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Identification and Biological Control of Fungal Pathogens Associated with Cactus Pear Diseases in Morocco

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Abstract

The cactus pear (*Opuntia* spp.) is a crop of major economic and ecological importance in arid and semi-arid regions. However, with its domestication and intensification, symptoms of fungal diseases have begun to emerge in different cultivation areas. This study was conducted to identify the pathogenic fungi associated with symptoms observed on cladodes in different regions of Morocco and to evaluate the effectiveness of bacterial and fungal antagonists. The study enabled the isolation and identification of several fungal agents from symptomatic cladodes, namely *Alternaria alternata*, *Alternaria tenuissima*, *Colletotrichum gloeosporioides*, and *Aspergillus tubingensis*. Among these pathogens, *A. alternata* proved to be the most aggressive and was therefore selected for in vitro and in vivo antagonism assays. Twelve bacterial isolates belonging to the genera *Bacillus* and *Pseudomonas*, as well as one isolate of *Trichoderma harzianum*, were evaluated for their antifungal activity. All antagonists showed significant inhibitory effects against *A. alternata* in vitro preliminary assay. However, the bacterial isolates *B. siamensis*, *B. halotolerans*, and *P. peli*, as well as *T. harzianum*, exhibited the highest efficacy. This efficacy was confirmed through direct confrontation tests in vivo on one-year-old cladodes for the three bacterial isolates. In contrast, *T. harzianum* showed significant pathogenic potential on cladodes of *O. ficus-indica* and *O. megacantha*. Investigation of the mechanisms of action of the three most effective bacterial isolates revealed their ability to produce antifungal volatile organic compounds. Enzymatic analyses showed differential production of amylase, chitinase, cellulase and protease among the three isolates, while genes associated with the biosynthesis of antifungal lipopeptides were detected only in *P. peli*.

Keywords: Morocco; cactus pear; fungal diseases; *Bacillus*; *Pseudomonas*; *Trichoderma harzianum*

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1. Introduction

The cactus pear (*Opuntia* spp.), belonging to the family *Cactaceae*, is a xerophytic plant native to Mexico and widely cultivated in arid and semi-arid regions worldwide due to its remarkable ability to adapt to extreme climatic conditions, poor soils, and water scarcity [1]. Owing to its fleshy cladodes rich in mucilage, extensive root system, and low water requirements, this species offers multiple uses, including fruit production, animal feed, cosmetic applications, erosion control, living fences, and industrial valorization [2].

In Morocco, this species has been progressively domesticated and intensively established in several drought-prone areas, becoming a strategic crop within national policies aimed at climate resilience and rural development [1]. Prior to 2014, the cultivated area exceeded 150,000 hectares, particularly in the regions of Fez–Meknes, Beni Mellal–Khenifra, Marrakech–Safi, and Casablanca–Settat. However, this expansion was abruptly halted by the accidental introduction of the cactus cochineal *Dactylopius opuntiae*, a highly destructive pest that caused the devastation of large areas of cactus pear plantations in Morocco and severely threatened the sustainability of the sector [3].

To limit the spread of the pest, an emergency action plan was launched in July 2016. This plan included a research program integrating several major management strategies, notably varietal resistance, the use of biopesticides, and the deployment of beneficial insects [4]. This approach reflects the increasing importance of biological control within crop protection programs in Morocco, particularly for cactus pear cultivation. Within this framework, several biological control methods have been evaluated against the cactus cochineal, focusing on different biocontrol agents such as predators including *Cryptolaemus montrouzieri* [5], entomopathogenic nematodes [6], plant-derived biopesticides [4,7], as well as entomopathogenic fungi and bacteria [8–10].

Following this crisis, recovery efforts led to the introduction of resistant varieties, the establishment of nurseries, and the resumption of plantations in several regions. Nevertheless, this recovery phase, often associated with intensive agricultural practices such as irrigation, fertilization, increased planting density, and the use of uncontrolled planting material [8–10], may favor the emergence of new phytosanitary problems that were previously considered negligible in the current context of cactus pear cultivation in Morocco. Worldwide, cactus pear, once considered a highly resilient crop, is now exposed to an increasing diversity of diseases affecting its various organs in production areas [11]. These diseases are mainly caused by biotic agents, including bacteria, yeasts, fungi, phytoplasmas, and viruses. However, fungi represent the predominant group of biotic agents and are responsible for significant damage to cladodes, roots, and fruits during both pre-harvest and post-harvest stages [12].

