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Efficiency of Marine Bacteria and Yeasts on the Biocontrol Activity of *Pythium ultimum* in Ancho-Type Pepper Seedlings

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Abstract: Ancho-type pepper (*Capsicum annuum* L.) is a crop susceptible to *Pythium ultimum*, which has already been controlled with synthetic fungicide applications; however, marine antagonist microorganisms could be an alternative source of control. The efficiency in vitro and in vivo of marine bacteria and yeasts was determined against *P. ultimum*. The inhibition of the radial growth of *P. ultimum* was quantified in vitro by the bacteria *Stenotrophomonas rhizophila* KM01 and KM02; *Bacillus subtilis* RBM01 and RBM02, *B. amyloliquefaciens* 2RLBF and 3R4CF; and *Pseudomonas* spp. 2R6BF and 2RE9CF, as well as the yeasts *Debaryomyces hansenii* 1R11AB, 1R11CB, and LL01 and *Cryptococcus laurentii* 2R3BF and 2R1CB. The β-1,3-glucanase activity of the marine microorganisms was quantified in the presence of the phytopathogen. The disease index (DI), growth parameters, and colony forming units (CFU) were determined in ancho-type pepper plants inoculated with marine bacteria, yeasts, and *P. ultimum*. The radial zone of the phytopathogen was inhibited by 80% and 75% by *S. rhizophila* KM01 and *C. laurentii* 2R1CB, respectively. *D. hansenii* LL01 and *S. rhizophila* KM02 showed the highest activity of β-1,3-glucanase, with 6060 U/mL and 47 U/mL, respectively. *B. subtilis* RBM02 protected 100% of the plants from the oomycete, and an increase was quantified in all the growth parameters and CFU. The use of these marine bacteria and yeasts are, therefore, an option for *P. ultimum* biocontrol in ancho-type pepper plants, thereby minimizing the application of synthetic fungicides.

Keywords: *Capsicum annum* L.; damping-off; marine microorganisms; oomycete

1. Introduction

Ancho-type pepper (*Capsicum annuum* L.) is an important crop worldwide, which is cultivated in greenhouses or open-fields. Damping-off disease is one of the major global factors affecting the germination of the seeds and plants of the different pepper germplasms [1,2]. *Pythium* damping-off has caused severe damage to the root systems of plants, with close to 70% plant mortality at the seedling stage or in the field, thus reducing the production, quality, and quantity of the pepper's harvest potential [3,4].

Pre- and post-emergence damping-off disease is caused by *Pythium* spp. in pepper; this species is economically very important worldwide [5,6]. The rapid sporangia germination of *Pythium* followed

by immediate infection makes management of this phytopathogen very difficult [7]. Several *Pythium* species, including *P. aphanidermatum*, *P. irregular*, and *P. ultimum*, are known to cause damping-off and root rot diseases in pepper [8–10]. Although synthetic fungicides have shown promising results in controlling damping-off disease, phytotoxicity and fungicide residues pose serious problems to human and animal health, as well as the environment. In this context, phytosanitary measures and the management of pepper cultivation should include the application of microorganisms as biocontrol agents to minimize the use of synthetic fungicides [11].

Bacteria and yeasts have a high capacity to control phytopathogens without causing damage to human and animal health and the environment [12,13]. Several mechanisms have been proposed for the biocontrol of phytopathogens by bacteria and yeasts, including competition for space and nutrients [14,15], lytic enzyme production such as β-1,3-glucanase [16,17], and induction of host resistance [18,19], among others. Both microorganisms have been isolated in different terrestrial ecosystems, mainly in plants, fruits, and soils; the main bacteria used as biocontrol agents are species of *Stenotrophomonas*, *Bacillus*, and *Pseudomonas*, which have already been reported for the control of *Fusarium proliferatum* in melon [20]; *Sclerotium rolfsii* [21] and *Rhizoctonia solani* in wheat [22]; and *F. solani* in cassava [23], among others.

On the other hand, among the yeasts that have been used as antagonists, the species that stand out are *Debaryomyces* and *Cryptococcus*, which have been efficient in the control of *Monilinia fructicola* in apple [24]; *Colletotrichum gloeosporioides* in mango [25]; and *Penicillium italicum* in citrus [26], among others.

