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Hardening Blueberry Plants to Face Drought and Cold Events by the Application of Fungal Endophytes

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Abstract: Harsh environmental conditions derived from current climate change trends are among the main challenges for agricultural production worldwide. In the Mediterranean climatic region of central Chile, sudden occurrence of spring cold temperatures in combination with water shortage for irrigation (drought) constitutes a major limitation to highbush blueberry (*Vaccinium corymbosum*) plantations, as flowering and fruiting stages are highly sensitive. Hardening crops may be achievable by boosting beneficial interactions of plants with microorganisms. Inoculation with symbiotic fungi isolated from plants adapted to extreme environments could be a good strategy, if they are able to maintain functional roles with non-original hosts. Here, we evaluated the effect of two Antarctic fungal endophytes (AFE), *Penicillium rubens* and *P. bialowienzense*, on the tolerance of *V. corymbosum* plants to cold events in combination with drought under controlled conditions. Inoculated and uninoculated plants were exposed for a month to one event of a cold temperature (2 °C/8 h) per week with or without drought and were evaluated in physiological, biochemical, and molecular variables. A complementary set of plants was kept under the same environmental conditions for two additional months to evaluate survival as well as fruit weight and size. There was an overall positive effect of AFE on plant performance in both environmental conditions. Endophyte-inoculated plants exhibited higher gene expression of the Late Embryogenesis Abundant protein (LEA1), higher photochemical efficiency (Fv/Fm), and low oxidative stress (TBARS) than uninoculated counterparts. On the other hand, plant survival was positively affected by the presence of fungal endophytes. Similarly, fruit diameter and fruit fresh weight were improved by fungal inoculation, being this difference higher under well-watered condition. Inoculating plants with fungal endophytes isolated from extreme environments represents a promising alternative for hardening crops. This is especially relevant nowadays since agriculture is confronting great environmental uncertainties and difficulties which could became worse in the near future due to climate change.

Keywords: plant-microorganisms interaction; water deficit; cold-stress; functional symbiosis

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1. Introduction

Most modern crop plants have been developed for growing under environmental conditions with high level of resources and low overall stress. However, as global climate change is associated with great climatic variability [1,2], crops are facing multiple stressful events at different stages of their production cycle [3,4]. Moreover, climate change models predict that some areas, such as Mediterranean Chile, would be subjected to reduced precipitations and increased temperatures, especially during the summers [5]. In addition to changes in rainfall and temperature, an increase in the frequency of extreme climatic events such as drought, flooding, heavy snowfalls, and peaks of extreme temperatures is also predicted [5]. In the last decade, for example, ecosystems of central Chile have been experiencing what has been considered a mega-drought [6]. These extreme events can have negative impacts on agriculture that can be even more harmful than the effects of gradual climate change [7]. The economic losses can scale up as the negative consequences of such stressful scenarios may not only impact crop yield (quantity and quality of the harvestable product) but also plant survival. Together with the increasing societal demand for environmentally friendly strategies, the use of bio-inputs (e.g., biopesticides, bio-fertilizers, growth-promoting endophytes) is proposed as a promising strategy for a new agriculture [8–11].

Symbiotic microorganisms are usually associated with improved performance in plants, a characteristic that seems to be more evident under stressful conditions such as drought, osmotic stress, and/or extreme temperatures [12–15]. However, great variation in the outcome of the symbiosis suggests that there are underlying shaping factors in each type of interaction that may depend on the evolutionary origin of the participating organisms and current environmental condition [16,17]. For example, class II fungal endophytes, broad-range plant-colonizing symbionts, can confer benefits in a habitat-adapted fashion [18]. That is, microorganisms isolated from a particular habitat characterized by the incidence of a given stress factors are expected to confer tolerance to that factor independently of the host species [10,18,19]. In fact, certain fungal endophytes can be isolated from plants living in harsh environments where more than one factor of abiotic stress co-occur such as is the case for drought and cold in Antarctica [15,20]. This poses a situation where the possibility of manipulating endophytic fungi can be used to extend the benefits on crop plants not just for one, but for two stress factors [21].

Root endophytes isolated from Antarctic plants may represent one of these cases as they live in a very harsh environment characterized, as mentioned above, by extremely low temperature and drought (note that most of the water is frozen and not available for plants) [22]. For example, experimental manipulation of Antarctic root endophytes has shown to improve the performance of the native plants *Deschampsia antarctica* and *Colobanthus quitensis* to cold, drought, and salinity when studied independently [20,23,24]. These positive effects have been also observed when those endophytes were inoculated in non-naturally occurring host species like *Notofagus alesandrii*, *Lactuca sativa*, *Solanum lycopersicum*, and *Capsicum annuum* [14,15,25,26]. Several reviews point out to an endophyte-improved antioxidant machinery and production of secondary metabolites with osmotic effects (e.g., proline) as the underlying mechanism [12,14,17]. Hence, it may be possible to improve plant responses to multiple stress factors in different crop species through the isolation and inoculation of mutualistic fungi, establishing novel symbiotic interactions between this kind of microorganisms and alternative plant-hosts [14,27].