The most frequently reported symptoms worldwide include black and brown lesions on cladodes, anthracnose, root rot, stem and crown rot, as well as charcoal spot. A wide diversity of fungal taxa is directly associated with these symptoms, particularly species belonging to the genera *Alternaria*, *Fusarium*, *Colletotrichum*, *Lasiodiplodia*, *Curvularia*, *Diplodia*, and *Pseudocercospora*. The management of these symptoms mainly relies on the removal and destruction of infected cladodes and plant material in order to limit the spread of the disease [13]. However, this practice quickly shows its limitations, particularly under conditions of severe infection. Therefore, several authors have evaluated a wide range of chemically active ingredients, despite the absence of registered chemical products for the control of these diseases [14,15]. Nevertheless, the excessive use of chemical fungicides represents a real threat to the environment and human health and may promote the emergence of resistant phytopathogenic fungi [16].

These risks justify the adoption of environmentally friendly alternative approaches, such as the use of chitosan, a natural and biodegradable compound widely employed in

the control of fungal diseases affecting agricultural crops, including cactus pear [11]. In addition, biological control using antagonistic microorganisms has shown strong potential across several cropping systems. The genus *Trichoderma* represents a particularly effective antagonist for the control of phytopathogenic fungi, and the efficacy of its species has been demonstrated against diseases affecting cactus pear (*Opuntia* spp. and *Nopalea* spp.) [16,17]. Moreover, bacteria belonging to the genus *Bacillus* have shown strong antagonistic activity against stem rot of grafted cactus caused by *Bipolaris cactivora*, one of the most important fungal diseases affecting ornamental cacti [18].

The objective of this study is to isolate and identify the fungal pathogens responsible for the symptoms observed in the field on cladodes of *O. ficus-indica* and *O. megacantha*. It also aims to subsequently evaluate the antagonistic effects of bacteria and fungi isolated from the cactus rhizosphere against these pathogens.

2. Results

2.1. Identification of Fungi Associated with Cactus Diseases

Based on macroscopic and microscopic observations, four fungal species were isolated and identified from symptomatic cladodes collected in the field. *Colletotrichum gloeosporioides* was associated with symptoms of cladode yellowing followed by necrosis and desiccation (Figure 1a). On PDA, it produced a dense, white, cottony mycelium with brown–orange acervuli, and microscopic examination revealed hyaline, septate hyphae and cylindrical, unicellular conidia (Figure 2a,b). Deeply penetrating black necrotic lesions and cladode perforations (Figure 1b,c). were caused by *Alternaria alternata*, which formed circular colonies with dense aerial mycelium ranging from grayish-green to black. Microscopically, this species produced brown, multicellular, ovoid to ellipsoidal conidia with transverse and occasional longitudinal septa, arranged in chains (Figure 2c,d).

Figure 1. Symptoms observed in the field. (**a**) Yellowing of cladodes progressing to necrosis and subsequently to desiccation, observed in a hedge of *Opuntia megacantha* near the city of Kenitra (34.40809° N, 6.52863° W). (**b**) Black necrotic spots of varying sizes penetrating deeply and causing perforations in the affected cladodes (**c**), observed in a hedge of *O. ficus-indica* near the city of Meknes (33.81479° N, 5.42496° W). (**d**) Black necrotic spots of variable sizes that do not progress deeply and dry out rapidly, leaving corky scars on the cladodes (**e**), observed in plantations of *O. megacantha* and *O. ficus-indica* near the city of Taza (34.42545° N, 3.98964° W).