Despite the proven efficiency of isolated terrestrial bacteria and yeasts for phytopathogen control, the search for new antagonists continues; one of the ecosystems that has been rarely studied or explored is the marine environment, which may contain more efficient microorganisms for phytopathogen control than those isolated from terrestrial ecosystems [27,28]. Marine bacteria and yeasts are now being considered as new sources of biological products that can be applied in different areas, such as agriculture [29,30]. Until now, no information has been available on the efficiency of marine microorganisms for the control of soil diseases in horticultural plants. Therefore, the objective of this study was to assess the efficiency of marine bacteria and yeasts in the biocontrol of *P. ultimum*, a causal agent of damping-off disease in ancho-type pepper seedlings.

2. Materials and Methods

2.1. Marine Antagonistic Microorganisms

The bacteria and yeasts studied belonged to the collection of marine microorganisms of the Centro de Investigaciones Biológicas del Noroeste (CIBNOR). The bacteria *S. rhizophila* KM01 and KM02, *B. subtilis* RBM01 and RBM02, *B. amyloliquefaciens* 2RLBF and 3R4CF, and *Pseudomonas* spp. 2R6BF and 2RE9CF, as well as the yeasts *D. hansenii* 1R11AB, 1R11CB, and LL01 and *C. laurentii* 2R3BF and 2R1CB, were assessed. The bacteria were cultured in a tryptic soy broth (TSB, Difco, BD, Franklin Lakes, NJ, USA), and the yeasts were cultured in yeast extract-peptone-dextrose (YPD, Difco, Sparks, MD, USA); both were incubated at 25 °C for 24 h. The concentration of the antagonists was adjusted to 1×10^7 cells/mL using a digital spectrophotometer (Thermo Spectronic Genesys 20, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for the bacteria, calibrated to 660 nm with an optical density of 0.8; the yeasts were adjusted using a hemocytometer.

2.2. Pythium Ultimum

The phytopathogen was provided by the Phytopathology Laboratory at CIBNOR. The oomycete was reactivated in a V8 medium prepared as follows: 160 mL of V8 vegetable juice was mixed with 3.5 g of $CaCO_3$ and then clarified by filtration; subsequently, 100 mL of V8 was diluted and clarified with 1000 mL of sterile distilled water, adding 20 g of agar at 25 °C for seven days. The concentration was adjusted to approximately 1×10^6 zoospores/mL using a hemocytometer.

2.3. Pathogenicity Test

Seeds of "Don Emilio" ancho-type pepper plants were disinfected for three minutes in 70% ethanol; subsequently, they were left for another three minutes in sodium hypochlorite at 5% and then washed three times with sterile distilled water. They were then sown in 200-well seedbeds containing a mixture of Sunshine® substrate (Agawam, MA, USA) and organic matter (3:1, v/v) previously sanitized with Anibac 580 liquid at a dose of 5 mL/L. One seedling was transplanted at 40 days of age after sowing into a plastic pot containing 60 g of substrate. At the moment of transplanting, the roots of each seedling were washed with sterile distilled water and submerged in a *P. ultimum* solution adjusted at 1×10^6 zoospores/mL for 15 s. As a control, a group of seedlings was not inoculated with the phytopathogen. Seedlings were incubated for three weeks within a controlled-environmental chamber (Conviron, Winnipeg, CAN) at 25 °C and 95% relative humidity (RH) under a light/dark photoperiod of 12/12 h. At the end of the experiment, root necrosis was quantified using the following scale [31]: 0 = plants with unharmed roots; 1 = < 1% of harmed roots; 2 = 1%–3% harmed roots; 3 = 4%–5% harmed roots; 4 = 6%–10% harmed roots; and 5 = >10% harmed roots.

The disease index (DI) was calculated with the following formula [32]: DI (%) = $\Sigma[(Ri \times Ni)/(Rt \times Nt)] \times 100$, where Ri is the number of plants in the category, Ni is the category degree, Rt is the infected plant with the highest scale, and Nt is the total number of plants assessed. The oomycetes of the infected plants were reisolated in the V8 medium to confirm the Koch postulates. Thirty seedlings were used per treatment, and the experiment was repeated twice.