We took this approach to assess the effect of Antarctic root endophytes in the response of highbush blueberry plants (*Vaccinum corymbosum* L.) to production scenarios characterized by cold temperature and drought. The environmental incidence of those factors has been associated with important production and economic losses in blueberry [28–30]. The shallow root system characteristic of this species compromises its ability for nutrient acquisition and makes it particularly sensible to drought stress [28]. In addition, despite the fact that blueberry plants can tolerate below-zero temperatures when dormant (in winter); during spring, even near-zero temperatures may inflict several injuries to the recently emerged reproductive organs [29,31,32]. Thus, the co-occurrence of these stress factors (i.e., cold and water deficit) can cause serious damage in blueberry plantations—especially if plants are at early flowering or fructification stage [32,33]. Besides the general warming trend and overall reduction in precipitations in some areas of the world [5], global change models predict a higher climatic variability with peaks of very low and very high temperatures in the cold and warm seasons, respectively [34]. Thus, more frequent—but unpredictable—cold events are expected to occur in late spring [35,36], which accompanied by a reduced availability of soil water, can cause damage in plant productions. This complex scenario for crop production is already happening in the Mediterranean region of Central Chile where farmers are having to deal with dry winters and high temperature variability during the growing season. Thus, most Chilean fruit farmers are now having to produce with less water and an unpredictable thermal environment [37].

In this study, we evaluated the effects of inoculating two fungal endophytes isolated from Antarctic plants on the tolerance responses of *V. corymbosum* to events of cold-shock stress under different scenarios of water availability. In real conditions of blueberry plantations, plants can be suddenly exposed to near-zero temperatures as well as different levels of water deficit which, depending on the magnitude, can impair the fruit quality and even cause plant mortality. Complementarily, two common symbionts of the Antarctic species *Colobanthus quitensis* (Caryophyllaceae) and *Deschampsia antarctica* (Poaceae), the endophytic fungi *Penicillium rubens* and *Penicillium bialowienzense* (see [25] for taxonomic redundancies), have been found to improve the performance of native and alternative (non-naturally occurring) plant-hosts [20,25], including crops [14,15,26]. Based on these antecedents and considering that functional roles of plant microbial symbionts depend on the environmental conditions of their original habitat [19], we predicted that the Antarctic fungal endophytes will improve the tolerance of *V. corymbosum* to cold and water stress. However, their relevance for the plant performance will be more evident under higher abiotic stress condition (e.g., drought). To evaluate this, we carried out a growth chamber experiment to assess the effects of a fungal endophytes on the physiological, biochemical, and molecular responses of blueberry plants under contrasting context of stress. Since there is an ever-increasing trend to environmentally friendly bio-inputs as a strategy in agriculture, we also evaluated the endophyte effects on aspects of harvestable fruit quality (size and weight).

2. Materials and Methods

2.1. Plant and Fungal Origin and Preparation

One hundred and forty 2-year-old highbush blueberry (*Vaccinium corymbosum* cv. Brigitta) plants were used in this study. Individuals were obtained from clonal propagation and from local certified producers, trying to ensure—as much as possible—a genetic and phenotypic homogeneity. In regard to the Antarctic fungal endophyte inoculum, it proceeded from fresh root tissues of *C. quitensis* collected at King George Island (South Shetland Archipelago) during the Antarctic growing season of 2015–2016, whose isolated cultures are now routinely maintained in the laboratory of Integrative Ecology at Universidad de Talca (Chile). For this experiment, fresh inoculums of both strains (*Penicillium rubens* and *Penicillium bialowienzense*) were obtained from single-conidia of fungal endophytes cultured on potato dextrose agar (PDA) medium diluted eight times, supplemented with 50 mg/mL of streptomycin, and incubated at 22 ± 2 °C with a photoperiod 14/10 h (day/night). After two weeks of incubation, conidia were harvested from plates by adding 10 mL of sterile water and gently scraping off the surface with a sterile glass slide. Conidia concentration was estimated by using a Neubauer chamber and adjusted to 1×10^5 conidia/mL. Mean conidia viability was >95% as determined following the methodology described by [38].

2.2. Experimental Design

We conducted two parallel experiments involving cold stress and soil water deficit, to study the effect of inoculation of Antarctic fungal endophytes (AFE) on the capacity of blueberry plants to grow and produce under these stressful conditions. In the first one, we monitored blueberry responses at the genetic, biochemical, and physiological level after four weeks of combined stress. Fruit development and plant survival was also assessed in a second set of plants after eight weeks of stress.

All the 140 plants were grown outdoors individually in 2 L plastic pots filled with a non-sterilized commercial potting substrate, without water deficit or plagues attack. Half of the plants ($n = 70$) were inoculated with the Antarctic root-fungal endophytes (AFE+) by incorporating in their watering a solution of fungal spores, while the other half were not (AFE−). Inoculation was confirmed using a light microscope in a subsample of roots (~10% of the experimental individuals) one week after inoculation and at the end of the first experiment (four weeks). Complementarily, through PCR detection on a subset of plants (2 per treatment/chamber) using specific primers for the referred fungal strains, we verified that all the evaluated plants effectively represented their symbiotic state (AFE+ or AFE−). Ten individuals per treatment were involved in the validation process, for which we ended with 60 plants in each inoculation group. Since the substrate on which plants were growing was not sterile, we cannot rule out other fungi were already there interacting with the plant roots.