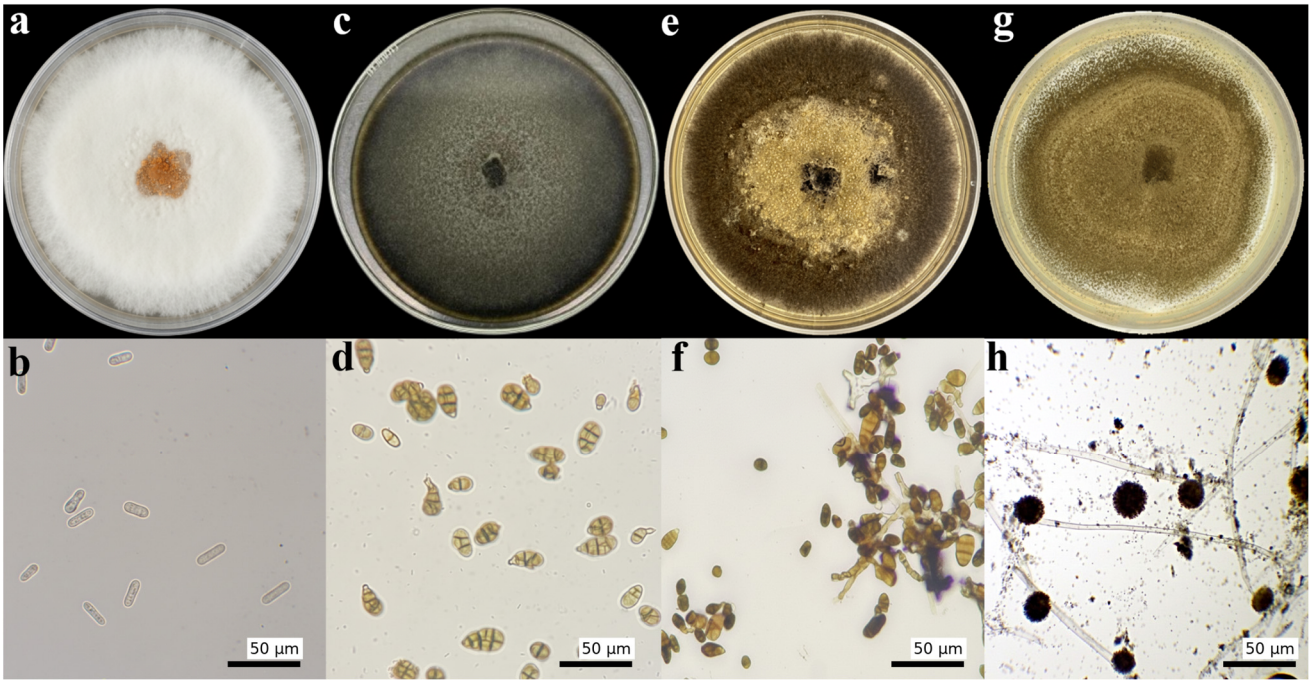

Figure 2. Representative macroscopic and microscopic (×100) observations of the five isolated fungi. (**a**,**b**): *Colletotrichum gloeosporioides*. (**c**,**d**): *Alternaria alternata*. (**e**,**f**): *Alternaria tenuissima*. (**g**,**h**): *Aspergillus tubingensis*. Field symptoms associated with each isolate are presented in Figure 1 and described in the text.

Rapidly drying black necrotic spots leaving corky scars were attributed to *Alternaria tenuissima* (Figure 1d,e). Colonies on PDA were olive-brown to dark gray with concentric zones, and microscopic observations showed dark, multicellular conidia with transverse and occasional longitudinal septa (Figure 2e,f). Postharvest soft rot of cladodes was associated with *Aspergillus tubingensis*, which exhibited fast-growing, dark greenish-black to brownish-black, powdery colonies on PDA and typical clavate conidial heads producing spherical, slightly roughened conidia (Figure 2g,h). Molecular identification of the four pathogenic agents is presented in Tables 1 and S2.

Table 1. Molecular identification of antagonistic and pathogenic microorganisms isolated in this study.