2.4. Antagonistic Test

Marine microorganisms were evaluated for their antagonistic activity against *P. ultimum* via a dual culture assay. Each marine bacterium and yeast was streaked approximately 2 cm from the edge of a Petri plate containing potato dextrose agar (PDA), and a 5 mm diameter agar plug of a seven-day-old culture of the phytopathogen was transferred to the center of a PDA plate and incubated at 25 °C for 10 days. A group of Petri plates was inoculated with the phytopathogen and the fungicide Captan at 2 g/kg. The control consisted of a Petri plate inoculated with a 5 mm diameter agar plug of the oomycete without marine microorganisms. The radial growth of the phytopathogen was measured in mm, and 10 replicates were used per treatment. The experiment was repeated twice.

2.5. Detection of Lytic Activity

2.5.1. Phytopathogen Cells and Culture of Marine Microorganisms

Pythium ultimum was cultured in a V8 medium (without agar) at 25 °C for 10 days. Subsequently, the phytopathogen was collected using sterile gauze and macerated in liquid nitrogen. The marine bacteria and yeasts were cultivated in a mineral salt medium (MSM) supplemented with 1 mg/mL phytopathogen cells and incubated at 25 °C for 15 days on a rotary shaker at 100 rpm. The supernatant was collected at 12 and 24 h to determine lytic activity. Ten replicates were used per treatment, and the experiment was repeated twice.

2.5.2. β-1,3-glucanase Activity

β-1,3-Glucanase activity was detected using laminarin, as indicated by Hernandez-Montiel et al. [30], in triplicate for each treatment; this step was performed twice. Briefly, the kit used for measuring glucose release was the Randox Glucose (GOD-PAP) method at pH 5.0 (37 °C). One unit (U) of β-1,3-glucanase was defined as the μmol of reducing sugar released/mg of protein per min at 37 °C and pH 5.0. The total protein content of the enzyme(s) solution was quantified by the method described by Lowry et al. [33] using bovine serum albumin as a standard.

2.6. Inoculation of Ancho-Type Pepper Plants with Marine Bacteria and Yeasts

The best marine antagonistic strains of the bacteria (KM01, KM02, RBM01, and RBM02) and yeasts (2R1CB, LL01, 1R11CB, and 2R3BF) were selected. The forty-day-old seedlings were deposited in plastic pot containing 60 g of a mixture of Sunshine® substrate (Agawam, MA, USA) and organic matter (3:1, v/v), which was previously sanitized with Anibac 580 liquid at a dose of 5 mL/L. At the moment of transplanting, the root of each seedling was washed with distilled sterile water and submerged in a *P. ultimum* solution previously adjusted (1×10^6 zoospores/mL) for 15 s. Subsequently, each seedling was inoculated with 1 mL of each suspension of a bacterium or yeast, which was previously adjusted (1×10^7 cells/mL). One seedling batch was inoculated only with the phytopathogen, and the other with the oomycete and fungicide Captan (2 g/kg). The seedlings were incubated for four weeks inside a controlled-environmental chamber (Conviron, Winnipeg, CAN) at 25 °C and 95% RH under a light/dark photoperiod of 12/12 h. At the end of the experiment, the disease index [32], plant height, radicle volume, leaf area, and biomass were quantified. Thirty plants per treatment were used, and the experiment was repeated twice.

2.7. Scanning Electron Microscopy Micrographs

Samples of fresh root were taken from the ancho-type pepper plants inoculated with marine microorganisms and *P. ultimum*. The samples were fixed by immersion using a 0.1 M phosphate buffered solution (pH 7.0) of glutaraldehide at 2.5% for 24 h. Subsequently, they were processed according to the methodology proposed by Usall et al. [34] and observed with a scanning electron microscope (S-3000N, Hitachi®). Five replicates were performed per treatment, and the experiment was repeated twice.

2.8. Root Colonization of Marine Microorganisms

The colony forming units (CFU) of the bacteria and yeasts were determined for each treatment following the methodology proposed by Swanson et al. [35], sowing the dilution in triplicate on plates with tryptic soy agar ((TSA) Difco, BD, Franklin Lakes, NJ, USA) for the bacteria and with PDA (Merck, DE) for yeasts. Ten replicates were performed per treatment, and the experiment was repeated twice.

