (Saint-Gobain Performance Plastics, Akron, OH, USA) from a 22.70-kg tank (Praxair, Seattle, WA, USA). The $CO_2$ gas was provided to two of the four chambers to elevate [$CO_2$] (ELE, approximately 800 ppm). The other two chambers remained at ambient [$CO_2$] (AMB, approximately 400 ppm). [$CO_2$] in the chambers was monitored every 30 min by diffusion type $CO_2$ probes (GMP343, Vaisala Oyj., Vantaa, Finland). Average [$CO_2$] of the AMB/ELE chambers was 407/843 and 437/886 ppm for Expt. 1 and 2, respectively.

A temperature and relative humidity probe (CS215, Campbell Scientific, Logan, UT, USA) was installed 20 cm from the tops of each chamber. Light intensity inside the chambers was monitored by a quantum sensor (SQ-100, Apogee Instruments, Logan, UT, USA), which was set up around the temperature/humidity probe. Data for temperature, relative humidity, and light intensity of the chambers were collected every 15 min and recorded into a datalogger (CR1000, Campbell Scientific). Supplementary artificial light (high pressure sodium 400 W single phase bulbs, Phillips Electronics North America Corp., Andover, MA, USA) was used to maintain consistent light intensity of the greenhouse in the daytime from 6:00 to 20:00 (14/10 hr of photoperiod) throughout both experiments. Average day/night temperature and relative humidity in Expt 1 were 23/19$^{o}$C and 57/64%, and were 23/19$^{o}$C and 60/66% in Expt 2. Average light

intensity and daily light integral in Expt 1 were 216.7 μmol $m^{-2}$ $s^{-1}$ and 11.6 mol $m^{-2}$ $d^{-1}$ photosynthetic photon flux density (PPFD) in Expt 1, and were 176.3 μmol $m^{-2}$ $s^{-1}$ and 9.1 mol $m^{-2}$ $d^{-1}$ PPFD in Expt 2, respectively.

Specifications for the $CO_2$ chamber system used in the current study can be also found in Kinmonth-Schultz & Kim (2011) and Nackley *et al.* (2016).

***Preparation of microbial materials and inoculation with endophytes***

Two select endophyte strains, PTD1 (*Rhizobium tropici*) and WP5 (*Rahnella* sp.), were used in Expts 1 and 2, respectively. PTD1 was isolated from hybrid poplar trees (*Populus trichocarpa* × *deltoides*), whereas WP5 was isolated from wild poplar trees (*P. trichocarpa*). Isolation processes and *in vitro* characteristics of the two strains, including production of indole-3-acetic acid and $N_2$ fixation abilities conferred by the *nifH* gene, were previously reported in Doty *et al.* (2005) and Doty *et al.* (2009), respectively.

The isolates had been stored in a 30% glycerol buffered solution in a −80ºC freezer prior to the beginning of both experiments. Before inoculation, the frozen endophytes were propagated on MG/L media plates (g $L^{-1}$: 5.0 tryptone, 2.5 yeast extract, 5.2 NaCl, 10.0 mannitol, 1.32 sodium glutamate, 0.50 $KH_2PO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, and 2 μg biotin at pH 7.0) (Cangelosi *et al.* 1991), and then cultured overnight in N-limited combined carbon media broth (NL-CCM) (g $L^{-1}$: Solution 1, 5.0 sucrose, 5.0 mannitol, 0.5 mL/L sodium lactate, 0.8 $K_2HPO_4$, 0.2 $KH_2PO_4$, 0.1 NaCl, 0.025 $Na_2MoO_4 \cdot 2H_2O$, 0.028 $Na_2FeEDTA$, 0.1 yeast extract; Solution 2, 0.2 $MgSO_4 \cdot 7H_2O$, 0.06 $CaCl_2$; a mixture of solution 1 and 2 was used after the two solutions were autoclaved) (Rennie 1981). The bacterial density ($OD_{600}$) of the culture was measured using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, USA) and adjusted to 0.1 (equivalent to approximately $1 \times 10^7$ cells) to create the inoculum by diluting the culture with sterile deionized water and N-free media broth (NFM) (g $L^{-1}$: 69.9 $KH_2PO_4$, 19.84 $K_2HPO_4$, 174.7 $K_2SO_4$, 119.7 $MgSO_4 \cdot 7H_2O$, 100 $MgCl_2 \cdot 6H_2O$, 219.8 $CaCl_2 \cdot H_2O$, 3.38 $MnSO_4 \cdot H_2O$, 0.5 $CuSO_4 \cdot 5H_2O$, 0.55 $ZnSO_4 \cdot 7H_2O$, 3.83 $H_3BO_3$, 0.24 $NaMoO_4 \cdot 2H_2O$, 0.11 $CoSO_4 \cdot 6.5H_2O$, and 35 Fe Sequestrine at pH 6.5) (Doty *et al.* 2009a).

***Preparation of plant materials and inoculation with endophytes***

Very-early to early-maturing Japonica M-206 rice plants (*Oryza sativa*) was chosen as our host plant species based on our previous study results (Kandel *et al.* 2015). In order to remove other microorganisms and contaminants, the rice seed surfaces were sterilized by soaking the seeds in 3% (v/v) NaOCl solution for 4 hours followed by rinsing five times with sterile water in a sterile hood. The seeds were then incubated on a thin, solidified layer of 1% (w/v) water agar in sterile and sealed petri dishes. 100% of the seeds germinated within two days in both experiments.

Seven days after germination (DAG), the rice seedlings were transferred to 1-gal and 3-gal pots filled with horticultural media Sunshine Mix #4 and #2 soil (SunGro, Bellevue, WA, USA) in Expts 1 and 2, respectively. The pots were cleaned by soaking them in commercial bleach for 30 minutes. Four seedlings were transplanted to each pot. Immediately after transplanting, 2 mL of the prepared endophyte inoculum were applied directly to the top of the crown half the rice seedlings using a micropipette, which were designated as endophyte-inoculated treatment groups (E+). The same amount of endophyte-free NFM solution was provided to the remaining seedlings, which were designated as mock-inoculated control groups (E−).

After inoculation, the pots were moved to the growth chambers. In the chambers, the pots were placed in plastic buckets (1.5-gal and 5-gal buckets for Expts 1 and 2, respectively) for easier supply of water and fertilizers, which could be poured in the buckets for absorption by soil media through drainage holes in the bottoms of the pots. A total of eight plants, four E− and four E+, were arranged in each chamber. For Expt 1, one set of AMB and ELE chamber pairs were used, for a total of four treatment replications. For Expt 2, two sets of AMB and ELE chamber pairs were used, for a total of eight treatment replications.

After 14 DAG, 200 mL of NFM fertilizer solution were supplied to the plastic buckets every week, with 0.640 g $L^{-1}$ and 0.160 g $L^{-1}$ $(NH_4)NO_3$ for Expts 1 and 2 to set high (HN) and low N (LN) regimes, respectively. These doses are equal to full and quarter strength Hoagland's solution (Hoagland & Arnon 1950), respectively. In addition to the fertilizer supply, the water in the bucket was refilled fully each week to maintain constant water supply.

***Simultaneous leaf gas exchange and chlorophyll fluorescence measurements***

Leaf gas exchange and chlorophyll fluorescence parameters – net $CO_2$ assimilation rate ($A$), atmospheric [$CO_2$] ($C_a$), intercellular space ($C_i$), chloroplast ($C_c$), stomatal ($g_s$), mesophyll conductance ($g_m$), transpiration rate ($E$), and electron transport rate (ETR) were determined on the second youngest fully expanded leaves at 65 and 100 DAG in Expts 1 and 2, respectively, at the panicle initiation stage. To measure these parameters simultaneously, portable infrared gas analyzers (IRGAs) (LI-6400XT, Li-Cor Inc., Lincoln, NE, USA) equipped with 2 $cm^2$-leaf chamber fluorometers (LI-6400-40, Li-Cor Inc.) were used. Measured variables were further processed to calculate intrinsic and extrinsic water use efficiency (iWUE and eWUE), which are defined by $A/g_s$, $A/E$ and $C_i/C_a$, $C_c/C_i$ ratios.

To examine the biochemical properties of photosynthetic $CO_2$ assimilation, $CO_2$ response curves ($AC_i$ curves) were constructed using a built-in program function of the gas exchange and fluorescence measurement systems. Net $CO_2$ assimilation rates at different $C_a$s ranging from 50 to 1400 μmol $CO_2$ $mol^{-1}$ were recorded along with the other gas exchange and fluorescence parameters. Leaf temperature was set to maintain 25ºC and RH was kept at 40-60% during measurements. Light intensity was set to 1500 μmol $m^{-2}$ $s^{-1}$ to provide a saturating light for the rice leaves. At first, $C_a$ was controlled to decrease stepwise from 400 to 50 μmol $mol^{-1}$, and then increase stepwise from 400 to 1400 μmol $mol^{-1}$ under ambient 21% [$O_2$] conditions. Minimum and maximum waiting time for the reading at each $C_a$ point was 1 and 2 mins, respectively, to reach the preset stability option. After that, the same protocol was used to construct the $AC_i$ curves under the low 2% [$O_2$] condition, inletting 2% [$O_2$] gas to the machine from a 2% [$O_2$] cylinder balanced with nitrogen gas.

To estimate internal conductance ($g_m$) of leaves, the Low [$O_2$] method developed by Bunce (2009) was used, combining two $AC_i$ curves constructed at different [$O_2$], 2% and 21%. First, for calculation of mitochondrial respiration rate ($R_d$) and $CO_2$ compensation point in the absence of $R_d$ ($\Gamma*$), three $AC_i$ curves at three different light intensities – 100, 200, and 800 μmol $m^{-2}$ $s^{-1}$ PPFD – were constructed at $C_a$ ranging from 50 to 250 μmol $mol^{-1}$ following the Laĭsk method (Laĭsk 1977). Second, using the photosynthetic parameterization established by Sharkey (2016), Farquhar-von Cammerer-Berry's biochemical properties of photosynthesis (FvCB, Farquhar *et al.*, 1980) – $V_{cmax}$, $J_{max}$, TPU, $K_c$, and $K_o$ of $A/C_i$ curves were estimated at 21% [$O_2$]. Finally,

these six variables – $R_d$, $\Gamma_*$, $V_{cmax}$, $J_{max}$, $K_c$, and $K_o$ – were plugged into the model parameterization of the Low [$O_2$] method to attain $g_m$ under the atmospheric [$CO_2$].

***Gene expression analysis by a dye-based qPCR***

In Expt 2, after a whole-plant physiological investigation was finished, leaf samples were harvested from the 11:30 am to 12:30 pm. The leaf samples were collected in 50-mL centrifuge tubes and immediately stored in a -80°C freezer until further analysis.

Total leaf mRNAs were extracted using an isolation kit (Spectrum Plant Total RNA Kit, Sigma-Aldrich Co., St. Louis, MO). The isolated mRNAs were mixed with a master reaction mixture containing fluorescent dyes (iQ SyBR Green Supermix, Bio-Rad Laboratories Inc., Hercules, CA) prior to a cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad Laboratories Inc.) and being loaded into a real-time qPCR thermocycler (Chromo 4 System, Bio-Rad Laboratories Inc.). The Pfaffl method was used to calculate relative quantity of *rbcS* gene expression(Pfaffl 2001). *rbcS* gene is translated into the small units of Rubisco complex, which is used as a proxy for the quantity of Rubisco in this study.

***Statistical analysis***

The experiment followed a split-plot design. The inoculation treatment (INOC) and the [N] treatment were regarded as fixed effects and the [$CO_2$] treatment (CO2) was regarded as a random effect. For Expt 2, since two pairs of chambers for the $CO_2$ treatment were used, the chamber effect on variation was tested and turned out to be insignificant at the $\alpha = 0.05$ level. As a result, all parameters from gas exchange and fluorescence measurements were subjected to a three-way analysis of variance (ANOVA) by fitting a linear model regression using R version 3.2.2 (R Core team 2016).

## Results

***Alleviation of photosynthetic down-regulation by endophytes under elevated $CO_2$ conditions***

$A/C_i$ curve analysis showed photosynthetic down-regulation by ELE under both HN and LN conditions at the panicle initiation stage in E- plants (upper and lower ELE panels in Figure 1). Yet, the $A/C_i$ curves of E+ plants have higher asymptotes than those of the E- plants.

For E- plants, all FvCB photosynthetic biochemistry parameters at the operational points of the $A/C_i$ curves – $V_{cmax}$, $J_{max}$, and TPU – were decreased by ELE, compared to E- plants under AMB conditions (Table 2). A 10, 3, and 21% decrease in $V_{cmax}$, $J_{max}$, and TPU, respectively, were observed in E- plants under HN conditions by ELE treatment. A 16 and 2% decrease in $J_{max}$ and TPU, respectively, were observed in E- plants under LN conditions for the ELE treatment. In contrast, for E+ plants, a reduction of the parameters was not observed. Compared to E- plants under ELE conditions, E+ plants under ELE conditions had a 5 and 14% increase in $V_{cmax}$ and $J_{max}$, respectively, under HN conditions. Under LN conditions, the extent of the increases was even higher than under HN conditions – a 33, 7, and 22% increase in $V_{cmax}$, $J_{max}$, and TPU, respectively. The increases in these parameters in response to endophyte inoculation were all significant (Table 2, $P < 0.01$). Significant INOC×CO2 interaction effects on $J_{max}$ and TPU were detected ($P = 0.031$ and 0.010, respectively).

Figure 2 also demonstrates the alleviation of down-regulation effects on photosynthesis. For E- plants, $A_{ele}/A_{amb}$ ratios were below 1.0 over the range of $C_i$, indicating down-regulation. However, endophyte inoculation shifted the ratios from below to above 1.0, indicating mitigation of down-regulation and further increases in photosynthetic capacity of E+ plants. The alleviation was observed under both HN and LN conditions over the range of $C_a$s from 400 to 800 μmol $CO_2$ $mol^{-1}$ ($P < 0.05$).

***Increases in ETR and $g_m$ under ELE with endophyte inoculation***

ETR at the operational points of $A/C_i$ curve responses were greater in E+ plants than in E- plants under ELE only (Figure 3). Under ELE conditions, endophyte inoculation increased 20 and 28% of ETR in HN and LN, respectively (Table 2). No changes were observed under AMB conditions in either N regime. There were significant INOC×$CO_2$ and $CO_2$×N interaction effects (Figure 3).

Mesophyll conductance showed contrasting responses to endophyte inoculation depending on [$CO_2$] (Figure 4 and Table 2). The INOC×$CO_2$ interaction effect was marginally significant ($P = 0.061$). The ELE treatment significantly reduced the amount of $g_m$ in E- plants, with a 29 and 70% decrease under HN and LN conditions, respectively, as opposed to a 1 and 27% decrease in E+ plants. Overall increases of $g_m$ under ELE conditions in response to endophyte inoculation

were 39 and 142% under HN and LN conditions. The increases in $C_c/C_i$ with endophyte inoculation under ELE by 4 and 58% correspond to these changes in $g_m$ (Table 2).

There were no significant INOC×N interaction effects in ETR and $g_m$.