Once the fructification process was started (mid-October 2017), all plants (i.e., Antarctic fungal endophytes added (AFE+) and Antarctic fungal endophytes absent (AFE−)) were exposed to cold-stress (8 h once per week at 2 °C overnight) to mimic abrupt spring cold events. Furthermore, to evaluate the additive effect of water deficit on the cold-stressed plants, half of the AFE+ plants and half of the AFE− plants ($n = 30$) were exposed to one of these two watering conditions: without water deficit (100% of field capacity; Cold/W+) or with water deficit (~55% of field capacity; Cold/W). Besides the weekly chilling night, common for all plants, temperature was maintained constant at 25° C the rest of the time. The two water contents in pots were achieved by watering every three days with 550 mL for the field capacity treatment or with 300 mL for the water deficit treatment, as determined in a previous calibration [39]. Ten plants from every treatment were randomly assigned to one of three blocks; each block consisted of an automated growth chamber (Bioref 38 L, PITEC, Chile) with a photosynthetic photon flux density (PPFD) of 371 μmol m^{-2} s^{-1} under a 16/8 h of day/night photoperiod. To avoid potential chamber effects, all plants of a given block were moved to a different chamber periodically as well as plant positions within the chamber were changed daily. Of the ten plants per treatment in each block, five were used to measure biochemical and physiological variables after one month, while the other five were maintained one additional month under the same conditions to characterize their survival and fruit development (size and weight).

2.3. Effect of the Inoculation on Blueberry Stress-Responses

Overall eco-physiological plant performance was assessed by estimating the photochemical efficiency through chlorophyll fluorescence parameters (maximum quantum yield of photosystem II [PSII], Fv/Fm), which has been widely used to characterize the response to stress in different plant species (e.g., [40]). All measurements were carried out at midday using a portable pulse-amplitude-modulated fluorometer (Pocket PEA FMS2, Hansatech UK). Three leaves per plant were measured to obtain a mean value per individual. All leaves were maintained in the dark for at least 30 min. before being measured by means of a leaf clip.

Foliar proline concentration was estimated as biochemical indicators of stress tolerance. Leaf proline concentration was determined following the protocol from [41] with slight modifications. Approximately 100 mg of frozen foliar tissue were ground in liquid nitrogen, homogenized with 2 mL of 3% sulphosalicylic acid, and centrifuged at 15,000× g for 20 min at 4 °C. From each sample, 1 mL of supernatant was added to 1 mL of ninhydrin reagent (2.5% ninhydrin in glacial acetic acid–distilled water—85% orthophosphoric acid [6:3:1]) and boiled in a water bath at 100 °C for 1 h. Samples were then cooled in ice to separate the chromophore by adding 2 mL toluene to each tube. Finally, the toluene fraction was extracted, and absorbance was measured at 525 nm using a spectrophotometer Jenway 6300 (Cole-Parmer, Cambridgeshire, UK). Proline concentration was assessed by comparing sample absorbance with a standard proline curve and expressed in μmol g^{-1} tissue fresh weight.

Complementarily, to assess the damage caused by the oxidative degradation of lipids in the plant cell membranes, we measured leaf concentration of malondialdehyde (MDA) by the thiobarbituric acid (TBA) assay [42]. To do this, 0.5 g of foliar tissue from each experimental plant was flash-frozen and pulverized in liquid nitrogen. The grinded tissue was added to 2 mL of trichloroacetic acid (TCA, 1%) and centrifuged at 10,000× *g* for 5 min. After this, 250 μL of each sample supernatant were mixed with 1 mL of TBA (0.5%) in TCA (20%) and incubated at 100 °C for 30 min in a dry bath Thermolyne 16,500 (Marshall Scientific, Hampton, NH, USA). After cooling to room temperature, thiobarbituric acid reactive substances content (TBARS) was determined on each sample by measuring its absorbance at 532 nm and non-specific absorbance at 600 nm [43]. Then, MDA content was determined by its molar extinction coefficient of 155 mM^{-1} cm^{-1} while the respective lipid peroxidation values were expressed as mmol TBARS g^{-1} of fresh weighted leaf tissues.

Finally, since the LEA protein family has been correlated with cellular tolerance to abiotic stress conditions such as cold, osmotic stress, and drought [44], and particularly the *LEA1* gene that has showed a significant induction in blueberry plants upon drought stress [39], we quantified the relative expression of *LEA1* gene in a subset of *V. corymbosum* plants (n = 5) per treatment. At the end of the fourth week, total RNA was extracted from leaves following a modified perchlorate method successfully used on this species (as described in: [39]). Briefly, a single-stranded cDNA was synthesized from 2 μg of total RNA (DNA-free) using oligo (dT) and the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). These cDNAs were, in turn, used for a quantitative real-time PCR analysis (qRT-PCR) to determine relative transcript abundance of *LEA1* coding gene. Each qPCR reaction contained 2 μL of diluted cDNA (50 ng), 10 μL of Maxima SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA), 6.4 μL of nuclease free water, and 0.8 μL at 10 mM of each forward and reverse specific primers (for details of primers see, [39]). Negative controls (nuclease-free water) were included for detecting any cross-contamination; positive controls for qPCR reactions were also included (*V. corymbosum* genomic DNA). Biological replicates were analyzed by triplicate using the Mx3000P qPCR system (Agilent, Santa Clara, CA, USA) under the following cycling conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 20 s [39]. To normalize qPCR data, *VcActin7* (*ACT7*) was set as internal reference, while relative expression levels between experimental conditions were calculated using the comparative $2^{-\Delta\Delta CT}$ method.