Isolate	Gene Bank Accession Number		Identity (%)	Dentification (Accession Number to Gen Bank)	
I1	*ITS*	PP944993.1	99.59	(OR810388.1)	*Alternaria alternata*
	β-tubulin	PX925246	100.00	(XM_018533191.1)	
I2	*ITS*	PX715475.1	100.00	(LC884982.1)	*Aspergillus tubingensis*
	β-tubulin	PX925245	100.00	(LC573675.1)	
I3	*ITS*	PX715474.1	99.57	(MT550036.1)	*Alternaria tenuissma*
	β-tubulin	PX925247	100.00	(MN752247.1)	
I4	*ITS*	PX715476.1	99.45	(MH636863.1)	*Colletotrichum gloeosporioides*
	β-tubulin	PX890883	99.01	(MT513061.1)	
Trichoderma sp.	*ITS*	PX715477.1	99.80	(OP935742.1)	*Trichoderma harzianum*
	β-tubulin	PX925248	99.62	(FJ884164.1)	
B1	PX260927.1		99.04	(JF804770.1)	*Bacillus siamensis*
B2	PX260541.1		99.03	(MN400022.1)	*Bacillus halotolerans*
B3	PX262059.1		99.55	(PX048790.1)	*Pseudomonas peli*

2.2. Pathogenicity Test

The pathogenicity test confirmed the pathogenic potential of the four isolated fungi on *Opuntia ficus-indica* and *O. megacantha* (Figure 3). Comparative pathogenicity assays showed similar disease symptoms and progression in both species, with no statistically significant differences detected. Consequently, data from the two cactus species were pooled for analysis and interpretation. Significant differences were observed among the tested isolates. *A. alternata* proved to be the most aggressive fungus, showing the greatest progression of the disease compared with the other three fungi, and induced lesions with a mean radius of 5.18 ± 1.03 mm. This was followed by *A. tubingensis*, *A. tenuissima*, and *C. gloeosporioides*, which produced lesions with mean radii of 4.08 ± 0.95 mm, 2.72 ± 0.71 mm, and 1.97 ± 0.92 mm, respectively (Table 2). Koch's postulates were fulfilled, as the fungi re-isolated from lesions on the cladodes during the pathogenicity test corresponded exactly to the four isolates initially obtained from field-collected samples (Figure 3).

