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Boron and Zinc Diminish Grey Necrosis Incidence by the Promotion of Desirable Microorganisms on Hazelnut Orchards

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Abstract: In the southern hemisphere, the commercial production of hazelnut has increased in recent years, with a concomitant detection of new pathogens associated with plant production, so-called emerging infectious diseases (EIDs). Gray necrosis (GN) is a hazelnut disease that causes 30% of economic losses in Europe. In this sense, we recently reported GN as an EID in Chile, the main hazelnut producer in the southern hemisphere. Therefore, control strategies are urgently required to avoid disease dissemination. In this study, the effect of boron (B) and zinc (Zn) fertilization on the incidence of GN was determined. Additionally, the community composition of microorganisms via Dendrogram Gradient Gel Electrophoresis (DGGE) was evaluated, and bacteria from internal tissue (endophytic) were isolated to study their bio-control traits under greenhouse conditions. The microbial occurrence and biocontrol ability was evaluated using MALDI-TOF/TOF. According to the results, B and Zn promote beneficial bacteria which may be able to diminish symptoms associated with GN. Thus, beneficial microorganisms, applied in combination with micronutrients, could be synergistically applied in sustainable agriculture.

Keywords: biocontrol of gray necrosis; bioproducts based microorganisms; *Corylus avellana*; fungal complex; sustainable agriculture

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1. Introduction

Hazelnut (*Corylus avellana* L.) is a native tree from Central Europe and western Asia. Currently, the commercial production of hazelnut has expanded worldwide as a result of the nutritional and culinary nut values [1]. In this respect, Turkey is the principal hazelnut producer, responsible for 75% of the global production, followed by Italy, Azerbaijan, the USA and Chile [2,3]. Therefore, Chile is the main hazelnut producer in the southern hemisphere [4], mainly in the southern regions. In fact, hazelnut production has significantly increased since 2012 by 97% in La Araucanía (from 37°34′59″ to 39°14′59″ south latitude), 95% in Los Ríos (from 39°14′59″ to 40°32′59″ south latitude) and even 275% in the Los Lagos region (from 40°32′59″ to 44°29′59″ south latitude). This increase in interest is due to the fact that hazelnut is an attractive option for farmers due to the relatively low costs of production and establishment, manual harvesting and simple seed (kernel) drying. In La Araucanía, hazelnut currently covers around 7000 ha, and cultivar Barcelona (BCN) covers 70% of the total area [5,6].

The introduction of new crop species is a common practice in agriculture, which regrettably increases the risk of introducing new pathogens and their vectors into production systems free of them [7–9]. Plant-assisted migration has clear potential to cause

the emergence of novel diseases by introducing potentially invasive pathogens, changing the composition of plant-associated microbial communities and altering environmental conditions [10]. The concept of emerging infectious diseases (EIDs) has been used to define diseases that influence host susceptibility, drive the host distribution, modify trade patterns and alter the virulence of pathogens and their geographical expansion [9,11]. Therefore, a better understanding of the factors affecting the disease triangle (environment, pathogens and host) that influences the onset of EIDs is an urgent necessity to contribute to the scientific knowledge on preventing the outbreak of phytopathogens.

We have recently published the first report of gray necrosis (GN) of hazelnut in Chile, detected during the productive stage of plants grown in the La Araucanía region. We revealed that this EID was caused by a fungal complex formed by *Fusarium sporotrichioides*, *Alternaria alternata*, *Diaporthe* sp. (its anamorph *Phomopsis* sp.) and *Neofusicoccum* sp. [12]. The main symptoms were evidenced as brown-grayish spots occurring at the bottom of the nut and enlarging upwards to the apex, and in severe infections, the bracts were also affected [12]. The high disease incidence was more important in cv BCN, especially when plants were not fertilized with boron (B) and zinc (Zn), suggesting that an adequate supply of these macronutrients could improve the defense mechanisms against plant infection caused by a pathogenic fungal complex [13–15].

The role of micronutrients is largely known to increase plant productivity [16,17]. For example, soils with low pH, as is the case of the acidic Andisol from southern Chile, B is usually present in low availability, affecting the yield of the crops. Therefore, the conventional agronomic management of commercial hazelnut orchards in Chile includes the foliar application of B and Zn. These micronutrients allow one to ensure the fruit set, reduce cluster drop and blank nuts, enhance yields, improve fruit quality and improve the plant nutritional status [18]. In addition, B application in combination with Zn promotes the vegetative and reproductive growth of the plant [19–21]. Boron and Zn application have also been shown to have an indirect effect on the yield and growth of some important crops by stimulating the functionality of some beneficial microorganisms associated with the plant. For example, it has been reported that B is required for rhizobacteria in the N fixing process, consequently causing an increase in N uptake and nitrate content in the soil–plant system. Similarly, B application can increase the soil microbial population, stimulate the rhizosphere metabolisms and improve the soil enzyme activities when applied alone or in combination with molybdenum [22]. In broccoli plants, B supplementation with beneficial microorganism showed an increase in yield and phosphorus (P) and potassium concentrations [23], whereas the application of B along with the B-tolerant bacteria *Bacillus* sp. increased nodulation, yield and grain B biofortification of chickpea grown in B-deficient soil [17]. On the other hand, the application of Zn along with beneficial bacterial strains resulted in greater yield, enzymatic activity and Zn^{2+} content in wheat grain [24]. Although there is some evidence describing the important role of B and Zn in promoting the activity of beneficial microorganisms associated with several crops, the role of these micronutrients in preventing the appearance of diseases, especially GN, in hazelnut has not been thoroughly studied. Therefore, (i) considering the economic importance of hazelnut, (ii) the significance of knowledge of IEDs, (iii) the role of micronutrients in plant performance and (iv) the importance of beneficial microorganisms on plant performance, this study was conducted to evaluate the effect of B and Zn fertilization on beneficial microorganisms to diminish the incidence of GN on hazelnut cv BCN.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was conducted in a hazelnut orchard located in Radal-Cunco, La Araucanía region, Chile (39°59′59″ S; 72°20′59″ W), during the 2018–2019 productive season (October–November). Plants were grown in Andisol soil from the Freire series (Typic Placudands), slightly acidic in a flat topography with gentle slopes and good drainage. From August 2018 to March 2019, the study area's minimum and maximum mean tempera-

tures were 6 °C and 20 °C, respectively, with a precipitation rate of 946 mm (data collected from meteorological measurements in the orchard using data logger CR1000, Campbell Scientific, Inc., Logan, UT, USA).