2.4. Effect of the Inoculation on Blueberry Survival and Fruit Development

A set of plants (n = 15 per treatment) was kept in the growth chambers for another four weeks. During this second month, plant survival was recorded weekly at the individual plant level (live/dead). At the end of the period, and just on the surviving plants, we characterized the produced fruits in terms of size and weight. On three matured fruits per plant, we measured diameter (mm) and fresh weight (g) using a ruler and a precise scale (±0.01 mm and 0.001 g, respectively).

2.5. Statistical Analysis

To compare the mean response of plants to treatments (fungal endophytes inoculation and environmental condition) in photochemical efficiency (Fv/Fm), lipid peroxidation state (TBARS), leaf proline concentration, and relative *LEA1* gene expression levels, we first fitted a linear mixed-effects model (LMM) for each variable to further perform an analysis of variance (ANOVA) on each model output. We used the mixed-effects model approach both to evaluate the random effect of the block and to deal with unequal group variances [45]. This was realized using the “lme” function from the *nlme* R-package [46].

A two-way analysis of variance (ANOVA) was performed to analyze the effect of the inoculation of the two Antarctic fungal endophytes (AFE+, AFE−), environmental condition (W+, W−), and their interaction, on variables of fruit development. In the case of significant interactions, *a posteriori* pair-wise comparisons were realized. For the LMM cases, we estimated and compared the least-square mean value for each group, as allowed by the *emmeans* package. For the case of the ANOVA models, we applied a Tukey Honest Significance Test (HSD). In both cases (LMM and ANOVA models), the normality of the respective residuals was verified using the Shapiro–Wilk test.

Finally, to determine the effect of treatments (fungal endophyte inoculation and stress condition) on survival probabilities of *V. corymbosum* plants, a Cox proportional-hazard model analysis was performed using the "coxph" function from the *survival* R-package [47]. The relevance of each factor was estimated by the significance of its hazard ratio parameter (HR), which describes the effect of a given factor on the mortality risk of the experimental individuals. Hence, a greater risk of mortality is related to values of HR >1, while HR < 1 means that mortality risk has decreased; logically, values that do not differ from 1 suggest that a given factor did not affect mortality risk. After the verification of proportionality between experimental factors with the "cox.zph" R-function, a pair-wise analysis of all results was performed between the experimental groups using the Peto and Peto modification of the Gehan–Wilcoxon test implemented in the "pairwise_survdiff" function of the *survminer* R-package [48].

3. Results

3.1. Effect of the Inoculation on Blueberry Stress-Responses

With overall photochemical efficiency (Fv/Fm) mean values below 0.7, the experimental design represented a stressful experience for all plant groups. However, for those *V. corymbosum* plants confronted to water deficit, this negative effect was exacerbated (Figure 1a). Nevertheless, this effect depended on the inoculation with fungal endophytes (Table 1); while water deficit caused on average a −13.5% reduction in the photochemical performance in AFE− plants, among AFE+ plants, this only represented a negative impact of just −4.9% (Figure 1a). A similar, but subtle, pattern was observed on the TBARS mean levels. Plants that experienced cold-stress and water deficit showed greater lipid peroxidation than plants without water deficit, but this negative effect was minimized with fungal inoculation status (Table 1; Figure 1b). In fact, even AFE+ plants under water deficit showed significantly lower membrane peroxidation than well irrigated AFE- plants (Figure 1b). The effect of endophytes on foliar proline was found to depend on the environmental scenario (Table 1). Nonetheless, great variation in proline content was observed in AFE+ plants in both stress treatments (Figure 1c). Complementarily, the relative expression of the *LEA1* gene was increased in plants exposed to cold events and water deficit together compared to plants that were kept at field capacity (Figure 1d). Overall, the expression of the gene was significantly higher in endophyte-inoculated plants than in non-inoculated ones (Table 1; Figure 1d).

Table 1. Analysis of variance (ANOVA) on the fitted Linear Mixed-effects Models (LMM) evaluating the effects of the presence/absence of two Antarctic fungal endophytes (AFE+ or AFE−) and Stress condition (cold without water deficit [Cold/W+] or cold and water deficit [Cold/W−]) on three physiological (green) and one genetic (grey) trait variables of blueberry plants. Significant factor effects ($p < 0.05$) are highlighted in bold.