***Increases in WUE with endophyte inoculation***

Marginally significant increases in iWUE and eWUE were detected in E+ plants ($P = 0.075$ and $P = 0.010$, respectively, Table 2). The increase in $C_c/C_i$ by 58% with endophyte inoculation is in accordance with this increase in WUE. Both iWUE and eWUE increased in response to ELE treatment in all cases regardless of N and INOC treatments (Table 2).

Except for the AMB and HN treatment combination, endophyte inoculation increased both iWUE and eWUE of the host plants. Under HN and AMB conditions, endophyte inoculation reduced iWUE and eWUE by 9 and 6%, respectively, whereas under HN and ELE conditions, an 11 and 18% increase in iWUE and eWUE were detected in E+ plants. Under LN and ELE conditions, endophyte inoculation increased iWUE and eWUE by 12 and 12 %, and by 25 and 20% in comparison to controls, respectively.

There were no significant INOC×N interaction effects in WUE.

***Increases in* rbcS *gene expression with endophyte inoculation***

Endophyte-inoculated plants grown under LN conditions showed a greater level of *rbcS* gene expression; however, the difference was only marginal ($P = 0.059$). Under AMB conditions, E+ plants had a 66% higher *rbcS* expression level compared to E− plants. Under ELE conditions, only a 7% increase of *rbcS* expression level was detected in E+ plants compared to E− plants. No $CO_2$ and INOC×$CO_2$ effects were observed (Figure 5).

## Discussion

In this study, we present mitigation of $C_3$ photosynthesis down-regulation in rice in response to long-term elevated [$CO_2$] exposure with symbiotic endophyte associations. The highlight of our study is that this phenomenon is likely derived from improvements on both the demand and supply sides of photosynthetic biophysical and biochemical processes. On the demand side, electron transport in the light reaction complexes was up-regulated, resulting in ETR increases

upon endophyte inoculations. Considering that $C_3$ photosynthesis is mostly limited by the provision of NADPH and ATP from the light reactions when under ELE conditions, the increases in ETR speak to the importance of the impact of endophytes on photosynthesis under future climate conditions. On the supply side, the velocity of $CO_2$ diffusion to the site of carboxylation was boosted upon endophyte inoculation under ELE conditions. Under ELE conditions, even though atmospheric $CO_2$ is sufficient for photosynthetic assimilation, biophysical and biochemical barriers – such as stomatal, cell wall, and liquid phase resistances – in leaves hinder photosynthetic $CO_2$ diffusion and further fixation. The rapid delivery of the substrate to the working site, therefore, is integral in improving the efficiency of the assimilation process. In this respect, the increases in $g_m$ triggered by endophytes are especially important. These photosynthetic adaptation responses in symbiotic plants were consistent over different N levels.

***Endophytes ameliorate photosynthetic down-regulation under elevated [$CO_2$]***

Signature responses to elevated [$CO_2$] in the down-regulation of $C_3$ photosynthesis are decreases in the initial slopes and decreases in the asymptotes of $A/C_i$ curves, as our results in Figure 1 show. The down-regulation of photosynthesis begins with accumulation of non-structural carbohydrates (NTCs), featuring starch accumulation in source tissue chloroplasts that are exposed to a long-term ELE treatment, which in turn signals a reduction in Rubisco turnover rates, finally resulting in decreases in Rubisco and associated enzyme content that operate $CO_2$ assimilation in the Calvin-Benson Cycle as a negative feedback reaction (Drake *et al.* 1997). Interestingly, however, E+ plants appeared to have higher values of FvCB $C_3$ photosynthetic parameters: $V_{cmax}$, $J_{max}$, and TPU were higher than those from E- plants under ELE conditions, regardless of N levels. This $A/C_i$ curve pattern apparently resembles legume photosynthetic responses to ELE conditions (Ainsworth & Rogers 2007). Fewer down-regulation symptoms than in other $C_3$ crop species were reported in 31 studies, shown in $A/C_i$ curves and parameterizations tested in Free-Air $CO_2$ Enrichment facilities across the world. The authors discuss the main two reasons behind the phenomenon: 1) N derived from BNF could be used to sustain Rubisco content and capacity under ELE conditions, and 2) increased sink strength imparted by the nodules symbiotic rhizobium bacteria created in their legume host roots (also well-reviewed in Leakey *et al.* 2009, focusing only on physiological $CO_2$ responses of legumes).

Regarding the second reason, Ainsworth *et al.* (2004) empirically tested this 'source-sink' relationship between plant hosts and symbiotic bacteria under higher $CO_2$ levels. They genetically modified sink strength of examined plant materials and found that a decrease in sink capacity in response to losing nodules in root tissues resulted in substantial down-regulation of photosynthesis compared to non-genetically modified controls. Furthermore, extra N fixed by endophytes themselves could be utilized to create sink tissues as building blocks of new biomass (Kim *et al.* 2003).

In this regard, a mechanistic explanation for the mitigation of down-regulation in the diazotrophic endophyte-inoculated plants can be found by analogy with the legume-rhizobium symbiosis. First, BNF by endophytes is a well-established functional trait of the plant-endophyte interactions (Doty 2017), as shown in our previous *in planta* study with Salicaceae endophytes (Knoth *et al.* 2014). Second, although the biological sink strength of endophytes has never been quantified through experimental approaches, endophytes are active symbionts that consume carbohydrates supplied by the plant host. Given that other symbiotic associations (e.g. rhizobium bacteria and mycorrhizal fungi) cost the host plant around 5-20% of total carbohydrates (Lambers 2008; Jones *et al.* 2009; Kaschuk *et al.* 2010), endophytes should drain a substantial amount of carbohydrates from the host, thereby serving as active biological sinks. Host plants become able to create and provide larger C food reserves when there are abundant environmental substrates, especially under ELE conditions.

### *The demand side: Endophytes facilitate photosynthetic electron transfer under elevated $CO_2$ conditions*

Although the underlying mechanisms that trigger increases in ETR are difficult to elucidate with our data (Figure 3), the response is consistent across diazotrophic endophyte inoculations under both N-sufficient and N-limited conditions. Likewise, according to previous research (Woodward *et al.* 2012), although *A* of the symbiotic and non-symbiotic plants were not significantly different, the photochemical efficiency ($\Phi_{PSII}$) increased in symbiotic tomato plants with fungal endophytes. The increases in $\Phi_{PSII}$ can be translated into the increases in ETR in the present study since the relationship between the two is positively linear (Baker 2008). This result implies that ATP and NADPH were produced more than in the non-symbiotic plants in the light

reaction of photosynthesis with the same number of quanta, they are consumed in the process of the $CO_2$ fixation, as seen in the increases in $A_{max}$ (Table 2).

Considering the fact that plants under elevated [$CO_2$] will encounter more RuBP regeneration limitation of photosynthesis (Ainsworth & Rogers 2007), the increases in ETR and pertinent increases in other PSII activities (data not shown, e.g. photochemical and non-photochemical quenching) by endophyte inoculation are promising results.

The carboxylation process by Rubisco was also slightly improved upon endophyte inoculation presented by the increases in *rbcS* gene expression in E+ plants (Figure 5). The response is in accordance with the steeper initial slopes of $A/C_i$ curves and the corresponding increases in $V_{cmax}$ in E+ plants (Figure 1 and Table 2, respectively). A greater amount of Rubisco with endophytes likely enhanced the carboxylation capacity of Rubisco in the Calvin cycle. Together, the improvements of the Rubisco capacity with endophytes would help the host plant alleviate the photosynthetic down-regulation under elevated $CO_2$ conditions. The impacts on the alleviation would surpass those found from the legume-rhizobium symbiosis under elevated $CO_2$ conditions since legumes do not seem to show the increases in the Rubisco capacity. Rather, symbiotic legumes maintain the same $V_{cmax}$ and $J_{max}$ under elevated $CO_2$ conditions (Ainsworth & Rogers 2007).

***The supply side: Endophytes promote internal $CO_2$ diffusion under elevated $CO_2$ conditions***

Higher $g_m$ facilitates the diffusion of [$CO_2$] through chloroplast cell walls and other layers on the path of $CO_2$ molecules from the atmosphere to the site of carboxylation. Therefore, plants with higher $g_m$ should have more $CO_2$ supplied along the diffusional pathway (Flexas *et al.* 2008).

The increases in $g_m$ only under ELE coincide with the increases in ETR (Figures 4 and 3, respectively). The coordinative mechanisms of photosynthetic electron transport in PSII of the thylakoid membrane, as well as the supply of bicarbonate to the thylakoid space, is reported and reviewed in Govindjee & van Rensen (1993) and van Rensen & Klimov (2005). $CO_2$ in the apoplastic spaces of leaves exists in the form of either bicarbonate ($CO_3^{2-}$) or carbonic acid ($HCO_3^-$). Under ELE conditions, plants produce more carbohydrates due to increases in the supply of carboxylation substrate (Ainsworth & Long 2005). Assimilated C as NTCs can be used by microorganisms that live in the intercellular spaces of the host plants. As endophytes consume

photoassimilates in the process of respiration, they release $CO_2$, which can be readily dissolved to bicarbonate ions. This may promote the light harvesting process in PSII by increasing ETR in coordination. With more $CO_2$ and NTCs, and therefore more microbial respiratory $CO_2$ release under ELE, there may be a better chance of symbiotic plants having more internal $CO_2$ available for assimilation. As a consequence, $g_m$ could be increased by this signal and further stimulate the entire assimilation process, as indicated by the increases in $A_{max}$ in our data.

A more efficient internal supply of $CO_2$ with endophytes under ELE conditions led to increases in WUE as the $C_i/C_a$ and $C_c/C_i$ data show (Table 2). $C_i/C_a$ decreased while $C_c/C_i$ increased when N was limited under ELE conditions. This indicates that the increases in WUE under ELE conditions with endophytes are attributable to the more rapid supply of $CO_2$ inside the leaf, rather than to the surface of the leaf by stomatal reactions. In addition, N supply affects this response. Under LN conditions, the increases in WUE were more significant than under HN conditions. This is a different mechanism of WUE increases by Salicaceae endophytes than the one previously reported in Rho *et al.* (2018), which showed that endophytes increased WUE by reducing stomatal aperture during afternoons and resultant water loss, while maintaining photosynthetic capacity under AMB conditions. Compared to this, under ELE conditions endophytes appear to modulate internal components of the leaf with more resources (i.e. carbohydrates) available. More fundamental approaches at the molecular scale will be required to elucidate a mechanistic understanding of the responses.

***Beneficial endophyte effects are greater under elevated $CO_2$ conditions***

Leaf level physiological characteristics were not noticeably altered with endophyte inoculation under AMB conditions. This corroborates the results from Rogers *et al.* (2012) in which hard-wood cuttings of *Populus deltoides* inoculated with *Enterobacter* sp. showed increased biomass, but no effects on photosynthetic parameters such as $A$, $g_s$, and photosynthetic WUE (i.e. iWUE or eWUE) were observed. The authors discuss that the increases in productivity are more related to the increases in leaf area at the whole plant physiological scale. Although varying effects were observed in some parameters, E+ plants in our study displayed similar responses to AMB.

Nevertheless, many photosynthetic parameters measured and analyzed in this study showed significant INOC × CO2 interactions, including $A_{max}$, ETR, $J_{max}$, TPU, $g_m$, $C_c/C_i$, and eWUE

(Table 2). This indicates that endophyte inoculation enhanced these photosynthetic properties, which may be more efficient under ELE conditions. Considering rice as a $C_3$ model crop, our results illustrate the potential for using diazotrophic symbionts as an adaptive solution for a variety of crop species under the future climate.

## Conclusions

In this study, select diazotrophic endophytes originally isolated from the Salicaceae family enabled their host plants to benefit from gaining higher capacities for photosynthetic $CO_2$ assimilation processes. The down-regulation of $C_3$ photosynthesis to elevated [$CO_2$] was ameliorated with endophyte inoculation, as observed in the $A/C_i$ responses. The electron transport in the light reactions and the $CO_2$ diffusion in the intercellular spaces were facilitated in rice upon endophyte inoculation, resulting in increases in the operational ETR and $g_m$ under HN and LN conditions. This pattern was only distinguished under ELE conditions where the plants underwent down-regulation of photosynthesis in response to $CO_2$ enrichment. The overall improvement of photosynthetic responses led to increases in WUE of the plants. Our findings suggest the potential of using endophytic microbial partners to promote plant growth under future climatic conditions. Further research is required to uncover the underlying mechanisms of the alleviation at the molecular biology scale.

## Author Contributions

Conceived idea and designed experiments: HR, SK. Conducted experiments: HR. Analyzed data: HR, SK. Provided materials and resources: SD, SK. Wrote the article: HR, SD, SK.

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## Tables & Figures

**Table 1** Experimental details of the study. INOC and CO2 stand for endophyte inoculation treatments and CO2 enrichment treatment, respectively.

| Category | Experiment 1 | Experiment 2 |
|---|---|---|
| Growing season | Spring to Summer (4/8 – 7/22, 2013) | Spring to Summer (3/21 – 9/2, 2014) |
| Growing duration | 105 days | 165 days |
| Experimental settings | 2 x 2 (INOC x CO2) | 2 x 2 (INOC x CO2) |
| Sample size | 4 (in one set of chambers) | 8 (in two sets of chambers) |
| Nitrogen fertilization* | 1X N (high N, HN) | ¼X N (low N, LN) |
| Average [$CO_2$] (AMB/ELE) | 407/843 μmol $mol^{-1}$ | 437/886 μmol $mol^{-1}$ |
| Average air temperature (day/night) | 23/19°C | 23/19°C |
| Average relative humidity (day/night) | 57/64% | 60/66% |
| Average instantaneous light intensity | 216.7 μmol $m^{-2}$ $s^{-1}$ | 176.3 μmol $m^{-2}$ $s^{-1}$ |
| Average daily light integral | 11.6 mol $m^{-2}$ $d^{-1}$ | 9.1 mol $m^{-2}$ $d^{-1}$ |
| Endophyte strains used** | PTD1 (*Rhizobium* sp.) | WP5 (*Rahnella* sp.) |
| Plant species | Rice (*Oryza sativa* 'M-206') | Rice (*Oryza sativa* 'M-206') |

* Compared to the standard dose of Hoagland's solution (Hoagland & Arnon 1950).
** Closest 16S rRNA match identified through the BLAST NCBI database (Doty *et al.* 2009a).