The hazelnut plants cv BCN were planted in 2009 at the spacing of 5 × 5 m with a total of 400 plants ha^{-1} with a multi-stem system. The combined dose of 800 and 400 mg L^{-1} B and Zn, respectively (fertilized, BCNf), were sprayed on foliage. Water was used in the control treatment (BCNc), with twelve plants in each treatment.

Plants were sprayed four times, since the phenological stage of ovary development to nut growth/premature stage (15 October until 30 November) every 15 days. The application was performed on clear days that were free from precipitation (72 h before and after application). The B was sprayed as Bortrac^{TM}150 (i.e., 15% sodium perborate, $Na_3BO_3 \times H_2O$ p/v, Yara Ltd., Grimsby, UK) and Zn as NutriZinc Plus (i.e., 16% zinc chloride, $ZnCl_2$ p/v, Cytozyme Laboratories Inc., South Salt Lake, UT, USA) with an output of 1500 L ha^{-1} of water, using a 15 L back sprayer (Cifarelli 1200, Cifarelli S.p.A-Voghera, Italy) with an output of 5 L min^{-1}. Three trees in each treatment (BCNf and BCNc) were selected, and 100 nuts of each were randomly collected and transported to the Biocontrol Research Laboratory (Universidad de La Frontera, Temuco, Chile). Nuts were stored at 3 °C for a maximum of 7 d before further analysis.

Composite soil samples (2 kg) were collected for chemical analyses before starting the study (August 2019) from a layer of 0–30 cm along the row at each studied quarter and stored in a cooler at 4 °C. For foliar analyses, 500 g of healthy and totally expanded leaves was taken from shoots in the middle of the tree canopy located in the four cardinal points [25], placed in polyethylene bags and stored in a cooler at 4 °C until analysis in the Soil and Plant Laboratory (Universidad de La Frontera, Temuco, Chile).

2.2. Determination of Soil and Foliar Chemical Analyses

The chemical properties were determined as follows: available P (P_{Olsen}) was extracted using 0.5 M Na-bicarbonate and analyzed using the molybdate method [26]. Organic matter (OM) content was estimated using wet digestion [27]. Soil pH was measured in 1:2.5 soil/deionized water suspensions. Exchangeable potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+) were extracted with 1 M ammonium acetate (CH_3COONH_4) at pH 7.0 and analyzed using flame atomic adsorption spectrophotometry (FAAS) [28]. Exchangeable aluminum (Al^{3+}) was extracted with 1 M KCl and analyzed using FAAS [29]. For foliar and nut analyses, element concentrations were determined using a simultaneous multi-element atomic absorption spectrophotometer (UNICAM 969 Atomic absorption Spectrometer, Cambridge, UK) [30]. All analyses were made in triplicate.

2.3. Structural Evaluation of Hazelnut Microbial Communities by Denaturing Gradient Gel of Electrophoresis (DGGE)

The structure of fungal and bacterial communities of hazelnut kernels obtained from each treatment were evaluated via DGGE using universally conserved bacterial and fungal primer sets. Thirty nuts from BCNf and thirty BCNc were randomly selected and surface sterilized via repeated immersion in 80% (v/v) ethanol for 5 min and 4% (v/v) NaOCl for 20 min, and then rinsed three times with sterile distilled water [31]. DNA was extracted from three replicates using an UltraClean Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions, where samples (1 g) were lysed via mechanical action on a vortex. From the lysed cells, the released DNA was bound to a silica spin filter. The filter was washed, and the DNA was recovered in certified DNA-free Tris buffer.

For the analysis of fungal communities, touchdown polymerase chain reaction (PCR) was followed by nested PCR using a Swift Maxi PCR thermal cycler (ESCO Technologies Inc., Hatboro, PA, USA). Firstly, touchdown PCR was performed with reagents supplied with GoTaq® R Flexi DNA Polymerase (Promega Co.) using the primer sets NS1/NS8 (Table 1). The PCR conditions were as follows: an initial denaturation at 93 °C for 3 min,

followed by 93 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min to 35 cycles, and finally by 72 °C for 5 min [32]. A second PCR with the primer sets NS7-/F1Ra was performed with 94 °C for 1 min, followed by 30 cycles of 55 °C for 1 min and 72 °C for 3 min, with a final extension at 72 °C for 7 min. The primer set NS1/NS8 amplifies a 1700 bp fragment of the 18S rRNA gene, and NS7-GC/F1Ra amplifies a 400 bp fragment nested within the NS1/NS8 target [32,33].

Table 1. Sequence of primers used in this study.