Trait	Factor	*F*	*p*
Fv/Fm	Intercept	100,036.43	**<0.0001**
	Antarctic fungal endophytes (AFE)	530.17	**<0.0001**
	Stress condition (SC)	219.23	**<0.0001**
	AFE × SC	39.94	**<0.0001**
Leaf proline concentration	Intercept	18,117.76	**<0.0001**
	Antarctic fungal endophytes (AFE)	11.16	**0.0015**
	Stress condition (SC)	13.63	**0.0005**
	AFE × SC	4.35	**0.0417**

Table 1. *Cont.*

Trait	Factor	*F*	*p*
TBARS	Intercept	10,782.45	**<0.0001**
	Antarctic fungal endophytes (AFE)	664.78	**<0.0001**
	Stress condition (SC)	287.23	**<0.0001**
	AFE × SC	9.81	**0.0028**
Relative *LEA1* expression	Intercept	8655.79	**<0.0001**
	Antarctic fungal endophytes (AFE)	188.51	**<0.0001**
	Stress condition (SC)	112.45	**<0.0001**
	AFE × SC	1.05	0.3137

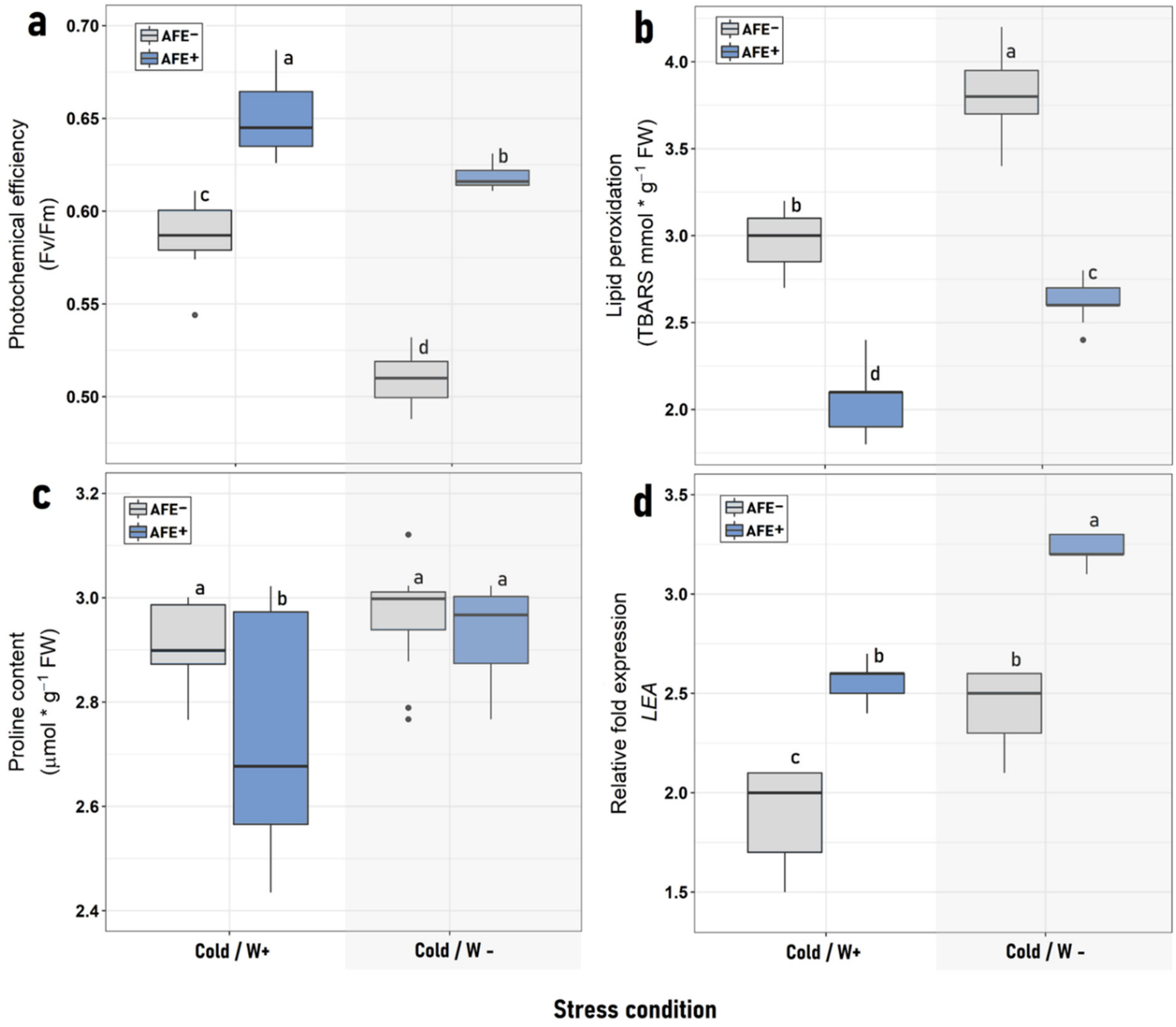

Figure 1. Box plots showing the effects of fungal endophyte inoculation (AFE+ or AFE−) and stress condition (cold without water deficit (Cold/W+) or cold and water deficit (Cold/W−)) on the physiological (**a**–**c**) and molecular (**d**) response variables of blueberry plants (*Vaccinum corymbosum*, cv Briggita). Different letters represent significant a posteriori differences (marginal mean pairwise comparisons, $p < 0.05$) assessed on the respective linear mixed-model (LMM) group estimations. The mid black line represents the data group median, while the box and bars represent the interquartile data distribution. Dark gray dots represent data outliers.