2.9. Statistical Analyses

Data were processed by a one-way analysis of variance (ANOVA) and Tukey's test with a significance level of 5%, using the STATISTICA software (version 8.0.360.0 StatSoft Inc., Tulsa, OK, USA) for Windows.

3. Results

3.1. Pathogenicity of Pythium ultimum

The ancho-type pepper plants showed a DI of 96% 21 days after oomycete inoculation. All the plants with *P. ultimum* showed damping-off at the base; no signs of disease were observed in the plants without the phytopathogen. *P. ultimum* was reisolated from the necrotized root, confirming the Koch postulates.

3.2. In Vitro Radial Growth of Pythium Ultimum

The radial growth of *P. ultimum* was inhibited 80% and 76% by the *S. rhizophila* strains KM01 and KM02, respectively (Figure 1). In the treatment with the oomycete and Captan fungicide, the phytopathogen was inhibited by 69%.

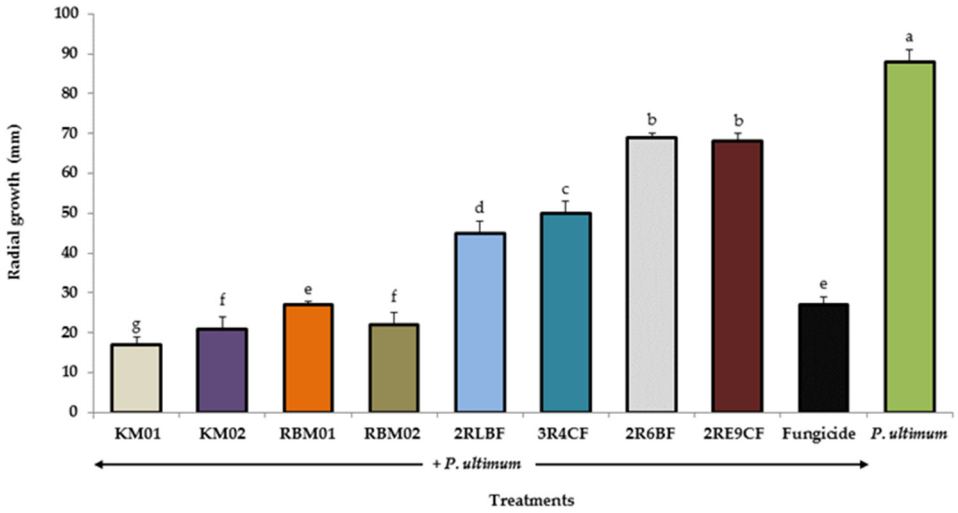

Figure 1. Effect of marine bacteria on the radial growth of *Pythium ultimum*. The bacteria are *Stenotrophomonas rhizophila* (KM01 and KM02), *Bacillus subtilis* (RBM01 and RBM02), *B. amyloliquefaciens* (2RLBF and 3R4CF), and *Pseudomonas* spp. (2R6BF and 2RE9CF). Data are shown as the mean ± standard deviation (SD) (n = 100). Columns with different letters were significantly different according to Tukey's test ($p < 0.05$).

With respect to the marine yeasts, *C. laurentii* 2R1CB inhibited 75% of the radial growth of the phytopathogen. In the treatment with the oomycete and Captan fungicide, the phytopathogen was inhibited by 66% (Figure 2).