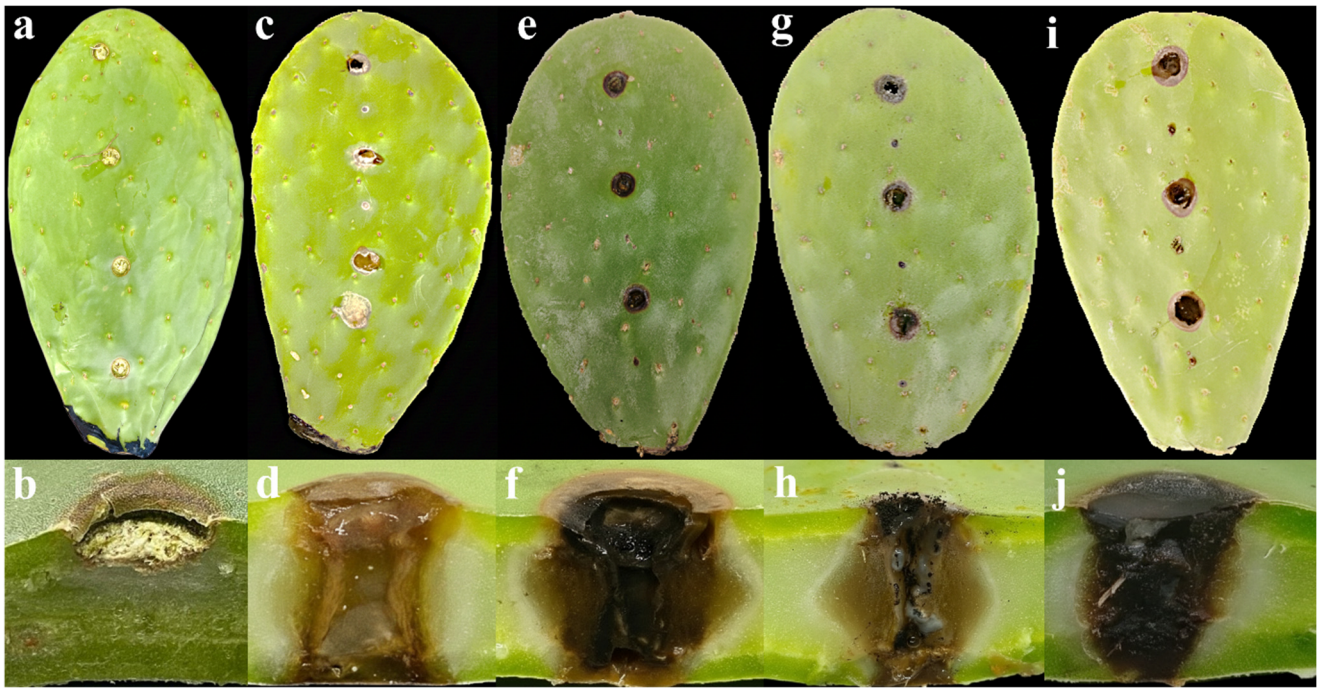

Figure 3. Symptoms caused by the four fungal isolates on one-year-old cladodes, 15 days after inoculation. (**a**,**b**): Control. (**c**,**d**): Symptoms caused by *Colletotrichum gloeosporioides*. (**e**,**f**): Symptoms caused by *Alternaria tenuissma*. (**g**,**h**): Symptoms caused by *Aspergillus tubingensis*. (**i**,**j**): Symptoms caused by *Alternaria alternata*.

Table 2. Pathogenic effect of the different fungal isolates expressed as the mean radius of lesion development in the plant material ± standard deviation.

Isolates	Mean Lesion Radius (mm)
Alternaria alternata	5.18 ± 1.03 ^{e}
Aspergillus tubingensis	4.08 ± 0.95 ^{d}
Alternaria tenuissma	2.72 ± 0.71 ^{c}
Colletotrichum gloeosporioides	1.97 ± 0.92 ^{b}
Control	0.00 ± 0.00 ^{a}
	p value < 0.001

Means followed by the same letter belong to the same homogeneous group according to Tukey test.

2.3. Biological Control of Cactus Pear Pathogens

2.3.1. Preliminary Screening Assay

A total of 12 bacterial isolates and one fungal isolate belonging to the genus *Trichoderma* were recovered from the rhizospheric soil of cactus plants. The antagonistic potential of all isolates was evaluated in vitro exclusively against *A. alternata*, which was identified as the most aggressive pathogen among the four fungal isolates. The dual culture assay revealed a statistically significant antagonistic effect for all tested isolates ($p < 0.001$). Notably, bacterial isolates B1, B2, and B3 exhibited significantly higher inhibition rates than the remaining isolates, reaching values of 77.90 ± 0.50%, 77.70 ± 1.48%, and 76.24 ± 0.88%, respectively, as indicated by the statistical groupings presented in Table 3. *Trichoderma* sp. also showed strong antagonistic activity, with an inhibition rate of 73.16 ± 4.35%. Representative images of the antagonistic interactions are shown in Figure 4. The most effective antagonists were subsequently selected for molecular identification and further experiments (Tables 1 and S1).