**Table 2** Descriptive and inferential statistics of photosynthetic parameters at the operational points of the $CO_2$ response curves. The means of four and eight replicated responses are provided with the standard errors in parentheses. *F*-statistics of ANOVA test results for inoculation effect (INOC), [$CO_2$] effect (CO2), [N] effect (N), and their interaction effects on various photosynthesis characteristics are presented with statistical significance codes: °, *, **, and *** for $P < 0.10$, 0.05, 0.01, and 0.001 levels, respectively. The corresponding *P*-values of the *F*-statistics are presented in parentheses.

| N | CO2 | INOC | *n* | $A_{max}$ ($\mu mol\ m^{-2}\ s^{-1}$) | ETR ($\mu mol\ m^{-2}\ s^{-1}$) | $V_{cmax}$ ($\mu mol\ m^{-2}\ s^{-1}$) | $J_{max}$ ($\mu mol\ m^{-2}\ s^{-1}$) | *TPU* ($\mu mol\ m^{-2}\ s^{-1}$) | $g_s$ ($mol\ H_2O\ m^{-2}\ s^{-1}$) | $g_m$ ($mol\ CO_2\ m^{-2}\ s^{-1}$) | $C_i/C_a$ (unitless) | $C_c/C_i$ (unitless) | *E* ($mmol\ H_2O\ m^{-2}\ s^{-1}$) | iWUE ($mol\ CO_2\ mol^{-1}\ H_2O$) | eWUE ($mol\ CO_2\ mol^{-1}\ H_2O$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HN | AMB | E- | 4 | 25.46 | 128.1 | 103.3 | 135.6 | 11.77 | 0.593 | 0.190 | 0.783 | 0.543 | 6.517 | 0.0046 | 0.393 |
| | | | | (0.476) | (5.246) | (2.570) | (10.94) | (0.851) | (0.091) | (0.023) | (0.032) | (0.028) | (0.329) | (0.0007) | (0.017) |
| | | E+ | 4 | 25.17 | 127.8 | 113.9 | 149.9 | 10.75 | 0.636 | 0.160 | 0.799 | 0.461 | 7.143 | 0.0042 | 0.369 |
| | | | | (0.029) | (5.017) | (7.645) | (5.545) | (0.321) | (0.047) | (0.007) | (0.016) | (0.028) | (0.335) | (0.0004) | (0.018) |
| | ELE | E- | 4 | 26.36 | 103.8 | 93.11 | 131.5 | 9.308 | 0.612 | 0.135 | 0.883 | 0.713 | 5.851 | 0.0047 | 0.473 |
| | | | | (0.481) | (2.431) | (3.539) | (3.155) | (0.364) | (0.095) | (0.009) | (0.020) | (0.020) | (0.702) | (0.0009) | (0.061) |
| | | E+ | 4 | 32.23 | 124.9 | 108.12 | 155.2 | 11.35 | 0.627 | 0.188 | 0.873 | 0.742 | 5.783 | 0.0052 | 0.558 |
| | | | | (2.437) | (9.380) | (6.902) | (10.90) | (0.816) | (0.041) | (0.027) | (0.009) | (0.013) | (0.044) | (0.0004) | (0.044) |
| LN | AMB | E- | 8 | 10.85 | 84.66 | 35.65 | 89.90 | 5.979 | 0.191 | 0.166 | 0.744 | 0.732 | 2.259 | 0.0058 | 0.484 |
| | | | | (0.797) | (7.882) | (3.059) | (4.460) | (0.326) | (0.016) | (0.031) | (0.014) | (0.066) | (0.191) | (0.0003) | (0.022) |
| | | E+ | 8 | 11.14 | 85.53 | 43.96 | 86.40 | 6.525 | 0.177 | 0.150 | 0.717 | 0.642 | 2.069 | 0.0065 | 0.540 |
| | | | | (0.940) | (5.339) | (5.147) | (5.405) | (0.362) | (0.021) | (0.049) | (0.011) | (0.056) | (0.139) | (0.0003) | (0.028) |
| | ELE | E- | 8 | 14.23 | 91.77 | 36.50 | 75.50 | 5.854 | 0.164 | 0.050 | 0.784 | 0.433 | 1.709 | 0.0097 | 0.844 |
| | | | | (0.663) | (6.745) | (2.529) | (2.553) | (0.162) | (0.026) | (0.014) | (0.022) | (0.076) | (0.116) | (0.0011) | (0.033) |
| | | E+ | 8 | 17.71 | 117.3 | 47.58 | 96.13 | 7.284 | 0.155 | 0.121 | 0.736 | 0.684 | 1.815 | 0.0121 | 1.013 |
| | | | | (1.348) | (7.909) | (5.825) | (5.819) | (0.392) | (0.018) | (0.022) | (0.020) | (0.069) | (0.204) | (0.0010) | (0.070) |
| **Treatment Effect** | | | | | | | | | | | | | | | |
| INOC df = 1 | | | | **9.203** (0.004)** | **5.610* (0.022)** | **9.581** (0.003)** | **8.774** (0.005)** | **7.701** (0.008)** | 0.006 (0.941) | 1.544 (0.222) | **3.077° (0.087)** | 1.499 (0.229) | 0.137 (0.713) | **3.337° (0.075)** | **7.310* (0.010)** |
| CO2 df = 1 | | | | **33.43*** (< 0.001)** | 2.636 (0.112) | 0.115 (0.735) | 0.059 (0.808) | 0.111 (0.740) | 0.293 (0.591) | **5.198* (0.029)** | **12.84*** (< 0.001)** | 0.077 (0.782) | **11.91** (0.001)** | **37.16*** (< 0.001)** | **104.8*** (< 0.001)** |
| N df = 1 | | | | **293.3*** (< 0.001)** | **22.99*** (< 0.001)** | **300.0*** (< 0.001)** | **202.9*** (< 0.001)** | **191.2*** (< 0.001)** | **241.3*** (< 0.001)** | **4.881* (0.034)** | **37.84*** (< 0.001)** | 0.010 (0.919) | **548.6*** (< 0.001)** | **42.70*** (< 0.001)** | **66.50*** (< 0.001)** |
| INOC x CO2 df = 1 | | | | **6.152* (0.017)** | **5.161* (0.028)** | 0.228 (0.635) | **4.938* (0.031)** | **7.230* (0.010)** | 0.012 (0.915) | **3.755° (0.061)** | 0.710 (0.404) | **7.936** (0.008)** | 0.009 (0.924) | 1.593 (0.214) | **3.184° (0.081)** |
| INOC x N df = 1 | | | | 0.728 (0.398) | 0.062 (0.803) | 0.179 (0.674) | 0.166 (0.686) | 0.562 (0.457) | 0.501 (0.483) | 0.188 (0.667) | 1.989 (0.166) | 2.013 (0.165) | 0.744 (0.393) | 1.622 (0.210) | 1.503 (0.227) |
| CO2 x N df = 1 | | | | 0.831 (0.367) | **9.040** (0.004)** | 1.934 (0.172) | 1.556 (0.219) | **3.893° (0.055)** | 0.267 (0.608) | 1.722 (0.198) | **4.014° (0.051)** | **15.70*** (< 0.001)** | 2.693 (0.108) | **12.88*** (< 0.001)** | **17.84*** (< 0.001)** |
| INOC x CO2 x N df = 1 | | | | 0.361 (0.551) | 0.022 (0.883) | 0.012 (0.911) | 0.563 (0.457) | **2.923° (0.095)** | 0.085 (0.772) | 0.001 (0.973) | 0.004 (0.951) | 1.660 (0.206) | 1.761 (0.192) | 0.121 (0.729) | 0.001 (0.971) |

**Table 3** List of abbreviations used in the manuscript.

| Abbreviation | Definition |
|---|---|
| AMB/ELE | Ambient/Elevated $CO_2$ treatments, [$CO_2$] of which were targeted 400/800 μmol $mol^{-1}$ with pure $CO_2$ gases |
| *A* | Net $CO_2$ assimilation rate (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |
| $A/C_i$ curves | *A* as a function the intercellular [$CO_2$], $CO_2$ response curves |
| $A_{max}$ | Light-saturated maximum *A* at the operational points of $A/C_i$ curves (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |
| BNF | Biological $N_2$ fixation |
| $C_a$, $C_c$, $C_i$ | [$CO_2$] in the atmosphere, chloroplast, intercellular air spaces (μmol $mol^{-1}$) |
| CO2 | $CO_2$ treatment effect |
| *E* | Transpiration rate (mmol $H_2O$ $m^{-2}$ $s^{-1}$) |
| E-/E+ | Mock-inoculated control/endophyte-inoculated plants |
| ETR | Photosynthetic electron transport rate between PSII and PSI in the light reactions (μmol electron $m^{-2}$ $s^{-1}$) |
| $g_m$, $g_s$ | Mesophyll, stomatal conductance (mol $CO_2$, $H_2O$ $m^{-2}$ $s^{-1}$) |
| HN/LN | High/Low [N] treatments |
| INOC | Endophyte inoculation effect |
| $J_{max}$ | Maximum ETR (μmol electron $m^{-2}$ $s^{-1}$) |
| $K_c$, $K_o$ | Michaelis-Menten constants for carboxylation, oxygenation of Rubisco |
| $R_d$ | Mitochondrial respiration rate (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |
| $V_{cmax}$ | Maximum carboxylation rate (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |
| eWUE, iWUE | Extrinsic, intrinsic water use efficiency, defined by $A/E$ and $A/g_s$ (mol $CO_2$ $mol^{-1}$ $H_2O$), respectively |
| TPU | Triose phosphate utilization rate (μmol $m^{-2}$ $s^{-1}$) |
| $^{O}$, *, **, *** | Statistically significant at $P$ = 0.10, 0.05, 0.01, 0.001 levels |
| $\Gamma^*$ | CO2 compensation point in the absence of $R_d$ (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |

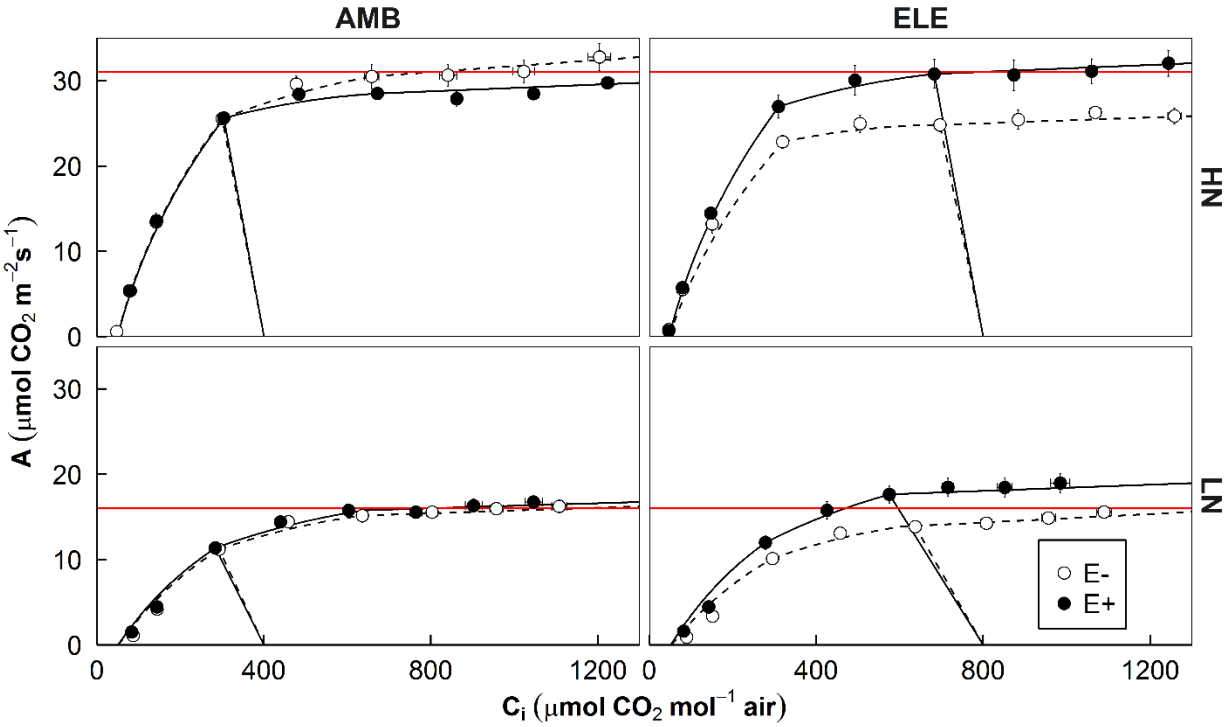


**Fig. 1** $CO_2$ response ($A/C_i$) curves of rice leaves at panicle initiation stage grown under ambient (AMB, app. 400 ppm) and elevated [$CO_2$] (ELE, app. 800 ppm). The top panels show the responses of the plants grown at full strength nitrogen condition (HN, $n$ = 4); while as the bottom panels show those at quarter strength (LN, $n$ = 8). Open and closed symbols indicate the mean responses from mock-inoculated control groups (E−) and endophyte inoculated groups (E+). Horizontal and vertical error bars display ± 1 S.E.M. of intercellular [$CO_2$] ($C_i$) and net $CO_2$ assimilation rate ($A$). The red reference lines show the asymptotes of the responses from E− plants under AMB conditions to highlight down-regulation under ELE conditions.

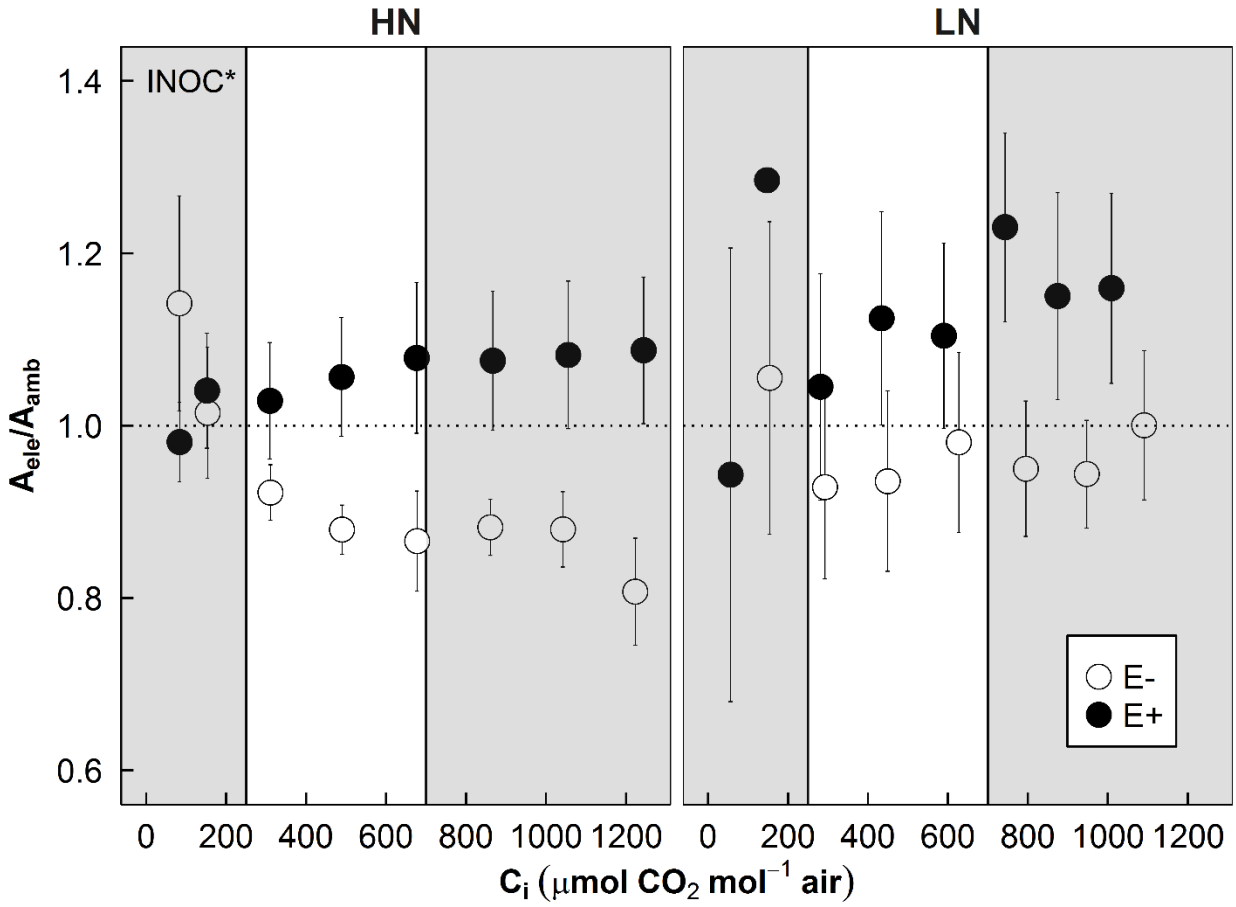


**Fig. 2** The ratio of $CO_2$ assimilation rates of rice leaves grown under elevated ($A_{ele}$, app. 800 ppm) and ambient ($A_{amb}$, app. 400 ppm) $CO_2$ chambers at the entire range of reference [$CO_2$] of the gas exchange measurement system. The metrics were derived from the $A/C_i$ datasets in **Fig. 1**. Open and closed symbols indicate the mean responses of mock-inoculated (E-) and inoculated (E+) samples. The left panel presents the responses of fully nitrogen supplied samples (HN), whereas the right panel presents those of quarter strength nitrogen supplied samples (LN). Error bars represent ± 1 S.E.M. ($n$ = 4 and 8 for HN and LN, respectively). The responses in the clear area (the reference [$CO_2$] ranges from 400 to 800 μmol $mol^{-1}$) show significant inoculation (INOC) effect on the ratio at a $P < 0.05$ level (*).