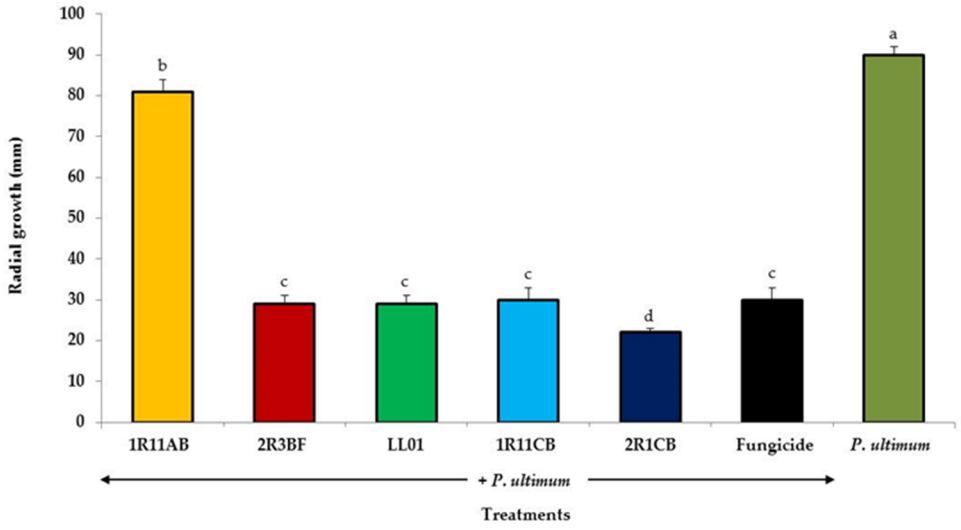

Figure 2. Effect of marine yeasts on the radial growth of *Pythium ultimum*. The yeasts are *Debaryomyces hansenii* (1R11AB, 1R11CB, and LL01) and *Cryptococcus laurentii* (2R3BF and 2R1CB). Data are shown as the mean ± standard deviation (SD) (n = 100). Columns with different letters were significantly different according to Tukey's test ($p < 0.05$).

Based on these results, the marine bacteria *S. rhizophila* KM01 and KM02 and *B. subtilis* RBM01 and RBM02, as well as the marine yeasts *D. hansenii* LL01 and 1R11CB and *C. laurentii* 2R3BF and 2R1CB, were selected for inoculation in ancho-type pepper plants.

3.3. Lytic Activity

The marine yeast *D. hansenii* LL01 and the marine bacterium *S. rhizophila* KM02 showed the highest activity of β-1,3-glucanase, with 6060 U/mL and 47 U/mL, respectively (Table 1).

Table 1. Lytic activity of β-1,3-glucanase in vitro with marine bacteria and yeasts.

Microorganism		Strain	Lytic Activity	
			β-1,3-glucanase (U/mL)	
			12 h	24 h
Bacterium	*S. rhizophila*	KM01	27 d*	41 e
		KM02	29 d	47 d
	B. subtilis	RBM01	7 g	17 g
		RBM02	9 g	14 g
	B. amyloliquefaciens	2RLBF	8 g	15 g
		3R4CF	11 f	21 fg
	Pseudomonas spp.	2R6BF	19 e	40 e
		2RE9CF	20 e	37 e
Yeast	*D. hansenii*	1R11AB	2589 b	5062 b
		1R11CB	2105 c	3562 c
		LL01	3907 a	6062 a
	C. laurentii	2R3BF	21 e	40 e
		2R1CB	19 e	37 e

* The values are the means ± standard deviation (SD) of 10 replicates. Different letters indicate a significant difference ($p < 0.05$) according to Tukey's test.

3.4. Protection of Ancho-Type Pepper Plants by Marine Bacteria and Yeasts

The marine bacterium *B. subtilis* RBM02 protected the ancho-type pepper plants by 100% against *P. ultimum*; the plants inoculated with the marine yeasts *C. laurentii* 2R1CB and *D. hansenii* LL01 showed the lowest DI (Figure 3).