3.2. Effect of the Inoculation on Blueberry Survival and Fruit Characteristics

The Cox-proportional-hazards model (*d.f.* = 2; Likelihood ratio test = 17.04; $p = 0.00019$) showed that the main factor positively influencing plant survival was the inoculation with fungal endophytes (Figure 2). Despite of the fact that after one month in the growth chambers plant survival fell constantly with time, it was not differentially affected by the water deficit. Accordingly, while water availability did not significantly change the mortality risk of the plants (W− vs. W+: $\beta = 0.21$; HR = 1.23; SE = 0.206; $z = 1.01$; $p = 0.312$); it was indeed reduced among inoculated individuals (AFE− vs. AFE+: $\beta = -0.85$; HR = 0.427; SE = 0.220; $z = -3.86$; $p = < 0.001$). In addition, after eight weeks of stress, plants under cold and water deficit produced smaller fruits than plants grown under cold and field capacity condition, but this negative effect of the combination of cold and water deficit was significantly alleviated by the inoculation with fungal endophytes (Table 2, Figure 3). Therefore, while AFE− cold-exposed plants experiencing water deficit reduced their plant fruit weight by 23.9% on average, AFE+ individuals only showed a 16.4% fruit weight loss (Table 2, Figure 3a). This trend was not observed on fruit diameter, which was larger in AFE+ plants relative to AFE− individuals within each watering condition. However, the effect of water deficit within each group was similar (Table 2, Figure 3b), representing a 12.1 and 13.8% of size reduction for AFE− and AFE+ plants, respectively.

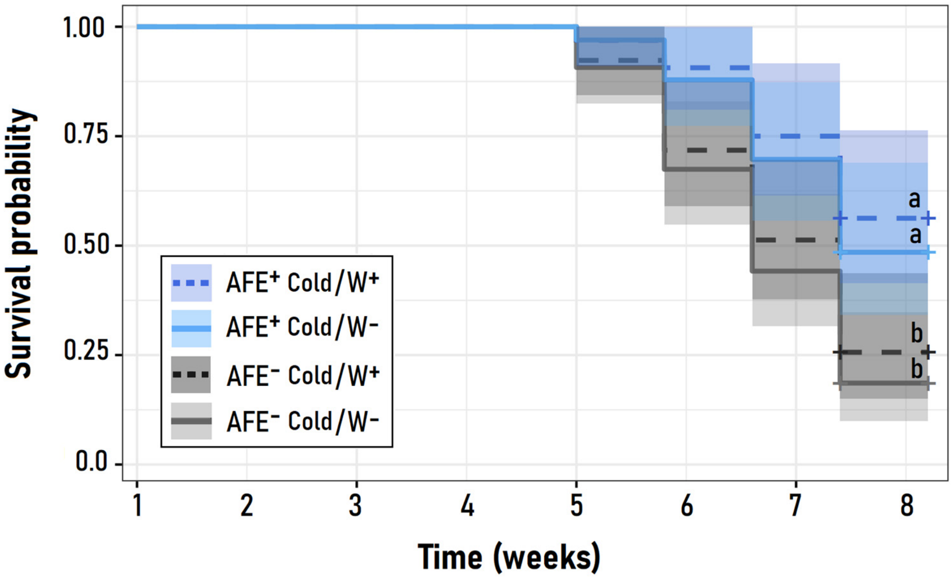

Figure 2. Effects of fungal endophyte inoculation (E+ or E−) and environmental condition (cold without water deficit (Cold/W+) or cold and water deficit (Cold/W−)) on survival dynamics of blueberry plants (*Vaccinum corymbosum*, cv Briggita). Different letters indicate different pairwise survival trends ($p > 0.05$), as estimated by the Gehan–Wilcoxon test.

Table 2. Two-way analysis of variance (ANOVA) testing the effect of the presence/absence of two Antarctic fungal endophytes (AFE+ or AFE−) and the stress condition (cold without water deficit [Cold/W+] or cold and water deficit [Cold/W−]) on size (mm) and weight (g) of fruits blueberry plants (*Vaccinum corymbosum*, cv Briggita). Significant factor effects ($p < 0.05$) are highlighted in bold, *d.f.*: degrees of freedom; *SS*: sum of squares; *MS*: mean squares, *F*: F statistic; *p*: probability value.

Variable	Model Term	*d.f.*	*SS*	*MS*	*F*	*p*
Fruit weight	Antarctic fungal endophytes (AFE)	1	0.224	0.224	160.225	**<0.0001**
	Stress condition (SC)	1	0.308	0.308	220.477	**<0.0001**
	AFE × SC	1	0.003	0.003	2.486	0.129
	residuals	22	0.031	0.001		
Fruit size	Antarctic fungal endophytes (AFE)	1	14.552	14.552	318.220	**<0.0001**
	Stress condition (SC)	1	20.410	20.410	446.322	**<0.0001**
	AFE × SC	1	0.353	0.353	7.722	0.0109
	residuals	22	1.006	0.046		