Application	Microbial Group	Primer Set	Sequence	Reference
DGGE	Total fungi	fNS1/rNS8 NS7-GC/f1Ra	5′-GTA GTC ATA TGC TTG TCT C-3′/5′-TCC GCA GGT TCA CCT ACG GA-3′ 5′-GAG GCA ATA ACA GGT CTG TGA TGC-3, GC-clamp: CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG/5′-CTT TTA CTT CCT CTA AAT GAC C-3′	[32]
DGGE	Total bacteria	EUBf933-GC/EUBr1387	5′-GCA CAA GCG GTG GAG CAT GTG G-3′/5′-GCC CGG GAA CGT ATT CAC CG-3′	[32]
Microbial identification	fungi	fITS9/ITS4	5′-GAACGCAGCRAAIIGYG-3′/5′-TCCTCCGCTTATTGATATGC-3′	[35]
Microbial identification	bacteria	27F/1492r	5′-AGA GTT TGATCC TGG CTC AG-3′/5′-TAC GGY TAC CTT GTT ACG ACT T-3′	[34]

For bacterial communities, analysis fragments of the 16S rRNA gene were amplified via touchdown PCR using the primer set EUBf933-GC/EUBr1387, which amplifies a 454 bp fragment of the 16S rRNA gene. The PCR amplifications were carried out with reagents supplied by GoTaq® Flexi DNA Polymerase (Promega Co., Madison, WI, USA) as follows: a hot-start was performed at 95 °C for 10 min, and annealing was initially set at 65 °C and was then decreased by 0.5 °C per cycle until 55 °C for 1 min, followed by extension at 72 °C for 3 min. Then, ten additional cycles were carried out at 55 °C (annealing) followed by denaturation at 94 °C for 1 min and primer extension at 72 °C for 3 min. The final extension step was 7 min at 72 °C [33]. DGGE analysis was performed using a DCode system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Twenty-five microliters of PCR product was loaded onto 6% (*w*/*v*) polyacrylamide gel with a 40–70% gradient (urea and formamide). The electrophoresis was run for 16 h at 75 V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co., Carlsbad, CA, USA) for 30 min and photographed on a UV transilluminator [3,33].

2.4. Isolation and Identification of Microorganisms from Kernels

Samples were surface sterilized via repeated immersion in 80% (*v*/*v*) ethanol for 5 min and 4% (*v*/*v*) NaOCl for 20 min, and then rinsed three times with sterile, distilled water [31] and put in potato dextrose agar (PDA, Sigma-Aldrich) + chloramphenicol (100 µg mL^{-1}) to prevent bacterial growth and in Luria Bertani (LB) + cycloheximide (100 µg mL^{-1}) at 30 °C for 5 and 2 days for fungal and bacteria isolation, respectively.

DNA was extracted using a microbial DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA), where microbial cells were added to a bead beating tube, followed by lysis solution. From the lysed cells, the released DNA was bound to a silica spin filter. The filter was washed, and the DNA was recovered in certified DNA-free Tris buffer. Fungal and bacteria identification was based on the partial sequencing of ITS and 16S rRNA gene, using PCR universal primer sets fITS9/ITS4 and 27f and 1492r, Table 1 [34]. After starting at 94 °C for 5 min, PCR amplification was performed for 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min. PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea). Sequences were deposited in the GenBank database and compared with those in the GenBank database.

Due to genetic affiliation of bacteria isolated from healthy plants (BCNf) to putative biocontrol activity, we formed a consortium to validate this trait.

2.4.1. Bacteria Consortium Formulation

For consortium formulation, four bacterial strains (*Pantoea* sp., *Ewingella* sp., *Pseudomonas* sp. and *Enterobacter* sp.) isolated from BCNf kernels were first evaluated to determine their putative antagonist effect among them [34].

Then, bacterial strains were separately grown in 800 mL of Luria Bertani (LB) media. After growth at 30 °C for 24 h with continuous shaking (150 rpm), the bacterial cells were collected via centrifugation at 5000× *g* for 10 min and rinsed twice with sterile saline solution (0.85% NaCl) and the suspended sterile distilled water. The bacterial cell suspensions were mixed in the proportion 1:1:1:1, and the bacterial mixture (bacterial consortium) was used as plant inoculum to evaluate its biocontrol ability in plants.

2.4.2. Biocontrol Ability of Bacteria Consortium under Greenhouse Conditions

To evaluate the biocontrol traits of the bacteria consortium, the fungal strains causing GN were inoculated in three healthy hazelnut plants (1 year old) cv BCN (BCNf). For this, each fungal strain was first grown onto potato dextrose agar (PDA) for 7 days at 25 °C and were put in PDA to determine the putative antagonist effect among them [34]. Then, fungi strains were grown in potato dextrose broth, and cells were harvested via centrifugation at 6000 rpm for 10 min, washed twice with 0.05 M phosphate buffer at pH 7.0 and resuspended in sterile, distilled water. Cell suspension was counted with a hemacytometer and adjusted to 1×10^8 CFUmL^{-1} with sterile distilled water. Plants were inoculated by spraying the water suspension of all fungi at same time. After one week, when initial symptoms were evidenced, the bacterial consortium was applied at a final concentration of 1×10^9 by pulverizing the suspension on the leaves of the plants. The inoculated plants were maintained under greenhouse conditions (16/8 h photoperiod at 25/16 °C (day/night) and relative humidity range of 60–70%) for 1 month. In order to evidence the effect of the bacterial consortium on the GN development plant symptoms, the protein profiles of plant, fungi and bacteria were studied using MALDI-TOF/MS.

2.5. Microbial Occurrence and Biocontrol Ability Validation Using MALDI-TOF/MS

The occurrence of inoculated microorganisms residing in plants was analyzed in a MALDI-TOF/MS platform by fingerprint comparing the protein profile due its ability to elucidate not only the interactions between microorganisms and their host plants but also those among different microbial taxa living in association with plants [36,37]. For this, previously inoculated leaves were suspended in 50 µL of formic acid (70%), and the tubes were vortexed for 2 min. The same volume of acetonitrile was added, and the suspension was mixed again and centrifuged at 13,000× *g* for 2 min. Then, 1 µL of the supernatant was spotted individually onto a MALDI plate and dried at room temperature. Protein extract was covered with 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) (saturated in ACN/TFA 2.5% 1:1). Protein spectra were acquired in a Bruker Daltonics (Leipzig, Germany) Autoflex Speed MALDI-TOF/TOF mass spectrometer in the positive ion mode. Protein profiles were acquired in the TOF linear mode and in the positive ion mode with a delayed extraction of 260 ns at 20 kV accelerating voltage. Each spectrum was manually collected as an average of 1000 laser shots in the range of m/z 2000–20,000. MS calibration was performed using protein calibration standard I (Bruker Daltonics, Leipzig, Germany, Leipzig, Germany). Spectra were acquired in duplicate using Flexcontrol Version 3.4 (Bruker Daltonics, Leipzig, Germany) and processed with MALDI Biotyper Compass Explorer Version 4.1 (Bruker Daltonics, Leipzig, Germany) in the range of m/z 2000 to 15,000. The samples were identified using a fungi database previously added to the database, and the mass spectra were clustered using a hierarchical method with distance measure as the correlation and the linkage algorithm as the average.