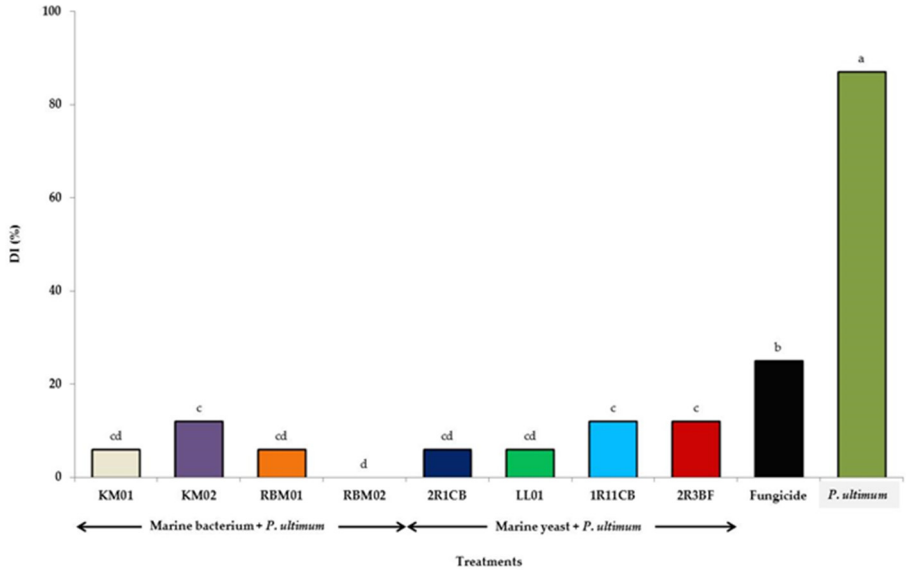

Figure 3. Disease index (DI) in the ancho-type pepper plants inoculated with *Pythium ultimum* and the marine microorganisms. The bacterial strains are *Stenotrophomonas rhizophila* (KM01 and KM02) and *Bacillus subtilis* (RBM01 and RBM02), and the yeasts are *Cryptococcus laurentii* (2R3BF and 2R1CB) and *Debaryomyces hansenii* (1R11CB and LL01). Data are shown as the mean ± standard deviation (SD) ($n = 30$). Columns with different letters were significantly different according to Tukey's test ($p < 0.05$).

The inoculated plants with the rest of the marine bacterial and yeast strains showed significantly lower DI levels, compared to the plants with the phytopathogen and Captan fungicide. Only *P. ultimum* produced a DI of 88%. Moreover, a significant increase was observed in all the growth variables in the plants inoculated with the marine bacterium *B. subtilis* RBM02 and *P. ultimum*, compared to the treatment with fungicide Captan and the phytopathogen (Table 2).

Table 2. Growth variables in the ancho-type pepper plants inoculated with marine microorganisms and *Pythium ultimum*.

Treatment	Height (cm)	Fresh Root Weight (g)	Dry Foliage Weight (g)	Dry Weight of Root (g)	Leaf Area (cm^2)	Radical Volume (cm^3)
Bacterium						
KM01	10.89 b	3.62 b	1.19 b	1.03 b	110.41 b	28.37 b
KM02	9.01 d	1.16 e	0.58 e	0.51 d	82.88 f	20.59 d
RBM01	10.71 b	3.56 b	1.18 b	1.08 b	107.75 c	28.75 b
RBM02	12.68 a	4.22 a	1.96 a	1.51 a	120.34 a	35.62 a
Yeast						
2R1CB	9.98 c	2.86 c	0.82 c	0.72 c	95.33 d	23.25 c
LL01	9.74 c	1.78 d	0.75 d	0.71 c	90.81 e	22.07 c
1R11CB	8.97 d	1.22 e	0.56 e	0.52 d	83.55 f	17.37 e
2R3BF	8.85 d	1.15 e	0.56 e	0.49 d	80.32 g	17.62 e
Fungicide	7.98 e	0.81 f	0.35 f	0.22 e	17.90 h	6.75 f
P. ultimum	5.07 f	0.49 g	0.19 g	0.11 f	4.77 i	3.11 g

* The values are the means ± standard deviation (SD) of 30 replicates. Different letters indicate significant difference ($p < 0.05$) according to Tukey's test.

3.5. Roots Colonized by Marine Microorganisms

At the end of the experiment, the largest population was quantified in the plants with the marine bacterium *B. subtilis* RBM02 (with 740 CFU/g) and with the marine yeasts *C. laurentii* 2R1CB and *D. hansenii* LL01 (with 430 and 421 CFU/g, respectively) (Figure 4). The micrographs performed on the roots showed an abundant presence of mycelium in the plants inoculated with *P. ultimum*, and the bacterial and yeast cells adhered to the mycelium of the phytopathogen (Figure 5).