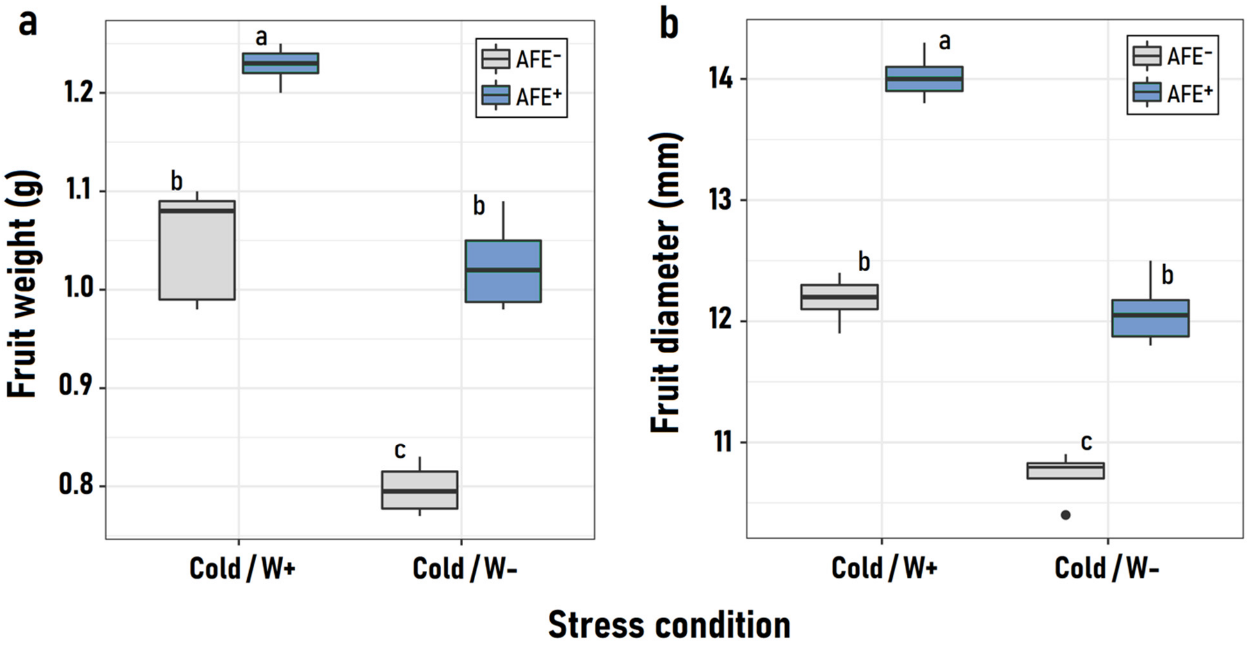

Figure 3. Box plots showing the effects of fungal endophyte inoculation (E+ or E−) and environmental condition (cold without water deficit (W+) or cold plus water deficit (W−)) on (**a**) fruit size (mm), and (**b**) weight (g) in blueberry plants (*Vaccinium corymbosum*, cv Briggita). Different letters represent significant a posteriori differences between means (Tukey's test, $p < 0.05$) assessed after significant ANOVA models were obtained. The mid black line represents the data group median, while the box and bars represent the interquartile data distribution. Dark gray dots represent data outliers.

4. Discussion

Our results point out to an improvement of ecophysiological and fitness-related traits in blueberry plants through root inoculation with the Antarctic fungal endophytes under scenarios combining sudden events of cold temperature and low water availability. First, experiencing the combination of cold and water deficit severely affected blueberry physiology and survival. Secondly, this negative impact was buffered by the presence of the selected Antarctic fungal endophytes. Interestingly, and similarly to other crop species [14,15,26], these fungal endophytes were able to develop a functional symbiosis as we observed a significant and positive effect of the inoculation on blueberry plants performance. Although our design lacks a proper control treatment for evaluating the effects of the recurrent cold events, it provides strong evidence suggesting that these class II fungal endophytes can establish functional symbiosis with a wide range of hosts and deliver benefits under different environmental conditions of stress.

Root inoculation with fungal endophytes improved some ecophysiological parameters of blueberry plants under both conditions of water supply. For example, endophyte-inoculated plants showed higher photochemical efficiency (↑Fv/Fm) and lower level of oxidative stress (↓TBARs) than uninoculated plants. Nevertheless, not all the assessed parameters associated with plant stress response showed clear-cut patterns. Foliar proline concentration, for example, did not show an important variation due to—but was increased by—the inoculation of plants with fungal endophytes. This later result was consistent and in the same direction as previously reported results with the same endophytes on *C. quitensis* when facing a water shortage [20]. An opposite result was observed in *Lolium multiflorum* plants in symbiosis with the foliar fungal endophyte *Epichloë occultans* under variable conditions of ozone [49]. Although proline can play roles as osmolyte and/or antioxidant [50], the attained physiological level may differentially depend on the factor of stress that triggers the response (and its intensity). Since endophyte presence is consistently associated with lower level of oxidative stress, other endophyte-modulated mechanisms can be playing a role in the plant response to stress and disrupting the relationship between the symbiosis, TBARS and proline.

