PLANT MICROBE INTERACTIONS



# **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<sub>4</sub> species gained more biomass than monocots and C<sub>3</sub> 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.

**Keywords** Bacteria/fungi/yeast · Drought/nitrogen/salinity stress · Effect size · Endophytes · Meta-analysis · Plant biomass

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## 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 micro-organisms is known as endophytes [1]. 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 [2].

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 [3–7]. 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 [8]. 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.

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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 [9–11]. 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 (http://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 [12] 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 [13]. 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 [14]:

$$d = \frac{\overline{X}^T - \overline{X}^C}{S} J \tag{1}$$

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

where  $\overline{X}^T$  and  $\overline{X}^C$  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.

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

$$95\% \text{CI} = 1.96 \times \sqrt{Vg} \tag{4}$$

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. benefits (or harmful effects) to the host

The strain used most in the analysis

The range of inoculum density used in the studies

Table 1 General statistical Category Overall Individual statistics information from the articles statistics (drought, nitrogen, salt) found on the SCOPUS (www. scopus.com) database about the The number of articles found in the search results 209 53, 121, 35 endophyte effects on plant fitness under drought, nitrogen, and salt The number of articles that met the selection criteria 108 30, 44, 34 stress conditions (as of October The number of articles actually used in the analysis 84 23, 37, 24 2016) The number of datasets analyzed 326 49, 125, 152 The endophyte genus that conferred maximum *Penicillium* (on *Cucumis sativas*, d = 26.89) benefits to the host *Neotyphodium* (on *Lolium perenne*, d = -0.830) The endophyte genus that conferred minimum

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 Electronic Supplementary Materials

The reciprocals of *Vds* 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:

$$M = \frac{\sum WY}{\sum W} \tag{5}$$

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

The SE of M (SEM) is,

$$SEM = \sqrt{\frac{1}{\Sigma W}}$$
(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 ( $n_{\rm fs}$ ) in order to estimate publication bias using "metafor" package in R [15]. If  $n_{\rm fs}$  is over 5n + 10, it is considered to be safe to ignore publication bias as described in Rosenberg [16], 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 with woody species, while group 2 did those on crops with non-crop species. Eudicot vs. monocot and  $C_3$  vs.  $C_4$  comparisons were conducted in groups 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.

 $2.0 \times 10^{5}$ - $1.0 \times 10^{9}$  CFU/mL inocula

Neotyphodium sp. (count, 7)

#### 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 with 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.

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



Most of (85%) the studies used single-strain inocula compared with 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 USA 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 one hundred eight searched articles had an incomplete experimental design (data not shown). For those completely designed studies, a paired t test to compare nonstressed 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 seven studies—followed by *Epichloe* and *Pseudomonas* with six 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; 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



**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

stress conditions. There was no publication bias in the cumulative endophyte effects under all three stress factors. The failsafety numbers of the drought/nitrogen/salinity stress cases were 989/9805/88,586, which were all greater than the criteria



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  $\pm 95\%$  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 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. \*\*P < 0.01, level of significance in the overall effect size panels showing paired *t* test results of endophyte effects under non-stressed vs. stressed conditions

(255/630/770). Even under non-stressed conditions from the same studies analyzed, the numbers were higher than these criteria (708/1730/3928).

Despite the smaller sample sizes, eudicot species (n = 4) in the category group 3 and C<sub>4</sub> species (n = 7) in group 4 under drought conditions showed superior performance



**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 Electronic supplementary materials (Fig. S1, "endo\_host\_heatmap.html"). The numbers of the endophyte strains and the host species examined were 94 and 42, respectively

when inoculated with endophytes according to their cumulative effect sizes (d = 4.697 and 5.091, respectively; groups 3 and 4—left panels in Fig. 3). Likewise, C<sub>4</sub> plants under salt stress conditions showed a greater effect size (d = 2.271; group 4—right panel in Fig. 3) than C<sub>3</sub> 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 with 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 were 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 greater 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 Z. 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 Electronic 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 3948 (endophyte strain  $\times$  plant species = 94  $\times$  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 metaanalysis 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. [17] 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 [18, 19] and elucidation of the mechanisms of molecular communication between the hosts and the endophytes [20, 21].

Compared with 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 [22] and have been successfully inoculated into a range of other host species [23-26]. 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 [10]. Unlike fungal and bacteria strains, only a few studies (6%, Fig. 2a) examined yeast endophytes for their ability to confer stress tolerance [24, 25].

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 [27] (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 metaanalytic reports [9–11]. 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<sub>3</sub> vs. C<sub>4</sub> comparison (Fig. 3). That is, C<sub>4</sub> plants benefited more by having endo-symbionts under drought and salinity stress conditions than C<sub>3</sub> plants did. C<sub>4</sub> species inherently have higher water use efficiency (WUE) than C<sub>3</sub> species through the specialized photosynthetic pathway [28]. 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. [29]. 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 [30], whereas endophytes do rather by providing phytohormones and inducing the defense related secondary metabolisms while residing in the plants [6]. 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 [29, 31]. 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., [19, 32, 33]) 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 3 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 [23, 34–37]. As Newsham [9] 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) [37–43]. Similarly, Nadeem et al. [44] 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 hostmicrobe 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 [45-50]. 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 anti-oxidant 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 [5, 49, 51–55]. 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 [56]. 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 also for restoring endangered species in native habitats as well [57, 58]. To meet this demand, developing an efficient screening tool for endophyte impacts on plants [59] 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. [23] 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 with this study, Kandel et al. [26] 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 with 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. Acknowledgments We gratefully acknowledge our previous and current graduate students in the lab at the time this meta-analysis was being prepared for publication: Matthew Flora-Tostado, Hannah Kinmonth-Schultz, Marlies Kovenock, Jennifer Hsiao, and Kyungdahm Yun. The USDA-NIFA grant 2012-68002-19824 supported this study.

Author Contributions HR and SHK conceived the idea. HR, MH, SLK, and JC designed the analysis and collected the data. HR performed the analysis. SLD and SHK provided materials and resources. HR, MH, SLK, SLD, and SHK wrote the paper.

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