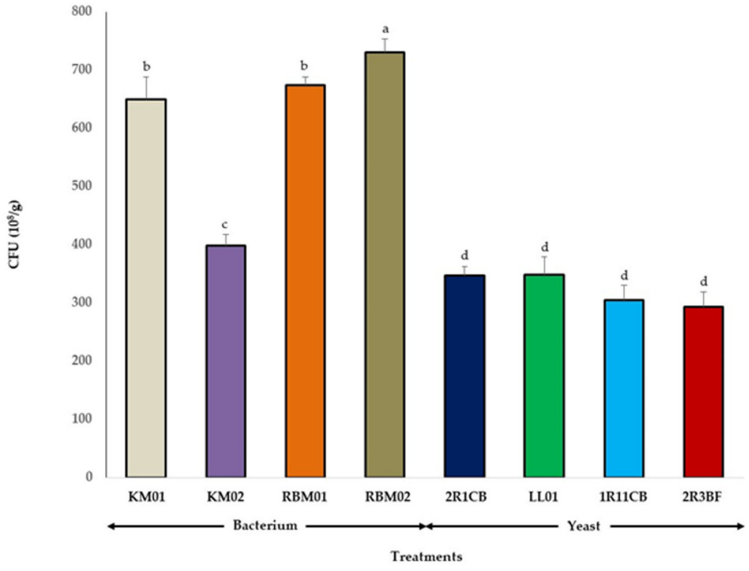

Figure 4. Colony forming units (CFU) in the roots of ancho-type pepper plants inoculated with different marine strains of bacteria and yeasts. The bacteria are *Stenotrophomonas rhizophila* (KM01 and KM02) and *Bacillus subtilis* (RBM01 and RBM02), and the yeasts are *Cryptococcus laurentii* (2R3BF and 2R1CB) and *Debaryomyces hansenii* (1R11CB and LL01). Data are shown as the mean ± standard deviation (SD) ($n = 10$). Columns with different letters were significantly different according to Tukey's test ($p < 0.05$).

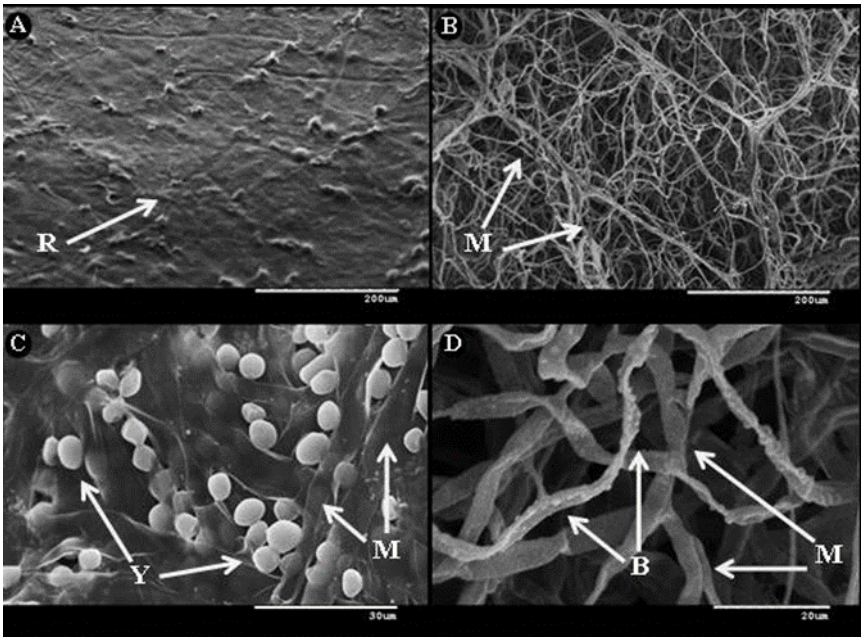

Figure 5. Micrographs from the scanning electron microscope of the inoculated roots of ancho-type pepper plants with *Pythium ultimum* and marine microorganisms. (**A**) Root (R) without microorganisms; (**B**) Oomycete Mycelium (M); (**C**) *Cryptococcus laurentii* (Y) 2R1CB; and (**D**) *Bacillus subtilis* (B) RBM02; both adhered to *P. ultimum* mycelium.