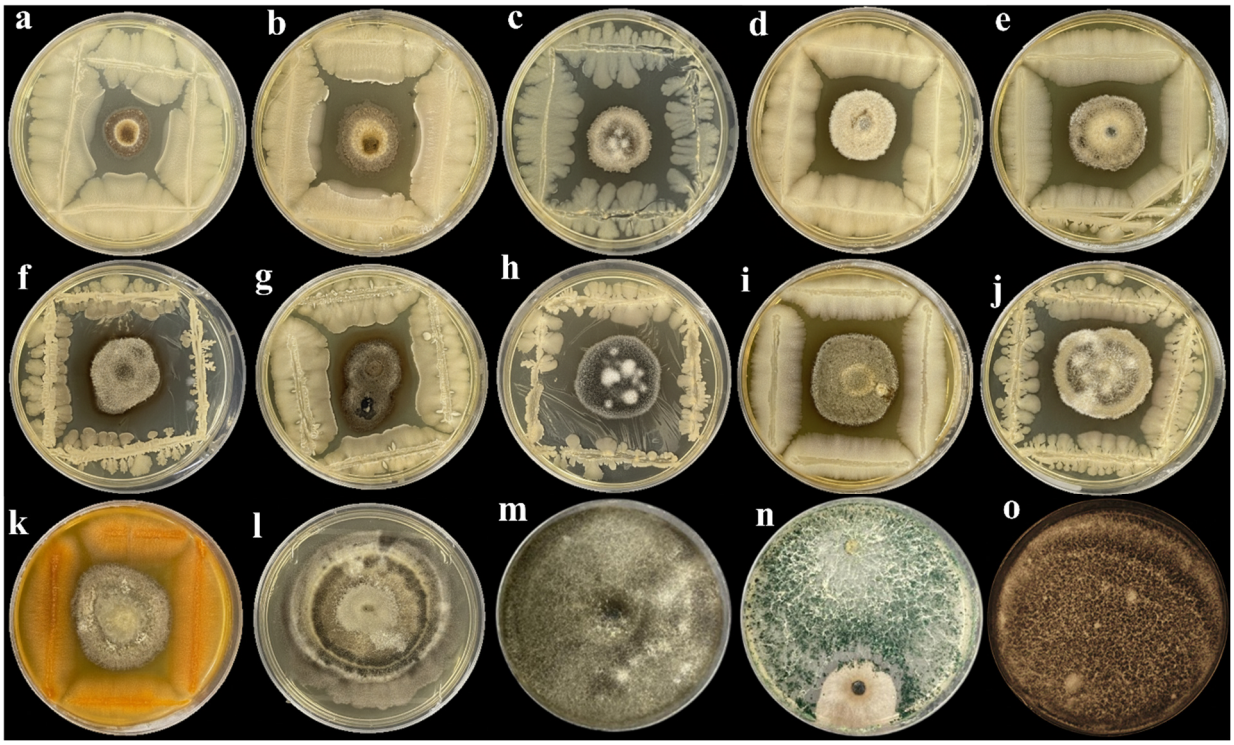

Figure 4. Representative images of the in vitro antagonistic effects of the isolates on the mycelial growth of *A. alternata* compared with the control. (**a**) Effect of bacterial isolate B1. (**b**) Effect of bacterial isolate B2. (**c**) Effect of bacterial isolate B3. (**d**) Effect of bacterial isolate B4. (**e**) Effect of bacterial isolate B5. (**f**) Effect of bacterial isolate B6. (**g**) Effect of bacterial isolate B7. (**h**) Effect of bacterial isolate B8. (**i**) Effect of bacterial isolate B9. (**j**) Effect of bacterial isolate B10. (**k**) Effect of bacterial isolate B11. (**l**) Effect of bacterial isolate B12. (**m**) Control for the bacterial assays. (**n**) Effect of *Trichoderma* sp. (**o**) Control for the *Trichoderma* sp. assay. Quantitative measurements of mycelial growth inhibition and the corresponding statistical analyses are presented in Table 3.

Table 3. Antagonistic effect of the different bacterial isolates and *Trichoderma* sp. against *A. alternata*, expressed as mean inhibition rate ± standard deviation.

Treatment	Inhibition (%)
B1	77.90 ± 0.50 ^{g}
B2	77.70 ± 1.48 ^{g}

Table 3. *Cont.*

Treatment	Inhibition (%)
B3	76.24 ± 0.88 [g]
Trichoderma sp.	73.16 ± 4.35 [f]
B4	72.40 ± 0.90 [e]
B5	70.77 ± 1.13 [e]
B6	70.23 ± 3.17 [e]
B7	69.82 ± 0.29 [d]
B8	69.79 ± 1.63 [d]
B9	69.33 ± 1.28 [d]
B10	68.88 ± 1.01 [c]
B11	68.77 ± 0.99 [c]
B12	46.80 ± 0.00 [b]
Control	0.00 ± 0.00 [a]
	p value < 0.001

Means followed by the same letter belong to the same homogeneous group according to the Tukey test.