2.6. Statistical Analyses

The similarities between fungal and bacterial communities between BCNf and BCNc obtained from DGGE analyses were visualized via non-metric multidimensional scaling

analysis (MDS), using Primer 7 software (Primer-E Ltd., Lutton, Ivybridge, UK), which showed a Bray–Curtis similarity index greater than 80% and 0.14 stress values (Clarke 1993). Values were given as mean ± standard errors. We considered differences significant when the *p* value was lower than or equal to 0.01. The in silico analysis was also used to estimate the microbial diversity by richness (S) and the Shannon–Wiener index and dominance by the Simpson Index (D), represented by $1 - D$ or $1 - \lambda$ [38]. The neighbor-joining trees (phylogenetic trees) were constructed with Molecular Evolutionary Genetic Analysis (Mega 7). To detect infection incidence, spectra of bacteria, fungi and plants were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, leading to 1000 laser shots per summed spectrum.

3. Results

3.1. Chemical Parameters of Soil and Leaf Tissues

Soil chemical analyses revealed that Andisol (Freire series) has some typical chemical characteristics of soils derived from volcanic ashes. These characteristics include low B concentration (0.39 mg kg^{-1}), low P availability (7 mg kg^{-1}), high content of OM (>21%) and Σ-bases around 9.5 cmol (+) kg^{-1} [39]. However, the soil pH was slightly acidic (pH = 6.1), with a concomitant low aluminum saturation (Al sat) of 0.32% as a result of the application of calcareous amendments (Table 2).

Table 2. Average values (±standard error) of chemical properties of Andisol (Freire series), where experiment was performed in hazelnut orchard. † Calculated as Al/cation exchange capacity [Σ (K, Ca, Mg, Na, and Al)] × 100, *n* = 3.

Soil Parameter	Soil Content
N (mg kg^{-1})	23.0 (±2.0)
P (mg kg^{-1})	7.0 (±0.6)
K (mg kg^{-1})	203.0 (±8.3)
pH (H_2O)	6.05 (±0.89)
Organic matter (%)	21.0 (±1.0)
K (cmol + kg^{-1})	0.52 (±0.03)
Na (cmol + kg^{-1})	0.04 (±0.00)
Ca (cmol + kg^{-1})	6.41 (±1.01)
Mg (cmol + kg^{-1})	2.37 (±0.09)
Al (cmol + kg^{-1})	0.03 (±0.00)
Al sat (%) †	0.32 (±0.00)
CICE (cmol + kg^{-1})	9.37 (±1.14)
Σ basis (cmol + kg^{-1})	9.34 (±1.13)
B (mg kg^{-1})	0.39 (±0.01)
Zn (mg kg^{-1})	0.69 (±0.02)
Cu (mg kg^{-1})	6.65 (±0.89)
Fe (mg kg^{-1})	61 (±2.03)
Mn (mg kg^{-1})	3.28 (±0.09)
S (mg kg^{-1})	27.00 (±1.03)
Ext. Al (mg kg^{-1})	967.0 (±23.1)
pH ($CaCl_2$)	5.17 (±0.03)

Foliar analyses of the leaf samples collected during the first week of October, before the application of B and Zn treatments, indicated average B concentrations of 20 mg kg^{-1} dry weight (DW). For Zn, we found 34 mg kg^{-1} DW. When B and Zn were applied (BCNf), the concentrations of these micronutrients in the leaf were substantially higher compared with BCNc (Table 3). In relation to other elements in the leaves, Ca, Mg, Na and K concentrations were uninfluenced by B + Zn treatments, and no significant differences between BCNc and BCNf were found (Table 3).

Table 3. Average chemical values (±standard error) in foliar samples of hazelnuts from cv Barcelona (BCNc and BCNf).

	BCNc	BCNf
B (mg kg^{-1})	19.8 ± 3.04	70.6 ± 13.4 *
Zn (mg kg^{-1})	34.4 ± 8.71	136 ± 31.5 *
Ca (mg kg^{-1})	0.66 ± 0.04	0.62 ± 0.03
Mg (mg kg^{-1})	0.19 ± 0.01	0.17 ± 0.00
Na (mg kg^{-1})	0.02 ± 0.00	0.02 ± 0.00
K (mg kg^{-1})	1.14 ± 0.06	1.15 ± 0.03

Samples BCNc and BCNf (columns) were analyzed using Student's *t*-test, and the difference was expressed by * (* indicates $p < 0.05$).

3.2. Microorganism Occurrence in Relation to B and Zn Fertilization

Around 50% of thirty nuts from BCNc showed symptoms of gray necrosis, evidenced as brown-grayish spots occurring at the bottom of the nut and enlarging upwards to the apex, whereas in the case of BCNf, this value was 5% (5 nuts). According to the results obtained from the non-metric multidimensional scaling (NMDS) analyses at 80% similarity, illustrated in Figure 1A, we found that both fungal and bacterial community structures were grouped independently in response to each treatment (BCNc and BCNf). The biodiversity index (Figure 1B) revealed a greater fungal diversity in BCNc, which was the treatment showing higher symptoms of GN. This greater biodiversity was expressed in higher species numbers (richness) and H′ index (richness and dominance). In the case of species dominance expressed by $1 - \lambda$, BCNc was more dominant. We also found greater bacteria diversity in BCNc, as expressed in richness with greater species numbers (S) and individuals (N), as well as H′ index. However, BCNf nuts had less species dominance ($1 - \lambda$).