4. Discussion

Biological control with microorganisms has long been considered a viable alternative for controlling phytopathogens [36], among which bacteria and yeasts are the main antagonists [11,37]. In this study, different bacteria and yeasts decreased *P. ultimum* growth significantly in vitro; however, those that stood out most were the marine bacteria *S. rhizophila* (strain KM01 and KM02) and the marine yeast *C. laurentii* 2R1CB. The main antagonistic mechanisms of the bacteria and yeasts in vitro against the phytopathogens are the production of hydrolytic enzymes, such as β-1,3-glucanase [38,39], which break the links of the glucan present in the cell wall of the phytopathogen, limiting their germination and growth. This enzyme has already been reported for *B. subtilis* [40], *S. rhizophila* [30,41,42], and *C. laurentii* [43]. The competence for space and nutrients is another mechanism through which the bacteria and yeasts limit phytopathogens in vitro because these microorganisms have a short period of exponential growth that allows them to exhaust carbon sources in their culture medium, thereby minimizing zoospore germination and phytopathogen growth [44,45].

Marine bacteria and yeasts decreased the disease index (DI) caused by *P. ultimum* in ancho-type pepper plants. This protection was measured by diverse mechanisms of action, such as the production of siderophores, which are metabolites of a low molecular weight that catch the available iron in the soil, limiting growth of the phytopathogens that occupy this micro-element in different metabolic pathways, as well as in DNA [46]. The host's resistance to induction through the expression of different proteins, such as the hydrolytic proteins related to pathogenicity (protein PR), antioxidants, and the genes related to plant defense, is another mechanism exerted by bacteria and yeasts to biocontrol phytopathogens [47]. The yeast *C. laurentii* and the bacterium *B. subtilis* have already been reported as inductors of salicylic acid (SA) and jasmonic acid (JA) in plants. First, both genes are related to the defense of tomato against *Botrytis cinerea* and *Alternaria alternata* [48]; second, they are related to the protection of grapevine against *B. cinerea* [49]. On the other hand, *Stenotrophomonas* species have induced PR proteins in potato, thus decreasing the severity of *Ralstonia solanacearum* [50].

An increase was quantified in all the growth parameters of the ancho-type pepper plants inoculated with marine bacteria and yeasts. This marine microorganism's capacity to increase plant growth

is related to different mechanisms, among which hormone production is important (of hormones such as indole ascetic acid (IAA) and gibberellins [51]), as well as phosphate solubilization [52] and atmospheric nitrogen fixation to soil [53], among others. With respect to the bacteria, several studies have reported the capacity of *S. rhizophila* to promote plant growth in cucumber [54], soy [55], and basil [29], as well as the different *Bacillus* species effects on the growth of tomato [56], wheat [57], and banana [58]. The action of yeasts is related to phytopathogen biocontrol. This study is the first report on the protection of ancho-type pepper plants against *P. ultimum* by applying marine bacteria (*S. rhizophila, B. subtilis, B. amyloliquefaciens*, and *Pseudomonas* spp.) and marine yeasts (*D. hansenii* and *C. laurentii*) (besides their roles as plant growth promoting microorganisms).

Finally, oomycete biocontrol efficiency and plant growth promoters are related to the capacity of the microorganisms to colonize and persist in plant roots and displace the host phytopathogen [59,60]. Therefore, it is important to select organisms that are capable of colonizing the plant rhizosphere rapidly and have prolonged persistence though time [61]. Further, in-depth studies should be performed on the mechanisms of action of marine microorganisms against *P. ultimum* to determine the minimum inhibitory concentration of each of them against the oomycete and to design agricultural management practices that limit the phytopathogens of soil under different agricultural cultivations.

5. Conclusions

The marine bacteria *S. rhizophila* and marine yeast *C. laurentii* showed the greatest antagonistic activity against *P. ultimum* in vitro and in vivo. The ancho-type pepper plants inoculated with the different marine bacterial and yeast strains showed the highest values in all the growth variables assessed. The disease index (DI) of the inoculated plants with *P. ultimum* and the different marine bacterial and yeast strains showed the lowest indexes in damage, surpassing the protective effect of the synthetic fungicide. The antagonism of marine bacteria and yeasts to phytopathogens is comparable with the biocontrol by microorganisms isolated from terrestrial environments. Therefore, marine microorganisms may have the potential to be used in plant bioprotection against phytopathogens and as plant growth promoters.

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