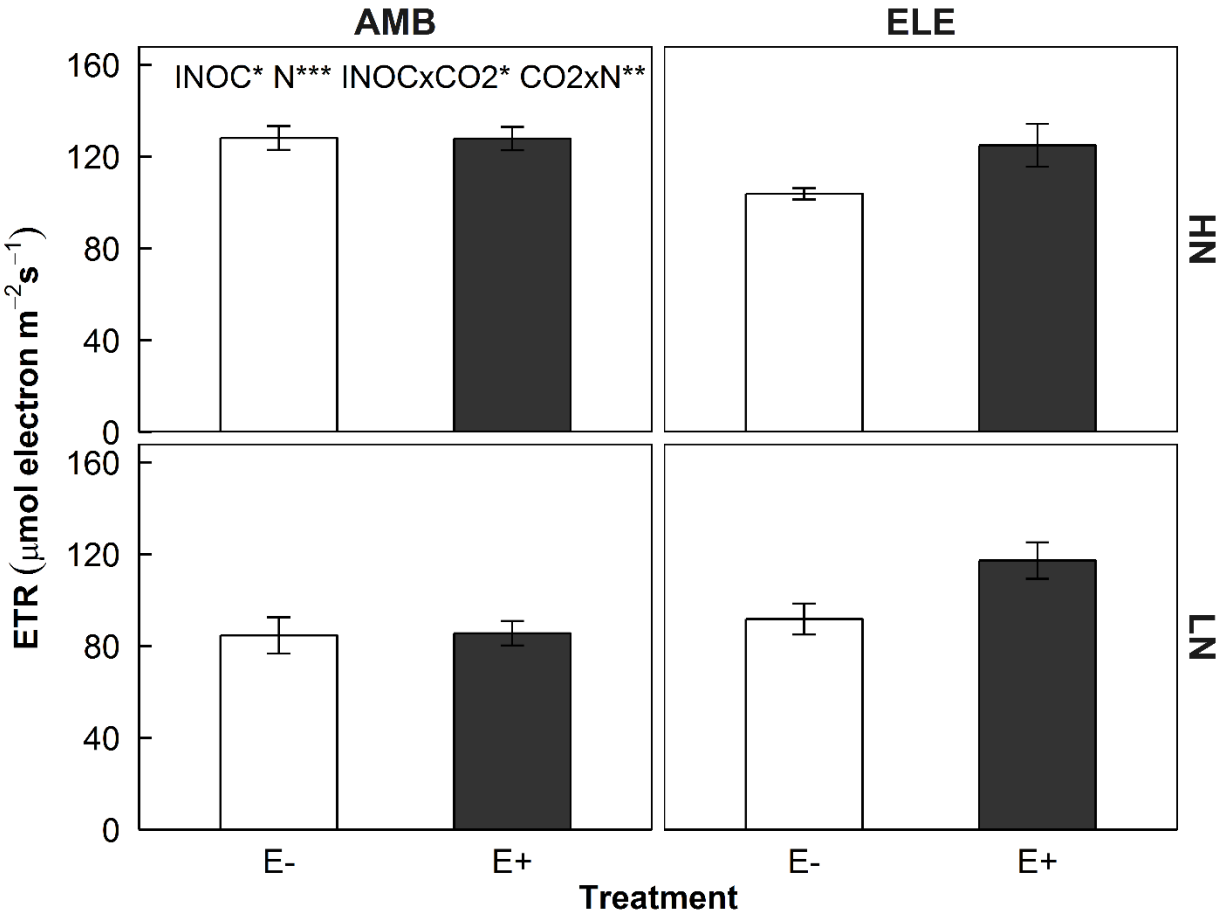


**Fig. 3** Electron transport rate (ETR) of rice leaves at panicle initiation stage grown under ambient (AMB, app. 400 ppm) and elevated [$CO_2$] (ELE, app. 800 ppm). The top panels show the responses of the plants grown at full strength nitrogen condition (HN, $n$ = 4); while as the bottom panels does those at quarter strength (LN, $n$ = 8). Open and closed symbols indicate the mean responses from mock-inoculated control groups (E-) and endophyte inoculated groups (E+). Error bars display ± 1 S.E.M. Significant treatment effects (inoculation, [$CO_2$], and [N] for INOC, CO2, and N) are provided with statistical codes: *, **, and *** for $P$ < 0.05, 0.01, and 0.001 levels, respectively. The data were retrieved from the operational points of the $A/C_i$ curves in **Fig. 1**.

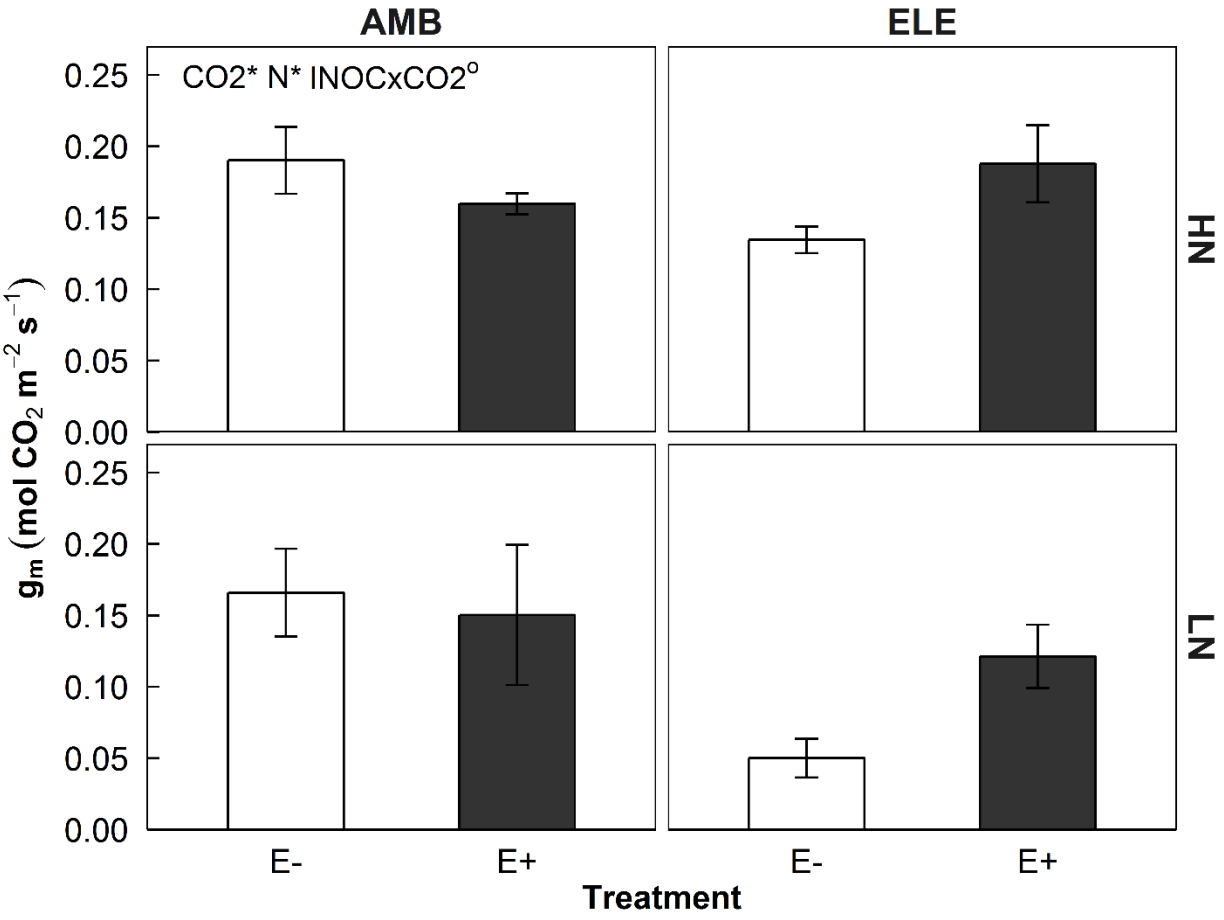


**Fig. 4** Mesophyll conductance ($g_m$) of rice leaves at panicle initiation stage grown under ambient (AMB, app. 400 ppm) and elevated [$CO_2$] (ELE, app. 800 ppm). The top panels show the responses of the plants grown at full strength nitrogen condition (HN, $n$ = 4); while as the bottom panels does those at quarter strength (LN, $n$ = 8). Open and closed symbols indicate the mean responses from mock-inoculated control groups (E-) and endophyte inoculated groups (E+). Error bars display ± 1 S.E.M. Significant treatment effects (inoculation, [$CO_2$], and [N] for INOC, CO2, and N) are provided with statistical codes: $^{o}$ and * for $P < 0.10$ and 0.05 levels, respectively. The data were retrieved from the operational points of the $A/C_i$ curves in **Fig. 1**.

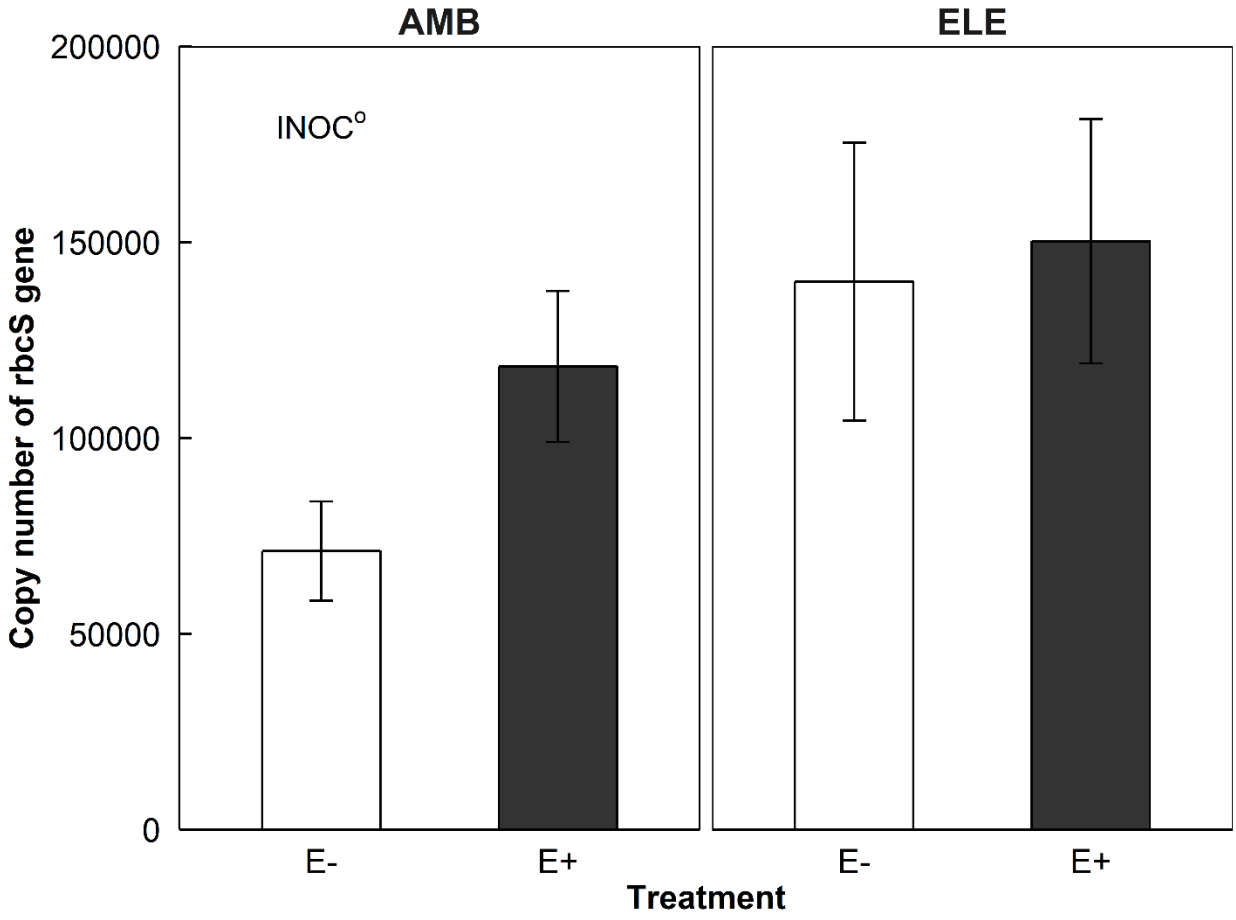


**Fig. 5** Gene expression levels of *rbcS* gene in rice leaves at panicle initiation stage grown under ambient (AMB, app. 400 ppm) and elevated [$CO_2$] (ELE, app. 800 ppm) conditions with low N supply. The actual copy numbers of rbcS was calculated by the Pfaffl method (Pfaffl 2001). Open and closed bars indicate the mean responses from mock-inoculated control groups (E-) and endophyte inoculated groups (E+) of replicates ($n$ = 6-8). Error bars display ± 1 S.E.M. A significant inoculation treatment effect (INOC) is indicated at a $P < 0.10$ level ($^{o}$).