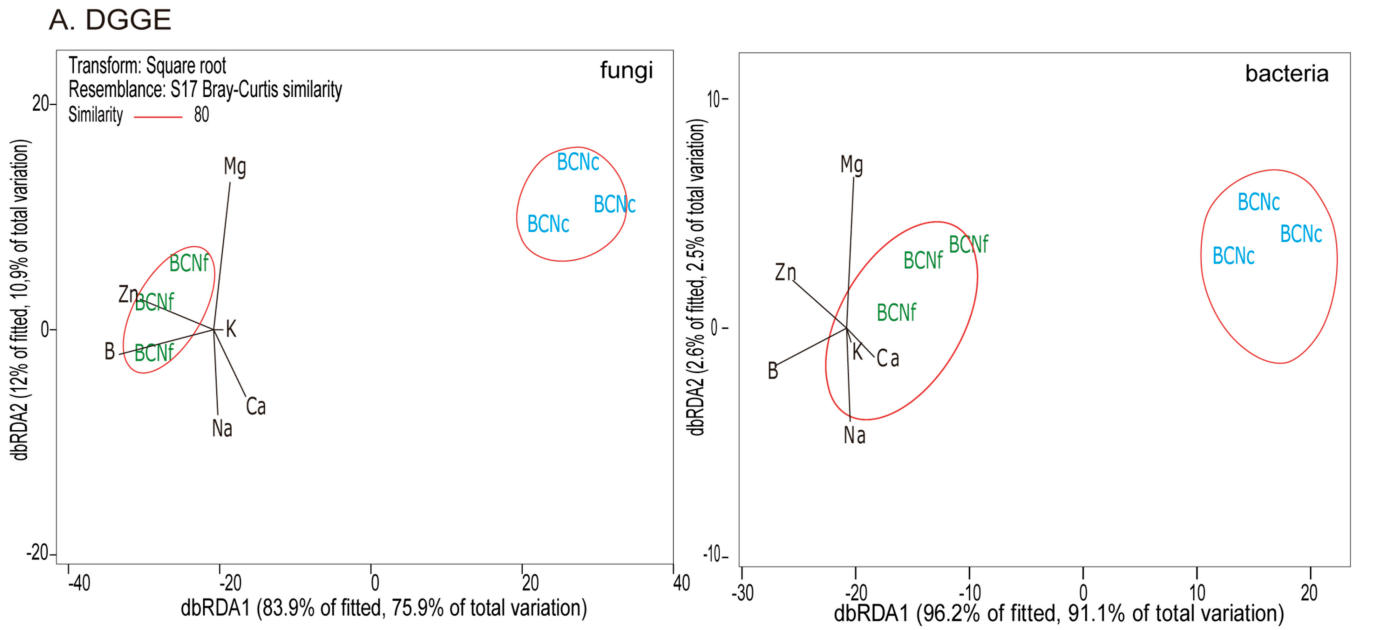
Interestingly, we noted that the fungal and bacterial community structures from BCNf were directly related with B and Zn content in leaves, as revealed by the NMDS ordination (Table 4A), whereas the most important correlation in the case of fungal communities was with B, B + Zn and B + Zn + Mg, as shown by correlation analyses between NMDS ordination and foliar parameters (Table 4B). Bacterial community structure was also related to B and Zn ($p < 0.01$, Table 4A), but the most significant correlation between NMDS ordination and chemical parameters of leaves was with B, B + Zn and B + Zn + Ca (Table 4B). No significant differences in BCNc were found (data not shown).

Table 4. (**A**) Significance level for NMDS ordination and environmental variables. two asterisks (**) represent statistically significant correlation ($p < 0.01$). (**B**) The most important correlation between NMDS ordination and foliar parameters of plants.

(A)		
Parameter	**Fungi**	**Bacteria**
B	0.001 **	0.001 **
Zn	0.001 **	0.002 **
Ca	0.435	0.462
Mg	0.190	0.400
Na	0.535	0.796
K	0.951	0.945

Table 4. *Cont.*

(B)			
Corr.	**Fungi**	**Correlation**	**Bacteria**
0.737	B	0.729	B
0.668	B, Zn	0.673	B, Zn
0.659	B, Zn, Mg	0.592	B, Zn, Ca
0.654	B, Zn, Ca, Mg	0.580	Zn
0.609	B, Mg	0.578	B, Zn, Mg
0.598	B, Zn, Ca	0.556	B, Zn, Ca, Mg
0.591	B, Zn, Mg, Ca	0.550	B, Zn, K
0.564	Zn	0.521	B, Zn, Na
0.554	B, Zn, K	0.506	B, Zn, Ca, K
0.551	B, Ca, Mg	0.504	B, Ca



B. Biodiversity index

fungi	S	H'	1-λ
BCNc	9±0.00^{a}	2.19±0.00^{a}	1.33±0.00^{b}
BCNf	3±0.00^{b}	1.10±0.00^{b}	1.58±0.00^{a}

bacteria	S	H'	1- λ
BCNc	18±0.00^{a}	2.87±0.00^{a}	1.24±0.00^{b}
BCNf	10±0.00^{b}	2.28±0.00^{b}	1.32±0.00^{a}

Figure 1. (**A**) Non-metric multidimensional scaling (NMDS) analysis of fungal (left) and bacteria (right) communities in fertilized nuts (green letters) and non-fertilized nuts (blue letters) from cv Barcelona. (**B**) Biodiversity indexes expressed as S (species number) Shanon–Wieneer (H′), Simpson (represented by $1 - \lambda$) of BCNc and BCNf. Tukey test was used to compare treatments means; values followed by the same letter do not differ at $p < 0.05$ ($n = 3$).