2.3.2. In Vivo Direct Confrontation Assay

The in vivo evaluation of the effect of the three bacterial isolates B1, B2, and B3 on the development of *Alternaria alternata* revealed a significant antagonistic activity for all isolates tested. The mean lesion radius in the positive control (pathogen alone) reached 4.92 ± 1.24 mm, indicating strong infection progression. Cladodes treated with isolates B2 and B3 showed no lesion development, indicating a complete inhibition of the pathogen, with a lesion radius of zero. In contrast, cladodes treated with isolate B1 exhibited slight pathogen development, with a mean lesion radius of 0.98 ± 1.02 mm, which was significantly greater than zero (Table 4 and Figure 5). These results demonstrate that pre-treatment with the selected rhizospheric bacterial isolates significantly reduced or completely inhibited disease development under in vivo conditions, thereby confirming their effective biocontrol potential against *A. alternata* as previously observed during the preliminary in vitro direct confrontation assay.

Table 4. In vivo effect of bacterial isolates B1 (*Bacillus siamensis*), B2 (*B. halotolerans*), B3 (*Pseudomonas peli*) and *Trichoderma harzianum* on *Alternaria alternata*, expressed as mean lesion radius on plant material ± standard deviation.

Treatment	Fungi Evolution
B3	0.00 ± 0.00 [d]
B2	0.00 ± 0.00 [d]
B1	0.98 ± 1.02 [c]
T. harzianum	5.71 ± 1.00 [a]
Negative control	0.00 ± 0.00 [c]
Positive control	4.92 ± 1.24 [b]
	p value < 0.001

Means followed by the same letter belong to the same homogeneous group according to the Tukey test.

Given its strong antagonistic potential observed in vitro, *Trichoderma harzianum* was also tested in vivo. However, the results revealed an ambiguous situation: soft rots accompanied by the marked development of a greenish fungus appeared around the inoculation wells. These symptoms do not correspond to those of *A. alternata*, which typically produces a dry, black rot, but are characteristic of *T. harzianum* itself (Figure 5c,d). Comparison of lesion radii caused by *T. harzianum* with those observed in the positive control showed a significantly greater lesion development under the experimental conditions tested (Table 4).

However, since Koch's postulates were not formally fulfilled, these findings should be interpreted with caution.