# CHAPTER 4

## Estimating Microbial Respiratory $CO_2$ from Endophytic Bacteria in Rice

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## Abstract

Endophytes are symbiotic microbes that live inside host plants. These endophytic symbionts receive photosynthesized carbohydrates from host plants while conferring symbiotic benefits to their host. During photosynthate-fueled respiration, endophytes release $CO_2$ into the intercellular spaces of their host plants in which they reside. We evaluated the possibility for host plants' re-assimilation of microbial respiratory $CO_2$. *In planta* and *in vitro* assays were conducted to examine respiratory characteristics of endophyte-symbiotic plants. Endophyte-inoculated plants had a greater *in planta* respiration rate. *In vitro* data demonstrated that respiration rates of endophytes are dependent on the total amount of endophytes and the concentration of carbohydrate supply. Assuming the host plant offers sufficient carbohydrates, we estimate that $CO_2$ produced during microbial respiration *in planta* accounts for about 15% of the $CO_2$ assimilated by the photosynthetic pathways of the symbiotic plant. This suggests that endophytes can produce significant amounts of $CO_2$, which could then be re-assimilated by host plants.

**Keywords:** endophytes[1], microbial respiration[2], photosynthetic assimilation[3], reassimilation[4], carbohydrates[5]

## Introduction

Endophytes are symbiotic bacteria, fungi, and yeast that reside in plant hosts (Dobereiner 1992). By hosting these mutualists, plants receive functional benefits including biological $N_2$ fixation (Knoth *et al.* 2014), phosphate solubilization (Wakelin *et al.* 2004), siderophore production (Costa & Loper 1994), phytohormone production (Xin *et al.* 2009), bio-control of pathogens (Kandel *et al.* 2017), and many other possible benefits. These symbiotic enhancements can lead to increased plant biomass (Kandel *et al.* 2015), conferment of plant environmental stress tolerance (Rho *et al.* 2017), or enhanced efficiency in plant water use (Rho *et al.* 2018). All these mechanistic processes encompass active communications between the plant host and its symbionts that take place in either the intercellular spaces or vascular tissue of the plant (Lemoine *et al.* 2013). Endophytes directly interact with host cells and molecules through this interface, potentially decreasing the distance of material transport, thereby increasing the possibility or frequency of signal transport (Reinhold-Hurek & Hurek 2011). Endophytes traffic

molecular signals and sucrose/hexoses in the apoplasm at the plant-microbe interface (Lemoine *et al.* 2013).

Endophytic microbes perform all these symbiotic functional activities as they multiply in their host plant. Thus, they require biochemical energy simply to live and to remain active (Kiers & Denison 2008). This energy is derived from the production of ATP and NADH during respiration, and requires metabolizing different forms of carbohydrates. The carbohydrates endophytic symbionts consume originate in the photosynthesis of the host plant, and a substantial amount of these is used to maintain symbiotic relationships (Kiers & Denison 2008; Kiers *et al.* 2011). For this reason, the metabolic cost of symbiosis can be expressed by the percentage of photosynthates plant symbionts consume during respiration (Baas & Kuiper 1989). For example, mycorrhizal fungi consume about 4-20% of host plant photosynthates (Lambers *et al.* 2008). This is based on measurements of plant respiration. However, respiratory characteristics of endophytic microbes have, to our knowledge, not been experimentally examined.

A further characteristic of microbial respiration may contribute to plant physiological processes. As an end-product of respiration, $CO_2$ is released. This $CO_2$ molecule can easily reenter the assimilation pathways for photosynthesis since it is more immediately accessible compared to external atmospheric $CO_2$. This is especially likely for endophyte respiration since they live in the intimate intercellular spaces of the plant.

Re-assimilation of respiratory $CO_2$ is not a new concept (Ogren 1984). Evidence supports that in addition to raw $CO_2$ diffused from the atmosphere, processed $CO_2$ from respiration – photorespiration included – can be a source for $CO_2$ assimilation. $^{13}C$-isotope assays have shown that up to 17.4% of $CO_2$ produced during poplar tree root respiration can be re-assimilated into other organ tissues of the trees (Bloemen *et al.* 2013b). One report shows that approximately 12% of the $CO_2$ produced by photorespiration can reenter the Calvin cycle and be reused in *Arabidopsis thaliana* (Xin *et al.* 2015). Also, as much as 24–38% of photorespired and respired $CO_2$ can be re-assimilated, resulting in an 8–11% increase in rice and wheat photosynthesis (Busch *et al.* 2013).

We hypothesize that the respiratory $CO_2$ can be more available to $CO_2$ fixation process at the site of carboxylation since it bypasses the $CO_2$ diffusional pathway from the atmosphere to the

intercellular space. Therefore, reducing the travel distance of $CO_2$ would benefit the plants. The symbiotic plants would not need to open stomata to take $CO_2$ up from the surrounding air. This is well demonstrated in Rho et al. (Rho *et al.* 2018). Diurnal trends of stomatal conductance showed stomatal conductance of endophyte-inoculated rice plants were lower than that of control plants due to faster accumulation of ABA during the afternoon. Yet, overall photosynthetic $CO_2$ assimilation was maintained with less opening stomata, indicating an extra source of $CO_2$ may exist. This gas exchange response benefited the host plants more when sufficient light for photosynthesis is available. This suggests that a certain level of sugars should support the endophytes' respiratory activities before the plants exploit it. To better understand the proposed mechanisms, an investigation on respiratory behaviors of endophyte-symbiotic plants and endophytes themselves should be required.

Microbial production of respiratory $CO_2$ accounts for a large portion of soil respiration (Cheng *et al.* 2011). Nevertheless, evidence has been lacking during *in vitro* level demonstration to determine if microbial respiratory production of $CO_2$ is quantifiable, and if so, if it can possibly be reused. The objective of this study is to estimate microbial respiratory $CO_2$ release inside the host plant and to assess the potential for re-assimilation of $CO_2$ produced in this manner.

## Materials and Methods

A series of *in planta* and *in vitro* assays were conducted at the University of Washington Center for Urban Horticulture (47°39′27″ N, 122°17′21″ W; 10 m elevation). The bacterial endophyte strain used in this study, WP5, was originally isolated from Washington State native poplar trees during our previous studies (Doty *et al.* 2009a). The isolate was identified as *Rahnella* sp. through a 16S rRNA sequence analysis and was known to contain the *nifH* gene. Other potential symbiotic traits of the strain are documented in Doty et al (Doty *et al.* 2009a) and Khan et al. (Khan *et al.* 2012).

### In planta *leaf gas exchange experiment*

*Preparation of inoculum*

The isolate was stored in a 30% glycerol buffered solution in a −80$^{o}$C freezer prior to the beginning of the experiment. Before inoculation, the frozen endophyte-containing solution was streaked on MG/L media plates (g $L^{-1}$: 5.0 tryptone, 2.5 yeast extract, 5.2 NaCl, 10.0 mannitol,

1.32 sodium glutamate, 0.50 $KH_2PO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, and 2 μg biotin at pH 7.0) (Cangelosi *et al.* 1991), and then cultured overnight in N-limited combined carbon media broth (NL-CCM) (g $L^{-1}$: Solution 1, 5.0 sucrose, 5.0 mannitol, 0.5 mL/L sodium lactate, 0.8 $K_2HPO_4$, 0.2 $KH_2PO_4$, 0.1 NaCl, 0.025 $Na_2MoO_4 \cdot 2H_2O$, 0.028 $Na_2FeEDTA$, 0.1 yeast extract; Solution 2, 0.2 $MgSO_4 \cdot 7H_2O$, 0.06 $CaCl_2$; a mixture of solution 1 and 2 was used after the two solutions were autoclaved separately) (Rennie 1981). The bacterial density ($OD_{600}$) of the culture was measured using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, USA) and adjusted to 0.1 (equivalent to approximately $1 \times 10^7$ cells) to create the inoculum by diluting the culture with sterile deionized (DI) water and N-free media broth (NFM) (g $L^{-1}$: 69.9 $KH_2PO_4$, 19.84 $K_2HPO_4$, 174.7 $K_2SO_4$, 119.7 $MgSO_4 \cdot 7H_2O$, 100 $MgCl_2 \cdot 6H_2O$, 219.8 $CaCl_2 \cdot H_2O$, 3.38 $MnSO_4 \cdot H_2O$, 0.5 $CuSO_4 \cdot 5H_2O$, 0.55 $ZnSO_4 \cdot 7H_2O$, 3.83 $H_3BO_3$, 0.24 $NaMoO_4 \cdot 2H_2O$, 0.11 $CoSO_4 \cdot 6.5H_2O$, and 35 Fe Sequestrine at pH 6.5) (Doty *et al.* 2009b).

*Preparation of plant materials and inoculation with endophytes*

Very-early to early-maturing Japonica M-206 rice (*Oryza sativa*) was chosen as our host plant species based on our previous studies in which this rice variety showed strong symbiotic impacts with the endophytes on biomass gain and physiological traits. (Kandel *et al.* 2015; Rho *et al.* 2018). In order to remove other microorganisms and contaminants, the rice seed surfaces were sterilized by soaking the seeds in 3% (v/v) NaOCl solution for 4 hrs, followed by rinsing five times with sterile water under a sterile hood (Model NU-425-400, NuAire Inc., Plymouth, MN, USA). The surface-sterilized seeds were directly sowed into horticultural media (Sunshine Mix #4, SunGro, Bellevue, WA, USA) in 10-gal pots. The pots were soaked in commercial bleach for 30 mins prior to adding horticultural media, for the sake of sterilization. Ten seeds were planted per pot and 30 pots were prepared in total. Approximately 95% of the seeds germinated within seven days.

Seven days after germination (DAG), 2 mL of the prepared WP5 inoculum were applied directly to the top of the crown half of the rice seedlings using a micropipette, which were designated the endophyte-inoculated treatment group (E+). The same amount of endophyte-free NFM solution was applied to the remaining seedlings, which were designated the mock-inoculated control group (E−).

Beginning at 14 DAG, 200 mL of NFM fertilizer solution were supplied to the plants once per week, with 0.160 g $L^{-1}$ $(NH_4)NO_3$ to set a low N regime. This dose is equivalent to quarter-strength Hoagland's solution (Hoagland & Arnon 1950). In addition to receiving fertilizer, the plants were fully irrigated once per week to maintain a constant water supply.

*Leaf respiration measurements*

Leaf samples were collected to measure both the release of $CO_2$ and the consumption of $O_2$, for the purpose of determining the rice plant respiration rates.

One-hundred DAG, one out of 10 plants was randomly selected per pot. Every leaf from the plant was harvested and scanned on a leaf area meter (LI-3100, Li-Cor Inc., Lincoln, NE, USA) to measure the total leaf area of the plant. After leaf area measurement, the leaves were transferred to a 100-mm petri-dish. The petri-dishes were covered with aluminum foils to prevent photosynthesis and to promote respiration by blocking light. After 30-min period of dark adaptation, the Petri dishes were placed into a soil flux chamber (6400-09, Li-Cor Inc.) of a gas exchange system (LI-6400XT, Li-Cor Inc.) to measure $CO_2$ efflux of the leaf samples. Three cycles with 405 ppm as a target CO2R concentration were repeated to estimate $CO_2$ flux of the samples, using the built-in measurement function of the instrument. The $CO_2$ release data from the measurements was divided by leaf area to estimate an area-based respiration rate.

Immediately after the $CO_2$ measurements, the leaf samples were excised using a cork borer to produce two 10-mm leaf disks. The leaf disks were transferred to an incubation chamber (OXY043A, Rank Brothers Ltd., Cambridge, UK) of the Clark type $O_2$ electrode (Digital Model 10, Rank Brothers Ltd.). The incubation chamber was filled with 3 mL of fresh 2% $NaHCO_3$ solution per sample and covered with aluminum foil to block light. To assess respiration rates, the consumption of $O_2$ was measured for 5 mins. The leaf disks were then collected and dried at 70°C over 24 hrs to measure dry leaf disk weight. Mass-based respiration rates were adjusted area-based using a previously determined specific leaf area (leaf area/weight).

**In vitro *bacterial gas exchange experiment***

*Microbial respiration measurements as a function of bacterial density*

To quantify endophytic respiration $CO_2$ release, we measured gas exchange of the growth media on Petri dishes where the endophytes were grown under high nutrient condition. The MG/L medium was used to promote fast growth of WP5 bacteria, and their respiratory $CO_2$ release was measured using a gas exchange machine (LI-6400XT, Li-Cor Inc.) equipped with a soil $CO_2$ flux chamber (6400-09, Li-Cor Inc.). A soil $CO_2$ flux chamber was also placed on Petri-dishes on which no bacteria were grown (control). WP5 endophytes were grown, and the quantity was assessed using a standard colony forming unit (CFU) count technique.

We prepared 25 mL of cell suspension culture MG/L broth with and without WP5 in 125-mL flasks. The flasks were shaken on an orbital shaker (DS-500E, VWR International, Radnor, PA, USA) for 5 days. After one day of growth, a 1-mL aliquot of each culture was collected in a microcentrifuge tube to measure optical density of the solution at 600 nm using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, USA). Another 1-mL aliquot was sampled under gnotobiotic conditions and spread on the MG/L plates. After 24 hrs, the Petri-dishes were measured with the aforementioned gas exchange measurement system. Three cycles with 405 ppm as a target CO2R concentration were repeated to estimate $CO_2$ flux of the samples. Five replicated samples were included in statistical analysis.

*Microbial respiration measurements as a function of carbohydrate supply*

A similar *in vitro* assay was designed to observe microbial respiration responses to different concentrations of carbohydrates.

To vary the level of carbohydrates in growing media, the amount of yeast extract and mannitol in MG/L plates was adjusted to 1/16X and 1/4X levels. WP5 were grown in MG/L broth for 5 days and the culture was diluted to $OD_{600}$ = 0.1. One mL of the culture was transferred to prepared 1/16X, 1/4X, and 1X MG/L plates and grown for 24 hrs. The same procedure was used to measure $CO_2$ efflux from the five replicated samples on the plates to determine respiration rates.

### *Quantification of endophytic bacteria* in planta

Combined with the *in vitro* respiration survey described in the previous section, bacterial density measurements of the plant tissue were needed to approximate the microbial respiratory $CO_2$ *in planta*.

For the quantification of endophytic bacteria, a separate experiment was designed and conducted under the conditions identical to those of the *in planta* assay. In short, the surface-sterilized rice seeds were propagated to 1-gal pots filled with horticultural media in the same greenhouse facility. A total of 20 pots were prepared. Half of the seedlings were mock-inoculated and the other half were inoculated with WP5 seven DAG. They were grown under fully irrigated conditions with a low N regime until reaching the panicle initiation stage. Sampling for bacterial extraction was done on 100 DAG.