3.3. Microorganisms Involved in Gray Necrosis Symptoms

After 10 days of growth in a moist chamber, fungal mycelium was observed in at least 50% of nuts from plants not supplemented with B and Zn (BCNc), in contrast to the supplemented plants (BCNf), where fungal growth was evidenced in less than 5% of the nuts, as confirmed using scanning electron micrographs (SEM, green arrows) (Figure 2).

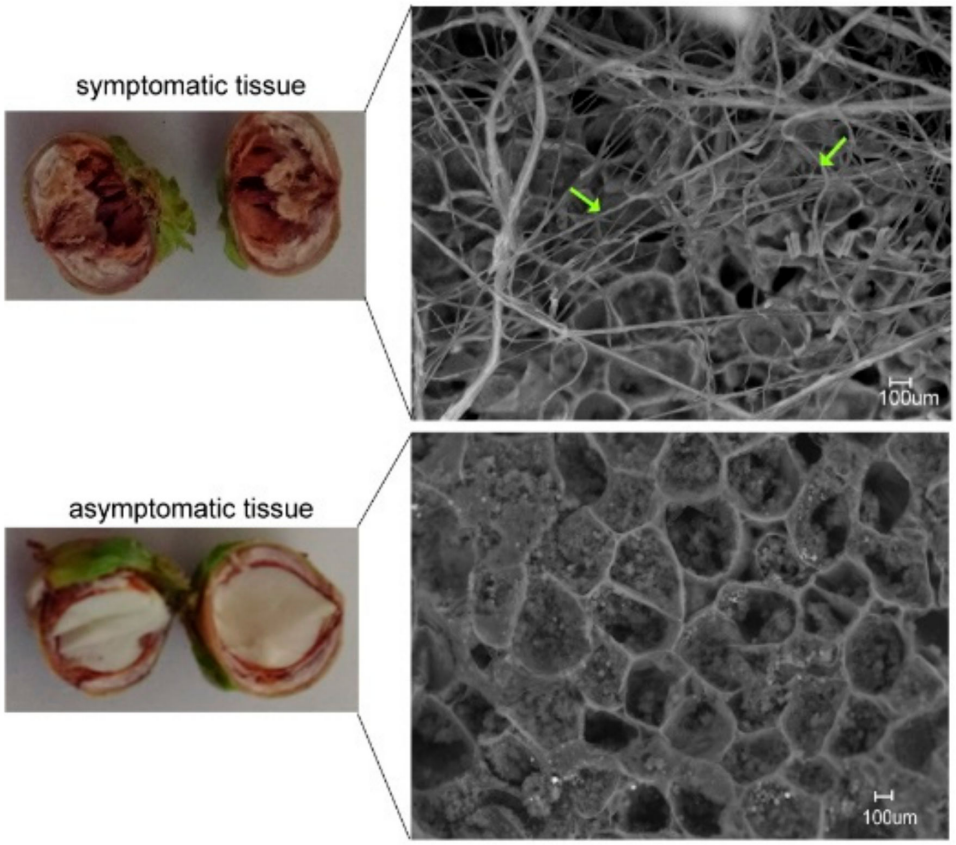


Figure 2. Scanning electron micrographs of BCNc (**above**) and BCNc (**below**) nuts.

A total of six fungal strains were isolated from the symptomatic kernel tissue of hazelnuts. Interestingly, we found that the fungi strains isolated from symptomatic kernels were phylogenetically associated with those reported to cause GN [40]. Thus, we evidenced the first report of GN in Chile [12]. All bacteria belonged to Proteobacteria phylum. Interestingly, all strains isolated from BCNf have been linked with biocontrol activity. Thus, four bacterial isolates from BCNf (*Pantoea* sp., *Ewingella* sp., *Pseudomonas* sp. and *Enterobacter* sp.) were selected to formulate a bacterial consortium in order to tested for their biocontrol ability in vivo. In contrast, only one bacterial strain was isolated from BCNc, which belonged to *Enterobacter* sp. BAG1 (Table 5).

Table 5. Phylogenetic affiliation of isolates based on partial sequencing of ITS2 and 16S rRNA genes.

Isolate	Closest Relatives or Cloned Sequences (Accession n°) †	Similarity	Accession N°
Fungi from BCNc			
Alternaria sp. HAB1.4	*Alternaria alternata* fungi colonizing after dieback (MF509751)	100%	MF629825
Phomopsis sp. HAB2.1	*Phomopsis* sp. dieback of *Pinus nigra* (KJ482539)	99%	MF629824
Neofusicoccum sp. HAB2.2	*Neofusicoccum arbuti* stem cancer and dieback on blueberries (EU856062)	99%	MF629823
Diaporthe sp. HAB2.3	*Diaporthe rudis* anamoph: *Phoma ridis* phytopatogen (KY964222)	99%	MF629822
Fusarium sp. HAB2.4	*Fusarium sporotrichioides* dieback in *Abies alba* (KU516465)	99%	MF629821
Diaporthe sp. HAB3.1	*Diaporthe australafricana* dieback in kiwi fruit (KU679315)	99%	MF629820
Bacteria from BCNc			
Enterobacter sp. BAG1.2	*Enterobacter* sp. phytate degrading bacteria, PGPR (JQ864388)	99%	MF623059
Bacteria from BCNf			
Pseudomonas sp. BAB1.2	*Pseudomonas poae* biocontrol against soybean cyst nematodes (KT695820)	99%	MF623063
Ewingella sp. BAB1.3	*Ewingella americana* endophytic bacteria with biocontrol activity (KY203802)	99%	MF623062
Pantoea sp. BAB2.1	*Pantoea agglomerans* biocontrol of fire blight of apple and pear (KY357286)	99%	MF623061
Enterobacter sp. BAG1.3	*Enterobacter* sp. biocontrol against *Rhizoctonia solani* (KM589029)	99%	MF623058

† Based on partial sequencing of 16S rRNA gene and comparison with sequences found in GenBank by using BLASTN.