Complementarily, *LEA* genes have been largely known for their role controlling plant responses to different abiotic stressors like drought, heat, salinity, and cold [51,52]. In consequence, its enhanced expression among AFE+ individuals in both environmental conditions might partly explain the better physiological response of inoculated *V. corymbosum* plants compared to uninoculated plants. This may suggest that some LEA-related biochemical compounds might be involved in the actual stress-response mechanisms [39]. In this context, since LEA proteins have been related to the osmotic plant regulation capacity, a condition that is strongly linked with both experimental stress factors, it is expected that plants with higher *LEA* gene expression also evidence a better physiological and fitness performance [52]. Indeed, the average increase in the expression of the *LEA1* gene among AFE+ plants was accompanied by a higher photochemical efficiency and a reduction in lipid membrane peroxidation level. Consequently, this may be, at least in part, behind the observed survival enhancement and better fruit development denoted by AFE+ plants.

A possible explanation for this observation is that it responds to the endophytes role in enhancing plant nutrient uptake rates, particularly nitrogen and phosphorous [53–55]. These fundamental nutrients are highly relevant for plants' amino acid and protein synthesis; therefore, any improvement of their supply would have a positive effect on plants' general performance [27]. The referred roles have been observed both in the native hosts plants of these endophytes (*Colobanthus quitensis*) and in alternative, non-host crop species like lettuce, tomato, and peppers [14,26], as mentioned earlier. Adding our results to the previous evidence, the studied Antarctic endophyte consolidates its generalist behavior in host selection, reaffirming that its positive effect is independent of the plant host species.

Under current global change scenarios, field crops are confronted with a great climatic uncertainty causing a negative impact on plant performance at multiple levels [4], especially in the case when climatic stress events occur during reproductive growth stages [56,57]. This environmental variability may increase, for example, the thermal oscillations during the seasonal transitions, rising the risk of stressful spring cold events [36]. As mentioned earlier, these sudden extreme events due to climate change can have a larger negative impact on agricultural systems compared to the slower decline in precipitation and increase in temperature [7]. Thus, crops in Central Chile may have to cope with an ever-increasing frequency of freezing events and severe drought during the growing season. As cultivated plants such as *V. corymbosum* may be sensible to different abiotic stressors [58,59], it is desirable to develop novel approaches aimed at increasing its tolerance to multiple sources of stress [32,57].

Established in the last decades, blueberry plantations have exported fruits for more than 100,000 tons, mainly from the Brigitta cultivar [60]. Berries of this cultivar are large, sweet, firm, and resistant to bruising and thus suitable for mechanical harvesting [30,61]. In addition, they present a long postharvest life retaining the desirable organoleptic properties and edible quality even after 8 weeks of storage [30,33]. However, all these positive qualities are counteracted by a reduced fruit set, possibly related with a high rate of abortions due to sub-zero temperatures during the spring season [33,62]. This condition can be worsened as plants of this cultivar are highly susceptible to drought, showing a severe impairment in physiological parameters when water availability is low [39]. Additionally, extreme low temperature events could be frequent during the winter and spring seasons in central Chile [63]. Since this condition, as stated earlier, can increase in frequency and intensity in the future [5,34], this can cause significant damages to diverse types of crops, including blueberries. Thus, blueberry is a suitable crop to search and test novel biotechnological solutions for dealing with abiotic stress, particularly drought and cold stress. Our results suggest that functional symbiosis with beneficial microorganisms like some endophytic fungi may be one of these solutions.

While there is evidence that abiotic priming could induce tolerance to different types of stress when convergent metabolic routes are utilized against different stressful factors, its occurrence may be rare since it entails a high resource cost aiming to produce multiple defensive-responsive compounds [56,57]. In accordance, our results suggest that priming with drought stress against an exposure to sudden events of cold negatively affected the performance of blueberry plants, since the combination of simultaneous stresses causes cumulative, amplified, and synergic detrimental effects [64]. Thus, the abiotic priming derived from a potential cross-tolerance stress response may not be a suitable strategy in this species. However, the less-severe stress damage reported for blueberry E+ plants exposed to cold and water deficit certainly suggests that a "biotic hardening" alternative, as provided by functional symbionts like *P. rubens* and *P. bialowienzense*, might be suitable for *V. corymbosum*. Further studies are needed to gain a deeper understanding of the underlying metabolic pathways and/or molecular mechanisms involved in the observed benefits before its scaling to field experiments. Nevertheless, by harnessing the beneficial effect of these Antarctic habitat-adapted symbiotic microorganisms (sensu [65]), this study provides new insights into using functional plant-microbe symbiosis as an alternative to cope with sudden cold events and water deficit in commercial fruit species like the highbush blueberry. This functional symbiosis could be essential for sustaining blueberry production in the coming years in the face of progressively worse climatic conditions due to climate change.

5. Conclusions

Here, we were able to improve the performance of blueberry plants under simulated production conditions, combining water scarcity and peaks of chilling temperatures, by means of inoculating their roots with Antarctic fungal endophytes. Since those endophytes were originally isolated from Antarctic native plants and inoculated to blueberry plants, our results strongly suggest their derived benefits are transferable to non-originally host plants. Therefore, fungal endophytes isolated from extreme habitats appear as a cost-effective bio-input to improve crop plants to face real production scenarios, especially in regions where those conditions are getting worse due to global change.

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