The bacterial extraction procedure was modified from Knoth et al. (Knoth *et al.* 2014). Five plants were randomly selected and harvested out of a total of 10 pots on site. Leaf, stem, and root tissues were separated and transferred into sterilized 100-mL glass vessels. The samples were moved to a laboratory and washed with a few drops of a Joy detergent in tap water. The vessels containing the washed samples were transferred to a biosafety cabinet for the assay under aseptic conditions. Surface sterilization of the samples was performed by incubating the samples in 0.6% NaOCl for 3 mins and 8 mins for leaf/stem and root tissue, respectively. The samples were rinsed with sterilized DI water four times. Approximately 100 mg of the samples were excised, torn, and transferred to 1.5-mL microcentrifuge tubes. Using a plastic mortar, the torn samples were further crushed and homogenized in 400 μL of NL-CCM solution inside the tubes. One-hundred μL of the mixed plant extracts were evenly spread on MG/L plates. Seventy-two hours later, CFUs of the extracts were counted to quantify the endophytic bacterial density in each plant tissue.

### *Calculation of microbial respiratory $CO_2$ in planta and comparison to photosynthetic $CO_2$ assimilation of the leaves*

*Estimation of microbial respiratory $CO_2$ in planta*

In order to establish our assumption that rice as a host plant provides endophytic bacteria with sufficient carbohydrates, average soluble sugar content (SSC) of the rice sap was collected at

noon and was 6.03°Bx from the same samples collected for bacterial density counts (referred to Rho *et al.* (2018)). We assumed that under favorable conditions for supplying substrate for respiration, carbohydrates are saturated for endophytic respiration in the host. Combined with the *in vitro* bacterial respiration survey data set, the estimates of microbial respiratory $CO_2$ release *in planta* ($R_{mic}$) was determined using the following equation. A more detailed calculation procedure is described in the supplementary section.

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

*Gas exchange measurements for net $CO_2$ assimilation*

The estimates were calculated tissue-by-tissue and compared with the net $CO_2$ assimilation rate ($A$) of the leaves, measured by a portable gas exchange system (LI-6400XT, Li-Cor Inc.), from the same plant host. For the measurements, $CO_2$ concentration, light intensity, and flow rate of a 2-$cm^2$ leaf chamber head (6400-40, Li-Cor Inc.) were set to 400 μmol $mol^{-1}$, 1500 μmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux density, and 300 μmol $mol^{-1}$. Leaf temperature and relative humidity of the chamber were maintained at 20°C and 40-60%, respectively. The leaf samples were clamped onto the chamber head and exposed to the environmental settings above for 4 mins before measurements.

***Statistical analysis***

A standard two sample *t*-test procedure was employed to test statistical significance of the differences in all measures. The blocking effects were removed in the analysis. All statistical analyses were performed using R version 3.2.2 (R Core team 2016).

## Results

*In planta* respiration results showed that E+ plants had a higher rate of $CO_2$ release ($R_c$) and $O_2$ consumption ($R_o$) when measured with a gas exchange measurement system and the Clark type electrode, respectively (**Fig. 1**). However, there was a significant difference in measurements between the two methods ($P < 0.001$). A significant 159% increase in respiration rate for plants inoculated with endophytes was detected when using the $R_o$ method ($P = 0.004$), while a 24% increase observed using the $R_c$ method was not significant ($P = 0.215$).

*In vitro* bacterial respiration rates increase linearly then saturation over the span of the density (**Fig. 2**). At a population size of about $1 \times 10^{18}$ CFU the maximum rate of bacterial respiration was 15 μmol $CO_2$ $m^{-2}$ $s^{-1}$. At inoculation level density ($OD_{600} = 0.1 \approx 1 \times 10^7$ CFU), the respiration rate was about 2.5 μmol $CO_2$ $m^{-2}$ $s^{-1}$.

Moreover, *in vitro* bacterial respiration rates were influenced by the amount of carbohydrate supplies, as shown in **Fig. 3**; there was a linear increase in $CO_2$ release by the bacteria corresponding to the concentrations of carbon compounds in MG/L media ($P = 0.028$). There were no changes in E− pure MG/L control plates. Setting E+ plates at 1/16X MG/L as the baseline for respiration rates, E+ plates on 1/4X and 1X MG/L media showed significant 53% and 113% increases in respiration rates, respectively ($P = 0.092$ and 0.031). From the SSC measurements, it was observed that 1/16X, 1/4X, and 1X MG/L media correspond to 0.13, 0.70, and 2.90°Bx.

Bacterial density of E+ plants was considerably greater than that of E− plants (**Fig. 4**). The endophyte inoculation effect (INOC) was highly significant for the bacterial counts in leaf, stem, and root tissue ($P = 0.001$). In total, there was a 3-fold increase in CFU with endophyte inoculation ($P = 0.011$).

Estimates of microbial respiratory $CO_2$ release showed that a marked amount of $CO_2$ can be released by endophytic microbes (**Fig. 5**). WP5 can produce 23% of the total C assimilated by its host plant during photosynthesis. In this study, the sum of respiration estimates was 3.479 μmol $CO_2$ $m^{-2}$ $s^{-1}$, while the actual photosynthetic assimilation was 14.829 μmol $CO_2$ $m^{-2}$ $s^{-1}$.

## Discussion

The *in vitro* gas exchange measurement data (**Figs. 2 & 3**) support the hypothesis in part that bacterial respiration is quantifiable. Bacterial respiration can potentially contribute to photosynthetic $CO_2$ assimilation when the bacteria are living inside plants. Together with *in planta* gas exchange assay results (**Figs. 1 &4**), these findings also offer a baseline estimation of microbial respiratory $CO_2$ release (**Fig. 5**).

*In planta* respiration is up-regulated upon endophyte inoculation (**Fig. 1**). However, it is not clear whether this is purely due to endophytic microbial respiration or to other impacts on metabolic

processes of the plant in response to endophyte inoculation. Typically, symbiotic plants are reported to have greater respiration rates due to their associated metabolic processes (Baas *et al.* 1989; Lambers *et al.* 2008). Clarifying how much of the increase is attributable to microbial respiration would enable a better understanding of the carbon costs of symbiotic associations. The linearity of the relationship between microbial respiration both microbial density and carbohydrate concentrations as resource availability, was confirmed (**Figs. 2 & 3**). These two *in vitro* characteristics of microbial respiration are biologically straightforward. Bacteria typically display strong attraction to carbohydrates, shown as chemotactic movements (Taiz *et al.* 2010). Thus, it is reasonable to presume that endophytic bacteria can detect and gain carbohydrates anywhere inside the host plant. Since the average SSC of rice sap collected at noon was 6.03$^{o}$Bx and that of 1X MG/L plants was about 2.90$^{o}$Bx, sufficient carbohydrates were always present for endophytic bacteria. These together suggest the host plant as a living habitat could supply sufficient food sources to the endophytes, and in return the micro-organisms could produce a significant amount of $CO_2$ that could be reused by plant photosynthetic assimilation to a degree.

Our hypothesis was originally derived from Bloemen et al. (Bloemen *et al.* 2013b) in which the authors employed a $^{13}C$ technique to estimate how much of the $CO_2$ respired by root tissues could be re-assimilated by photosynthetic processes. They found up to 22% of $CO_2$ transported by xylem transpiration streams was reused by the upper leafy tissues. Likewise, according to Busch et al. (Busch *et al.* 2013) photorespired $CO_2$ in $C_3$ plants can be incorporated into photosynthetic assimilation processes. These findings all provided a firm foundation for our prediction.

Generally, a bacterial microbiota population is estimated to be approximately $1 \times 10^6$-$10^7$ cells $cm^{-2}$ leaf area (Bulgarelli *et al.* 2013). Our microbial count information, as represented by CFU, also showed a similar density range of microorganisms living in host plants (**Fig. 4**). By drawing on our results, we estimated the bacterial respiration in rice plants at 3.479 μmol $CO_2$ $m^{-2}$ $s^{-1}$, which could be a significant contribution to the $CO_2$ assimilation/production cycle in the plants. Plants lose some respired $CO_2$ in root and stem tissue during mass transport, such that only 20% of transported $CO_2$ could be re-assimilated (Bloemen *et al.* 2013a). Yet it is possible to mitigate this inefficiency; an additional 1.461 μmol $CO_2$ $m^{-2}$ $s^{-1}$ can possibly be available through microbial respiration and utilized for photosynthesis in leaves. This is still about 10% of the total

assimilation. The difference in i*n planta* respiration rates by method supports the possibility of the re-assimilation of microbial respiratory $CO_2$ (**Fig. 1**). The difference between the respiration rates of E− and E+ plants by the $R_c$ method (24%) was not as great as that by the $R_o$ method (159%). This indicates that the carbohydrates were being oxidized, but not all the $CO_2$ was being released from E+ plants. It is possible that some portion of the respiratory $CO_2$ could reenter the photosynthetic assimilatory pathways and be incorporated in the plant before being released. This accounts for the decreases in the amount of the $CO_2$ release in E+ plants that was detectable by the $R_c$ method.

However, there are potential drawbacks of the estimates that were established on our assumptions. First, the actual population size of endophytes would be different even during the day considering fast growth and reproduction of bacteria. The measured bacterial density of the plant extracts used in the estimation would not be correct. Second, the carbohydrate supply to endophytes fluctuate as plants have a strong diurnal cycle of the photosynthetic carbohydrate production (Tallman 2004).

Considering these, the respiratory behavior would be dynamic during the day. Endophyte-inoculated rice plants showed faster accumulation of leaves ABA during the afternoon, which seemed to facilitate stomatal closure of the leaves (Rho *et al.* 2018). In the meantime, plants would have more carbohydrates produced by photosynthesis than in the morning, and the amount of the photosynthate would become sufficient for the endophyte respiration. Temporary increases in microbial respiratory $CO_2$ would follow and the possibility of the re-assimilation would increase with the closed stomata. Thus, the afternoon could be the time when the host plant would most benefit from the suggested microbial respiratory responses and interactions.

Further empirical evidence is required to verify this hypothesis. Employing the $^{13}CO_2$ method to differentiate the photo-assimilates would be one way to corroborate this hypothesis.

## Conclusions

*In planta* assays of plant respiration revealed that endophytic bacteria increased respiration rates of host rice plants, while *in vitro* microbial respiration assays confirmed two important fundamental respiratory characteristics of pure WP5 bacteria cultures. Respiration rates of these

microbes are dependent on both the number of bacterial cells in the culture and the concentration of carbohydrates in the growing media. The concentration of carbohydrates present in phloem sap would be sufficient to support endophyte growth. Endophyte inoculation increased the overall bacterial density within the host plant. Together with the *in planta* and *in vitro* survey data, the estimate of microbial respiratory $CO_2$ release in the intercellular spaces of the host plant was approximately 15% of total assimilated $CO_2$.

## Author Contributions

Conceptual development and experimental design: HR, SK. Conduction of experiments: HR. Data analysis: HR, SK. Provision of materials and resources: SD, SK. Article composition: HR, SD, SK.


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## Tables & Figures

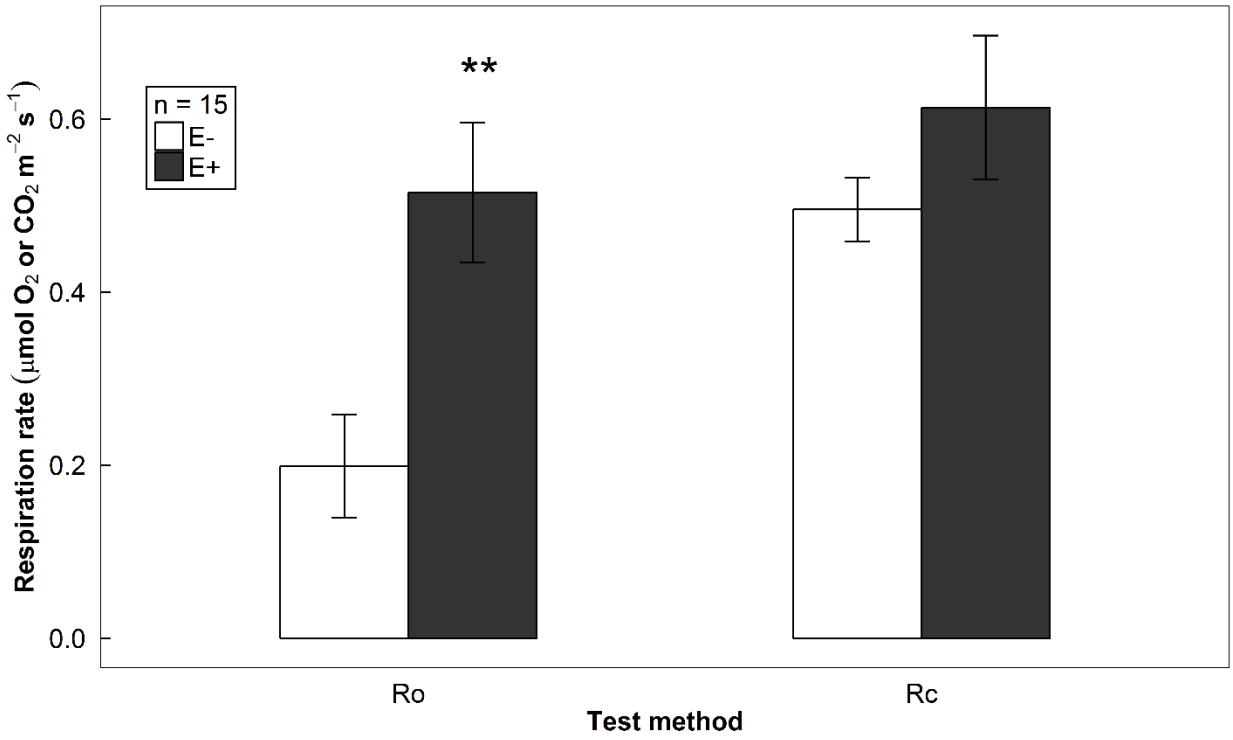


**Fig. 1** *In planta* respiration rates of rice leaves at the panicle initiation stage. Respiration rates were determined by measuring the consumption of $O_2$ (Ro) and the release of $CO_2$ (Rc). Bars present the mean responses of mock-inoculated control (E−, open) and endophyte-inoculated (E+, closed) plants. WP5 (*Rahnella* sp.) was used to inoculate the plant samples. Error bars show ± 1 S.E.M. ($n$ = 15). Asterisks indicate significant difference between E− and E+ plants at the $P < 0.01$ level.