3.4. Biocontrol Capacity of Isolated Bacteria from BCNf Healthy Plants

The results obtained from MALDI-TOF/MS analyses of kernels tissues revealed the presence of *Diaporthe* sp. (distance 0.25) and *Neofusiccoum* sp. (distance 0.03), as demonstrated by the similar protein spectra profiles to those of the pure fungal strains (Figure 3A), (Figure 3B). Plants showed evident symptoms of dieback (Figure 3C). Interestingly, when plants were inoculated with pathogenic fungus and bacteria associated with biocontrol (green letters), the signal was inhibited (Figure 3B), which was confirmed in plant symptomatology (Figure 3C, green letters), confirming the presence of *Diaporthe* and *Neofusiccoum* in inoculated plants, and similarly, the effect of biocontrol of bacteria was also evidenced.

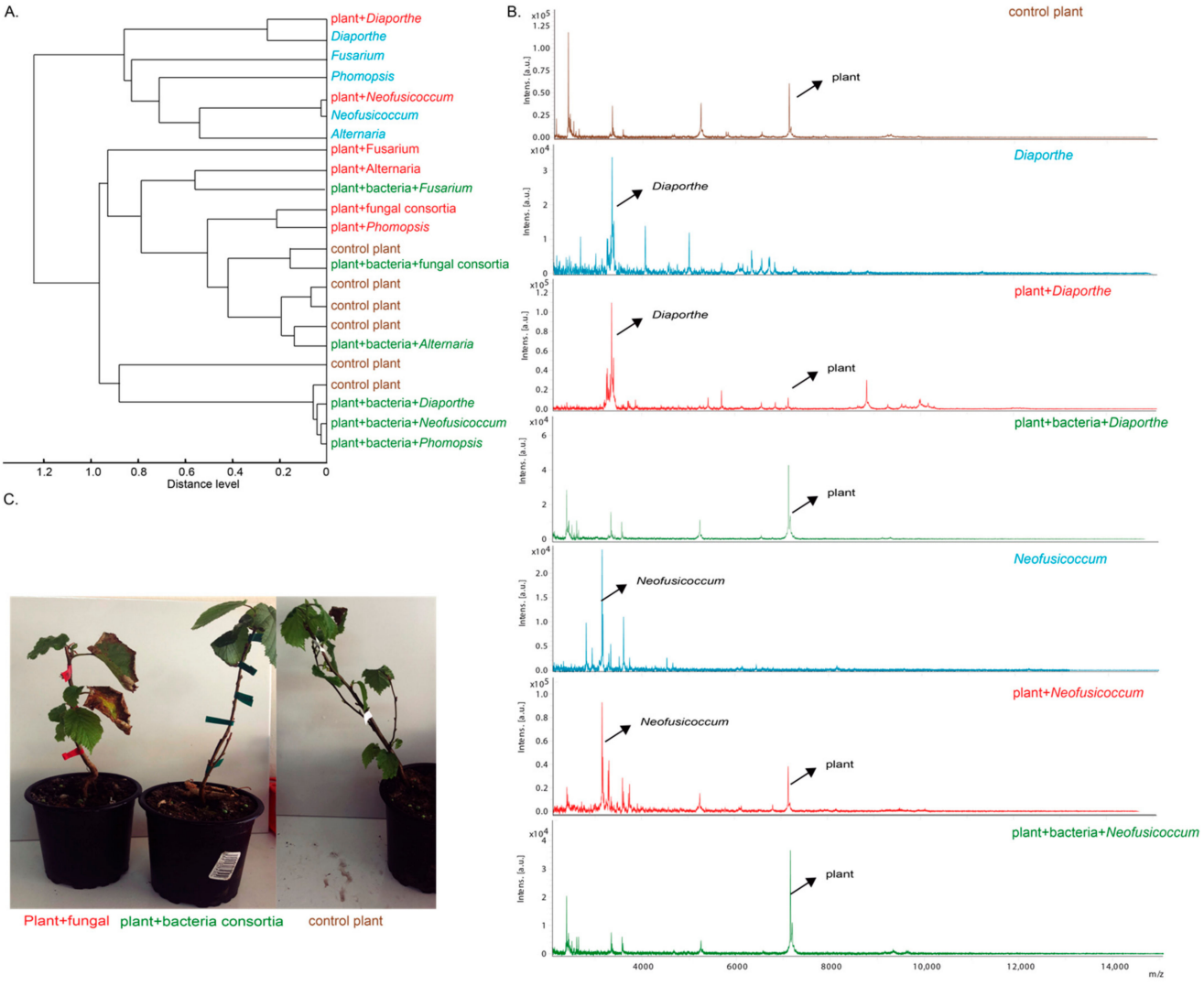


Figure 3. Hazelnut plants cv Barcelona analyzed via MALDI-TOF/MS. (**A**) Dendrogram and (**B**) spectra of protein profiles associated with fungal, plant, plant + fungal and plant + fungal + bacterial consortium, $n = 3$. (**C**) Symptoms of dieback in hazelnut plants (plant + fungal) and effect of inoculated bacteria (plant + fungi + bacteria consortia) and control healthy plant (without inoculation) $n = 3$.

4. Discussion

Gray necrosis is a fungal disease principally caused by *Alternaria* spp., *Diaporthe* spp., *Neofussicoccum* spp. and *Fusarium* spp. This fungal co-existence is defined as a complex, and their diagnosis and control are very complicated as a result of their interactions and synergistic colonization strategies [41]. This fungal complex causes around 30% of crop losses in Italy [40]. Our group recently published the first report of GN affecting hazelnut

in Chile [12]. We also observed that the disease incidence was especially important in CV Barcelona and when plants were unfertilized with B and Zn (BCNc). For this reason, this study is complementary with the first report of GN and was conducted to evaluate the effect of B and Zn fertilization on beneficial microorganisms to diminish the incidence of GN, as well as on the study of the microbial diversity communities associated with kernels of hazelnut plants.