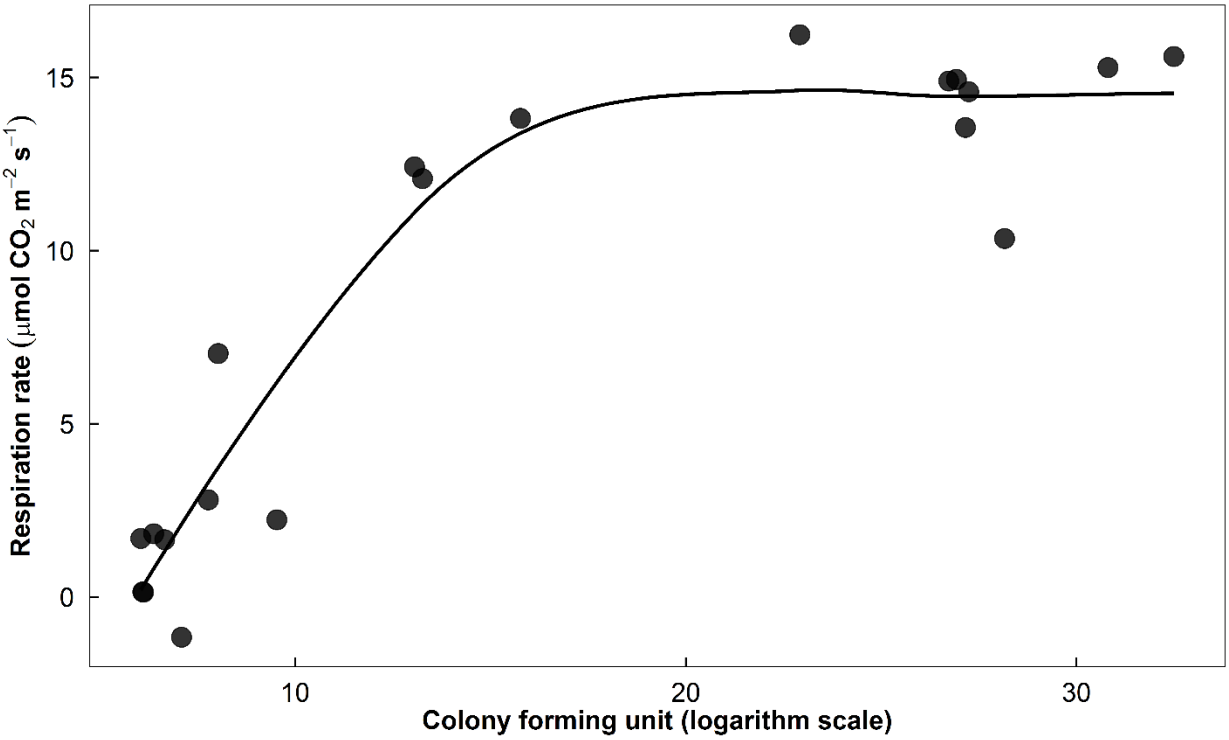


**Fig. 2** *In vitro* respiration rates of WP5 (*Rahnella* sp.) bacteria used in the study over different density of the population ($n$ = 30). The cultured bacteria were grown in MG/L media plates. The bacterial density was measured using a spectrophotometer and converted to the corresponding colony forming unit (CFU).

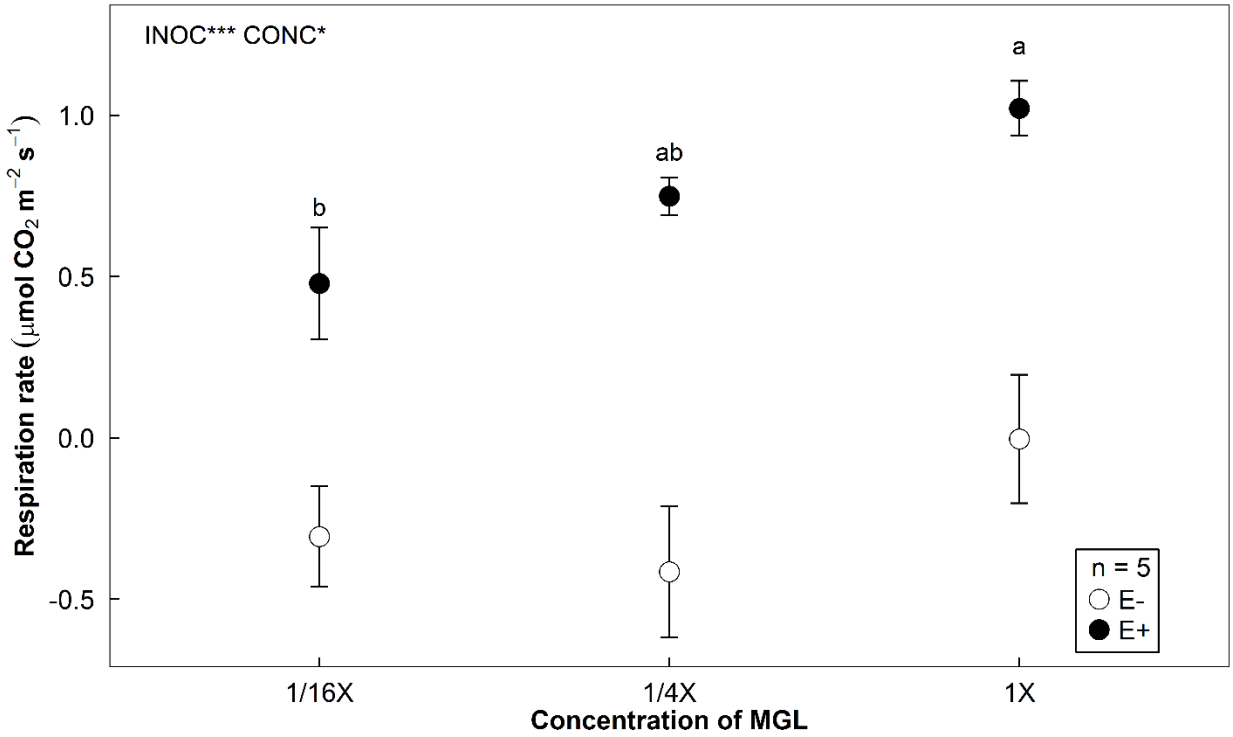


**Fig. 3** *In vitro* respiration rates of WP5 (*Rahnella* sp.) bacteria grown in different concentrations of MG/L media. The carbohydrate contents of MG/L media were adjusted to 1/16X, 1/4X, and 1X. Open symbols show the mean responses of control plates without WP5 (E−) while closed symbols does those of WP5 bacteria-growing plates (E+). Error bars indicate ± 1 S.E.M. ($n$ = 5). Two-way ANOVA results are provided on the top-left side. Asterisks, * and ***, mean significant differences between E− and E+ plates at $P < 0.05$, 0.001 levels, respectively. Letters on the symbols represent significant differences between varying concentrations of MG/L.

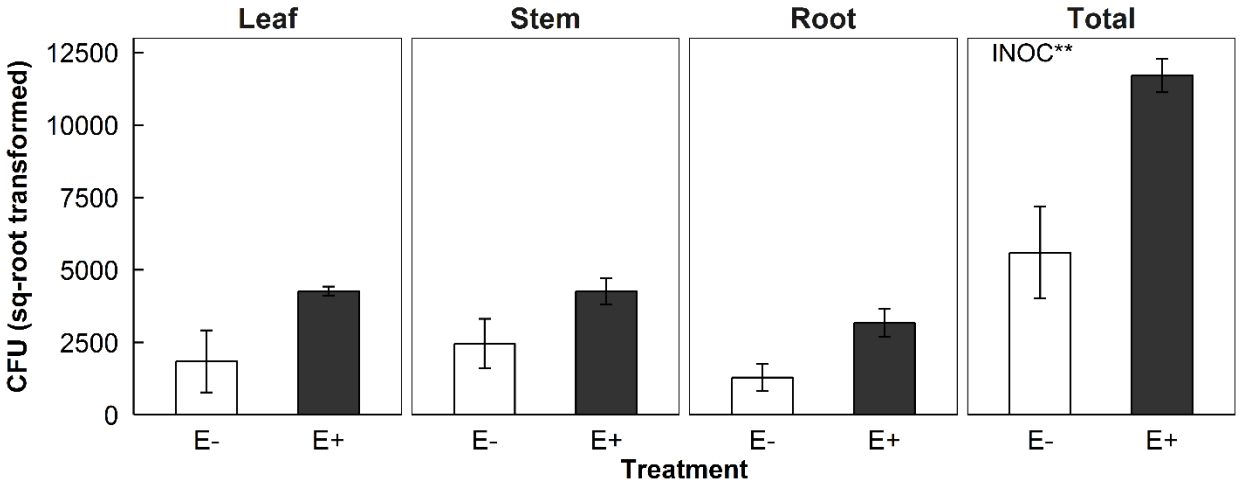


**Fig. 4** Bacterial density of the plant tissue expressed by colony forming unit (CFU). Bars represent the mean responses of mock-inoculated (E-, open) and WP5 (*Rahnella* sp.) endophyte-inoculated (E+, closed) rice plants, respectively. Error bars indicate ± 1 S.E.M. ($n$ = 4). The measured values were subject to the square-root transformation and analyzed using a 2-way ANOVA to examine the effects of endophyte inoculation (INOC), tissue, and interaction between the two. INOC is significant at the $P < 0.01$ level (**).

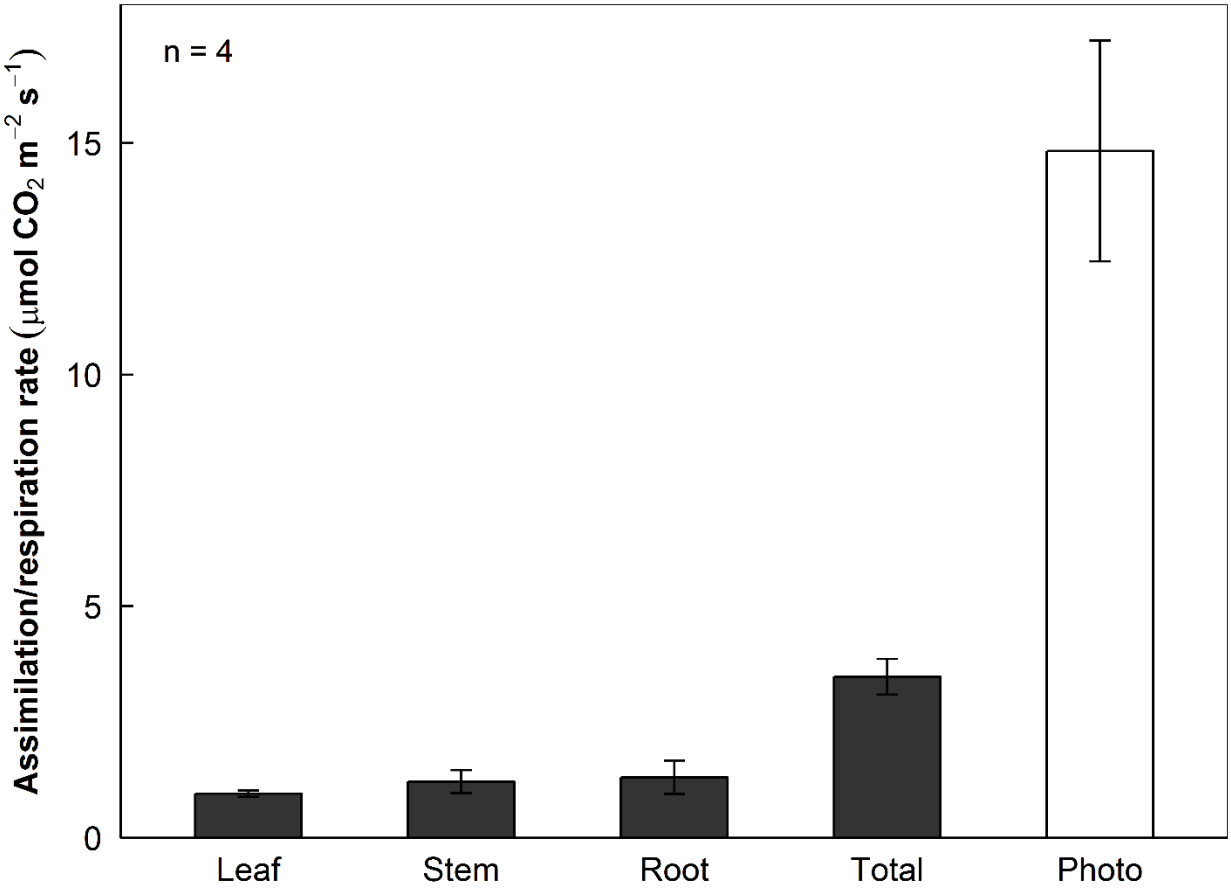


**Fig. 5** Estimates of endophytic microbial respiratory $CO_2$ release *in planta* separated by tissue (Leaf, Stem, Root, and Total, closed bars). The data are compared with photosynthetic $CO_2$ assimilation of the leaves in the main graph (Photo, open bar). Bars indicate the mean responses of WP5 (*Rahnella* sp.) endophyte-inoculated rice plants. Error bars indicate ± 1 S.E.M. (*n* = 4).

# CHAPTER 5 Conclusions and Future Directions

## Conclusions

The overarching goal of the experiments was to evaluate the symbiotic impacts of the Salicaceae endophytes on plant eco-physiology of rice (*O. sativa*) plants and to answer scientific inquiries of plant-microbe interactions. The focus of the investigation into the physiology was carbon metabolism and related water relations. The impacts of endophyte establishment on water relations, photosynthesis, and respiration were subject to experimentation based current knowledge.

The first chapter provides an overview of current understanding of plant-endophyte symbioses. A meta-analysis of endophyte effects on plant biomass gain over 209 articles showed increasing interests in application of this technology over the past two decades. Generalized responses of endophyte inoculation to a variety of host plant species point to the benefits of increased biomass gain under drought, nitrogen-limited, and salinity stress conditions. Even without the stress factors, endophytes increased biomass gain of the plants. Diversifying plant species-endophyte strain combinations in studies would facilitate the utilization of this technology in the field of agriculture. Another meta-analysis over 84 articles was conducted to measure endophytes impacts on plant physiological functions. Photosynthesis-associated parameters – chlorophyll content, maximum photochemical efficiency, and net $CO_2$ assimilation rate – were all improved with endophytes. Water relations-associated functions – stomatal conductance, transpiration rate, and water use efficiency – varied over different host plants inoculated with diverse endophyte strains. It was suggested that these dynamic responses should be interpreted in an experimental context-dependent way. Overall, the literature imparts positive impacts of endophytes on plant fitness and physiology.

The second chapter elucidated alterations of water relations and water use efficiency in rice with endophyte inoculations. From *in vitro* characterization and *in planta* assays in the prior studies, Salicaceae endophytes showed several potentially advantageous traits to host plants. This includes production of ABA by select Salicaceae endophytes. ABA is a phytohormone that plays a crucial role in governing stomatal responses to the environment. It was hypothesized that the host plant can capitalize on this potential symbiotic trait from the endophytes. A diurnal

examination of stomatal conductance showed endophyte-inoculated rice plants displayed decreased stomatal conductance in the afternoon. The decreases in stomatal conductance were correlated with the increases in ABA accumulation during the day in the inoculated rice leaves. Also, endophyte-inoculated rice plants had lower stomatal density. These led to decreases in transpiration and further water consumption over the growing period. In the meanwhile, photosynthetic capacity of the inoculated plants was maintained, and biomass did not change. As a result, endophyte inoculation significantly enhanced water use efficiency in rice.

The third chapter elaborated photosynthetic improvements of rice with endophyte inoculations. $C_3$ plants experience photosynthetic down-regulation or acclimation in response to long-term exposure to atmospheric elevated $CO_2$. The features of the down-regulation include a reduction of Rubisco capacity, a diminution of photosynthetic enzyme activities, which leads to lowering apparent $CO_2$ assimilation rates, and further decreases in biomass gain of $C_3$ plants. Nitrogen limitation is known to accelerate this response. It was speculated that Salicaceae endophytes would mitigate the down-regulation due to their ability to biologically fix atmospheric di-nitrogen similarly to nodule-forming symbiotic rhizobia in legumes. $CO_2$ response curves of endophyte-inoculated rice plants displayed the mitigation of photosynthetic down-regulation under elevated $CO_2$. Biochemical properties of photosynthetic enzymes were all improved by endophyte inoculations under elevated $CO_2$. In addition, internal $CO_2$ diffusion at the site of carboxylation was facilitated in the inoculated plants grown under elevated $CO_2$ conditions. Subsequent increases in water use efficiency were detected in the inoculated plants. The improvements were more substantial under nitrogen limited conditions.

Finally, the last chapter explored the possibility of re-assimilation of endophytic microbial respiratory $CO_2$. This chapter ties up the second and the third chapters in a way to provide an explanation of the increases in water use efficiency by not losing photosynthetic $CO_2$ assimilation capacity while closing stomata. It is well-established hypothesis in the literature that respiratory or even photorespiratory $CO_2$ can reenter to the photosynthetic $CO_2$ assimilation pathways. Endophyte inoculations increased respiration rates of the host plant. *In vitro* characterization of microbial respiration showed microbial respiration rates were positively correlated to the number of microbial cells and the carbohydrate supplies to the microbes. Bacterial density of the inoculated plant tissues was significantly greater than that of the control

plant tissues. Assuming the carbohydrate supplies from the host plant to the endophytes are saturating, the estimate of microbial respiratory $CO_2$ in the plant was about 15% of the $CO_2$ assimilated by photosynthetic pathways. This amount of $CO_2$ could be sufficient to compensate for the less uptake of the atmospheric $CO_2$ by stomatal closure triggered by endophytes.

## Limitations of the Study and Future Improvements

Many areas of the experiments conducted in the study could have been improved if the following elements had all been considered, strengthening the interpretations of the results.

### *Unrealistic sterile control plants*

Experimental controls do not represent plants in natural conditions. Therefore, careful attention is required in interpreting the results of the study when considering application in an agricultural field. This is totally due to the nature of the study on plant-microbe interactions. To better compare and so highlight the treatment effects of endophyte inoculations, the control plants were constructed by sterilizing the seed surfaces and reducing any possible encounters with other microbes in experimental systems. In natural conditions, however, plants interact with a plethora of microbes in either the rhizosphere or the atmosphere. On top of that, microbes themselves interact with other microbes, and these comparative interactions might affect host plants' physiology in an unexpected way. It is implausible to assume that pure sterile controls exist in agricultural ecosystems. This provides a foundation to believe that the treatment effects would have been overestimated.

### *Seasonal variability of the responses*

There were significant variations in the responses between the plants grown in different growing seasons. These deferred plant assays and framed limited timelines of the project. Even in the same rice-Salicaceae endophytes model experimental system, plant-endophyte interactions as measured by physiological benefits or symptoms were various. Most of the results shown in the present study were obtained from experiments conducted in spring or summer growing seasons. Those from fall or winter growing seasons did not produce quality data to analyze, and even negative effects of endophyte treatments were observed. The main reason for the huge variation is probably the differences in light intensity among the seasons, affecting the amount of photosynthates created by the host plants. The aggregate of photosynthates as a carbon pool, in

turn, affects the supplies of carbohydrates to endophytes in the plants, which might determine their capacity to provide symbiotic functions to the host. Even with the supplementary lighting system in the greenhouse, due to the weather conditions in Seattle – scattered sunlight and many overcast days plants – do not grow at their full potential. This point on light intensity is delivered in part through a discussion in Chapter 2.

***Limited available resources***

In the present study, we used only one host species with multiple strains of bacteria/yeast endophytes due to limitations of resources. Rice was used as a model $C_3$ crop and the best performing strains of Salicaceae endophytes, the *nifH* mix, were used. Bearing host specificities of plant-microbe interactions in mind, this would limit the extrapolations of the study in the context of application in agriculture. If resources were available, there would been more space to conduct experiments using more diverse host species, which would reinforce conclusions of the study.

Regarding the findings and the limitations in Chapter 2, further investigation should be conducted to test the endophyte-produced ABA effects on stomata at the biochemical and molecular scales. Gene expression analysis of host plants associated with ABA biosynthesis will be required to tease apart the endophyte producing ABA *in planta* and the plant producing ABA stimulated by endophyte inoculation. A mutant analysis will also be useful. Genomic analysis of the ABA production pathways of endophytes will accelerate the construction of ABA-mutant strains. Together, we will be able to test the importance of ABA biosynthesis by the endophyte strains for the observed impacts on the plants.

The physical characteristics – stomatal density – of the leaves affected by the ABA-producing endophytes was partly investigated. However, more evidence should be provided. A follow-up analysis of the relationship between the decreased stomatal density and the decreased stomatal conductance, and their relationship to the fast ABA accumulation in the inoculated plants will provide a clear understanding of the structural alteration. Stomatal index, the size of stomata and physical measurements of the aperture will be points of future investigation.

Although Chapter 4 explored the possibility of re-assimilation of $CO_2$ produced by endophytes inside the host plants by estimating microbial respiration, this remains as a preliminary

assessment. Further experimental approach will be required to confirm the findings and to add significance to the study. If resources – instruments and time – were allowed, this could have been possible in the project timeline. As the re-assimilation of microbial respiratory hypothesis is a key to bridge the water relations (Chapter 2) and the photosynthesis (Chapter 3) responses of the plants to endophytes through the control of stomatal reactions, a closer investigation will uncover a mechanistic layer of the interplay between the host and the endo-symbionts. The recent advance of isotope tracking technology such as NanoSIMS would be a tool that allows this investigation (Ceh *et al.* 2013).

## Future Directions

This dissertation project has posed many interesting points of future investigation into plant-microbe symbiotic interactions. Based on the findings from the present study, further research will focus on forming a better mechanistic understanding of the interactions. This will facilitate a translation of the interactions into a field scale application in the end, which should be set as an ultimate aim of the research.

### *Source-sink dynamics of plant-microbe symbiotic interactions*

Plant-endophyte interactions vary depending on environmental conditions (Schulz & Boyle 2005). The variability determines the effects of endophytes on the host plant physiology. I assume the reason for the variability comes from the production of photosynthates, as they are the sources of the exchange currency between the host and the symbionts. Thus, understanding of source-sink dynamics on the interactions stands its importance in discovering underlying mechanics.

The plant photosynthetic responses to endophyte inoculation differed among the environmental conditions that might alter source-sink relationships such as low temperature, N supplies, elevated [$CO_2$], and long/short day conditions (**Fig. 1**). The dynamic responses of plants to endophytes due to altered source-sink relationships involve changes in leaf gas exchange and photochemical properties including electron transport rate of the light harvesting complexes, photosynthetic net $CO_2$ assimilation rate, stomatal and mesophyll conductance, and the resulting water use efficiency as shown through Chapters 2 and 3. I suppose these sets of responses are coupled with the C supply from source leaves to endophytes. Therefore, the leaf physiological

changes with endophytes relate to any environmental cues that might affect instant photosynthetic capacities of the plants. For example, light intensity or daily irradiance determines how many photosynthates are produced during the day. In turn, these fill up the C pool of the plants and C loading/unloading allocation starts in order to provide resources to build new biomass in sink tissue. This is the point at which, I suppose, the interruption of endophytes is engaged. If there are a good amount of sugars, the endosymbionts will get more actively involved in trafficking the food. As a consequence, the microorganisms will produce more $CO_2$ molecules via respiration as demonstrated in Chapter 4. I presented and discussed the endophyte effects on the decreases in water consumption through stomatal closure in response to the increases in integral light intensities over the growing period from three different experiments in Chapter 2.

***Cost-benefit analysis of plant-endophyte symbiosis***

To comprehend the interactions from the plant physiological perspective, a cost-benefit approach of the symbiosis would also be advantageous in a mechanistic study. Research on sink-source relationships with an emphasis on C and N metabolisms would provide a useful insight (White *et al.* 2016). In this respect, C and N relations of the plants would be a key to interpret plant-microbe interactions (**Fig. 2**). Therefore, plant-diazotrophic endophyte interactions serve as an ideal platform of the future research on the cost-benefit aspect of plant symbioses. This is because the amount of biologically fixed nitrogen forwarded to the plant host represents the quantifiable benefits of the symbiotic interactions in exchange for the quantifiable costs of carbohydrate.

Carbohydrates are central for the relationships between host plants and microbial symbionts. The demand/supply of carbohydrates between those two entities portray the interactions of plant-endophyte associations. To unlock the fundamental interactive mechanics, therefore, understanding carbohydrate metabolisms with emphasis on photosynthetic C assimilation can be crucial. Carbon assimilation processes then can be determined by the demand/supply of $CO_2$ from/to the site of carboxylation. The demand can be determined by source-sink balance and the supply can be determined by the diffusion of $CO_2$ which are actively governed by stomatal opening/closure or other sources of internal $CO_2$. Endophytes can possibly impact these two aspects by acting as lively biological sinks and by producing respiratory $CO_2$ consuming

carbohydrates for their diet. To uncover both ends, estimation of the C cost/benefit of hosting endophytes in plants requires imminent investigation. This C exchange can be affected by the source-sink balance of plants, which is a potential key to measure the symbiotic effects of endophytes on plant physiology.

In return, the benefits of the symbiosis can be represented by the N supply through biological N fixation by diazotrophic endophytes. An example of this trade-off approach can be found in plant-mycorrhizae symbiosis. Researchers use the trade-off concept to interpret the exchanges of C and P in the model plant-mycorrhizae system. Likewise, the symbiotic benefits can be substituted with the amount of N supplied to the host plant in exchange for a unit C consumed by the symbionts. This tightens the connections between the source-sink relationship and the C:N ratio of the plants.

While bearing these two perspectives in mind, molecular studies to delineate mechanisms underlying the controls associated with C and N metabolisms and stomatal reactions should be followed to outline details of the interactions. Together, we will be able to conclude the reasons for improvement of water use efficiency in host plants with endophytes that were found in the present examinations described in Chapters 2 and 3.

***Applications to agriculture***

Modern agriculture is heavily dependent on resource intensive, non-sustainable agricultural practices and tools. Farmers have been using a tons of N fertilizers in agricultural ecosystems, which may have made crops lose symbiotic associations in relation to N uptake. Breeding programs have aimed to select varieties that are more responsive to N fertilizers, which may reduce the ability of crops to naturally host symbionts over time. Likewise, all resource intensive agriculture practices may have made many crops detached from symbiotic associations.

A study demonstrated significant differences in structure, variation, and assembly of soil microbial diversity between organic farms and conventional farms (Edwards *et al.* 2015). This highlights that intensive agricultural practices would reduce the chances of crops' encounters with fluent and healthy mutualists from the rhizosphere in the field. Only not resource-superfluous and sustainable environment would guarantee the recovery of using plant partnerships.

With sustainability in mind, utilizing the current knowledge about plant-microbial interactions can provide a unique means to mitigate climate change impacts on agricultural ecosystems. It should be set as a long-term goal of this subject area. Using beneficial microbes in many areas of agriculture will transform the industry. This includes biocontrol of pathogens by microbes, use of biofertilizer, enhancement of plant stress tolerance by symbionts, and breeding for the ability to host symbionts. This should be a gradual change, not a rapid movement.

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## Tables & Figures

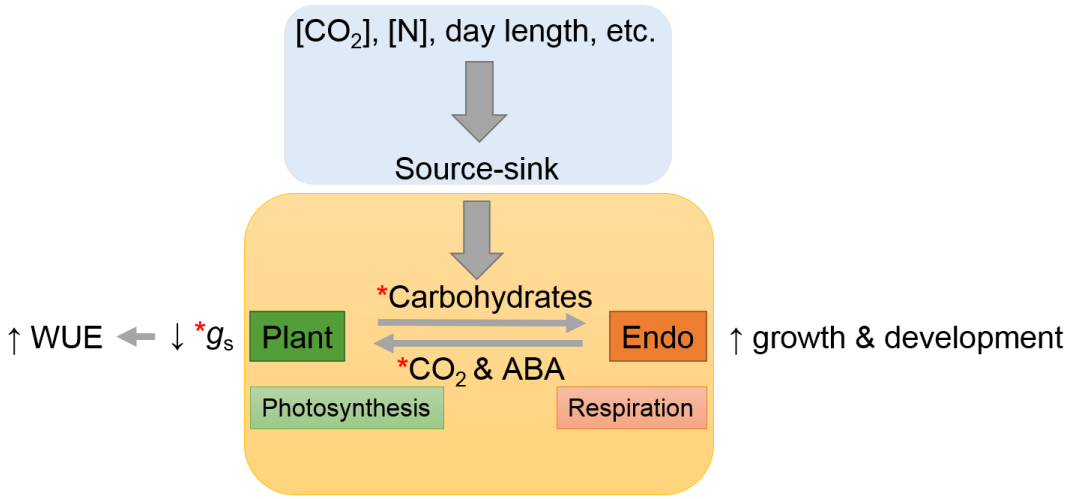


**Fig. 1** A schematic diagram of the proposed future study areas. The scope of the future study should be on the source-sink relationships affecting the symbiotic interactions to uncover a mechanistic layer of the responses found in the current study.

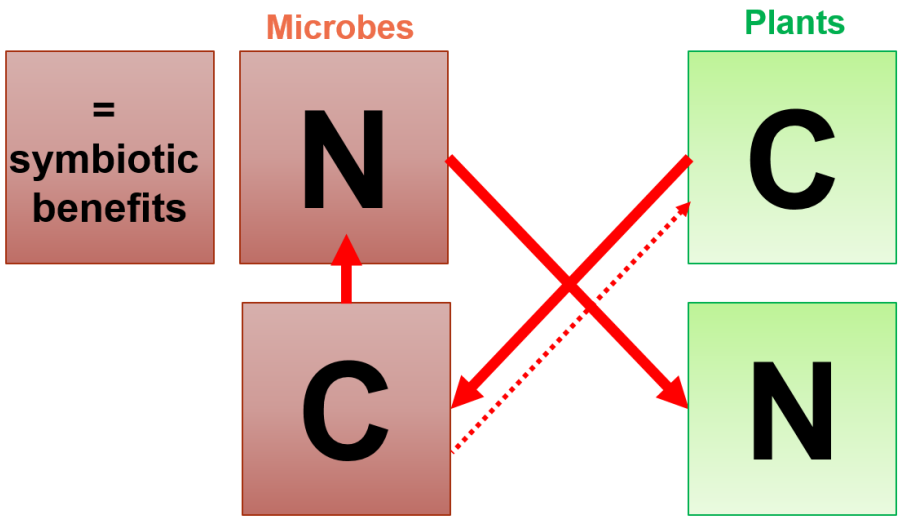


**Fig. 2** Interactions between plants and microbes in relation to C and N metabolisms of each. Understanding C and N relations of the plant hosting symbiotic microbes would find its importance in the cost-benefit analysis of the symbiotic microbial interactions.

**END OF TEXT**