According to the results, we noted that plants supplemented with B and Zn (BCNf) showed the most important correlation in the microbial (both fungal and bacterial) structure, suggesting that an adequate supply of these micronutrients can improve the mechanisms of defense against the pathogenic fungal infection of plants as previously reported for *Alternaria grandis* in potato [13], *Alternaria solani* in tomato [14] and *Hemileia vastatrix* in coffee [15]. This could be attributed to the relation with plant growth promotion, mainly in synergy with Zn [19,20]. For example, in internal tissues of symptomatic nuts from treatment without B and Zn, more fungal species richness and greater dominance (less diversity) was evidenced, detecting the presence of eight putative phytopathogenic fungi previously associated with the onset of GN. This is in concordance with previous studies carried out by Zhang et al. [42] and Trivedi et al. [43], where the diversity of the plants and citrus rhizosphere decreased significantly with increased disease severity of powdery mildew and huanglongbing (hlb) of citrus. In contrast, in healthy nuts (supplemented with B and Zn), less fungal richness was detected. This is an important aspect to consider, as fertilization strategies could be a determinant factor in GN management and control. The protective effect of B against fungal diseases has already been evidenced in rice [44]. In a similar way, B has also been linked with diminished spore germination, germ tube elongation and mycelia spread, as well as with higher membrane disruption of the *Botrytis cinerea* [45,46], whereas Zn has an effect in diminishing the intensity of some fungal diseases such as rhizoctonia, take-all and smut in maize and powdery scab and yellow leaf spot in tomato [47]. The application of Zn in plants has also been linked to an improved response to oxidative damage by detoxicating superoxide radicals [48] and to the increased production of antibiotics by bacterial biocontrol agents [47]. In this context, it is interesting to note that nuts from BCNf showed the presence of endophytic bacteria species previously described for their biocontrol activity. These interesting results should be deepened, since they indicate that supplementation with B and Zn may allow the recruitment of beneficial microorganisms in hazelnut kernels.

MALDI-TOF/MS has potential in the elucidation not only of the interactions between microorganisms and their host plants, but also those among different microbial taxa living in association with plants [36]. The presence and infectivity ability of phytopathogenic fungi associated with GN was checked in both inoculated and no inoculated plants by comparing the protein spectra via MALDI-TOF/MS. Thus, we only confirmed the presence of *Diaporthe* sp. and *Neofusiccoum* sp. causing dieback in young plants (1 year). This could be attributed to the fact that strains from fungal consortium could infect different phenological stages. For example, *Diaporthe* spp., the dominant genus causing GN, has an increased incidence from the early to full ripening of hazelnut orchard [49,50], whereas *Alternaria* sp. and *Fusarium* has been rather described as a secondary colonizer with higher incidence in the latter stages of the disease [12,40,51], which would explain why it was not detected with MALDI-TOF/MS.

In order to corroborate the biocontrol ability of the isolated bacteria (*Pseudomonas* sp., *Pantoea* sp., *Ewingella* sp. and *Enterobacter* sp.), these were inoculated as a consortium in healthy plants along with the fungal strains causing GN. According to the results, plants inoculated with the consortium had considerably diminished symptoms associated with GN. Interestingly, the protein spectra of pathogenic fungi evidenced with MALDI-TOF/MS was inhibited by all of at least one strain of the bacterial consortium, mainly decreasing the presence of *Diaporthe* sp. and *Neofusiccoum* sp. Other studies also reported the biocontrol effect of *Pseudomonas putida* strain THJ609-3 against *Diaporthe citri,* one of the world's most important diseases in citrus orchards. The authors attributed the disease reduction

to the direct antifungal activity of the bacterial strain on the leaf surfaces [52]. Similarly, *P. fluorecens* strain UM270 has been implicated in the inhibition of important fungal phytopathogens including *Diaporthe phaseolorum* [53]. The biocontrol role of *Pantoea* spp. has also been reported against *Erwinia amylovora* in apple flower stigmas [54], *Botrytis cinerea* in grapevine [55], postharvest diseases in citrus fruit [56], black rot in sweet potato [57], *Penicillium expansum* in apples [58], etc. *Ewingella* spp. and *Enterobacter* spp. have also been extensively reported as biocontrol and as plant growth promoting bacteria (PGPB) [59–65].

Finally, we suggest that B and Zn supplementation is a promising strategy for GN control as it may promote beneficial endophytic microorganisms to GN biocontrol. Further studies under field and greenhouse conditions are needed.

5. Conclusions

In this study, we evaluated the effect of boron (B) and zinc (Zn) fertilization on fungi associated with gray necrosis (GN) in hazelnut. More than 50% of unfertilized plants were symptomatic to GN, evidencing the presence of the fungal complex causing the disease. In contrast, 5% of fertilized plants showed symptoms of GN. Thus, B and Zn may be involved in the occurrence of endophytic bacterial strains associated with biocontrol ability, such as *Pantoea* sp., *Ewingella* sp., *Pseudomonas* sp. and *Enterobacter* sp. The effective biocontrol ability was evidenced in one-year-old plants, mainly against early colonizers such as *Diaporthe* sp. and *Neofussicoccum* sp. Thus, our study suggests that B and Zn may enhance the presence of beneficial microorganisms to GN biocontrol. Further studies on fungal complex co-existence are still necessary to develop effective and applicable biocontrol strategies, in order to monitor emerging infective diseases (EIDs). Thus, beneficial microorganisms applied in combination with micronutrients could be a better choice for farmers or the bioproducts industry to produce plants of superior quality, making agriculture more productive.

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