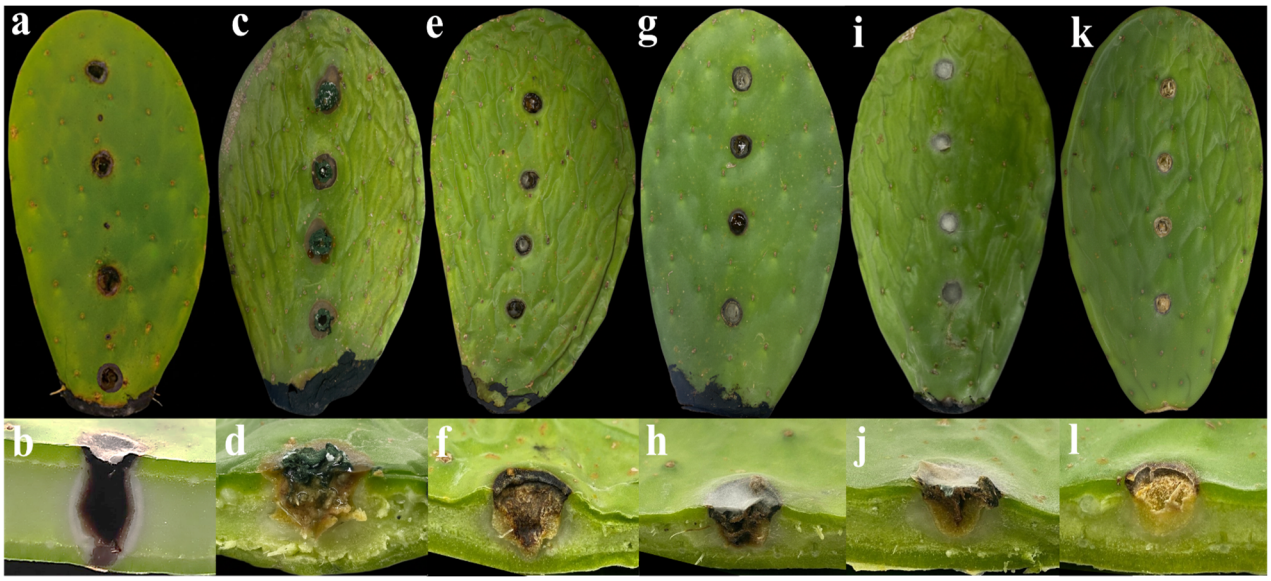

Figure 5. Representative images of in vivo antagonistic effects on cladodes. (**a**,**b**): Positive control. (**c**,**d**): Symptoms caused by *Trichoderma harzianum* on cladodes. (**e**,**f**): Effect of bacterial isolate B1 (*Bacillus siamensis*) on the in vivo development of *Alternaria alternata*. (**g**,**h**): Effect of bacterial isolate B2 (*B. halotolerans*) on the in vivo development of *A. alternata*. (**i**,**j**): Effect of bacterial isolate B3 (*Pseudomonas peli*) on the in vivo development of *A. alternata*. (**k**,**l**): Negative control. Quantitative analysis of lesion development and corresponding statistical results are presented in Table 4.

2.4. Modes of Action of Antagonistic Microorganisms

2.4.1. Indirect Confrontation Assay

The test revealed a significant reduction in the growth of *A. alternata* during indirect confrontation with the three bacterial isolates compared to the control. This inhibition suggests the secretion of volatile organic compounds with inhibitory effects on the growth of *A. alternata*. The Tukey test showed a significant difference among the three bacterial isolates tested, identifying isolate B3 as the most effective, with a mycelial growth inhibition rate of 47.34 ± 1.38% (Table 5).

Table 5. Growth of *Alternaria alternata* during indirect confrontation with different bacterial isolates (B1 (*Bacillus siamensis*), B2 (*B. halotolerans*), B3 *(Pseudomonas peli*)) (mean inhibition rate ± standard deviation, %).

Isolate	Inhibition (%)
B3	47.34 ± 1.38 [a]
B1	42.95 ± 2.98 [b]
B2	35.12 ± 4.43 [c]
Control	0.00 ± 0.00 [d]
	p value < 0.001

Means followed by the same letter belong to the same homogeneous group according to the Tukey test.

2.4.2. Evaluation of Enzymatic Activity of Antagonistic Bacteria

The evaluation of enzymatic activity of the three bacterial isolates selected for their strong antagonistic potential revealed a marked variability depending on the type of enzyme studied ($p < 0.001$) (Figure S1). The highest amylase activity was observed in isolate B2 (0.47 ± 0.05), followed by B1 (0.44 ± 0.05), while B3 exhibited the lowest activity

(0.34 ± 0.04). Regarding chitinolytic activity, isolate B1 was distinguished by a significantly higher value (0.60 ± 0.02), followed by B2 (0.45 ± 0.07), whereas B3 showed the lowest activity (0.18 ± 0.05). For protease activity, isolates B1 and B3 were the most active, with respective indices of 0.61 ± 0.04 and 0.67 ± 0.03. Finally, the results showed that all three bacterial isolates exhibited low cellulase activity, with indices of 0.07 ± 0.03, 0.09 ± 0.04, and 0.04 ± 0.06 for B1, B2, and B3, respectively. These statistically significant differences are explicitly indicated in Table 6.

Table 6. Enzymatic activity of three antagonistic bacteria (B1 (*Bacillus siamensis*), B2 (*B. halotolerans*), B3 (*Pseudomonas peli*)).

Isolate	Amylase	Chitinase	Protease	Cellulase
B1	0.44 ± 0.05 ^{b}	0.60 ± 0.02 ^{c}	0.61 ± 0.04 ^{b}	0.07± 0.03 ^{a}
B2	0.47 ± 0.05 ^{b}	0.45 ± 0.07 ^{b}	0.45 ± 0.18 ^{a}	0.09 ± 0.04 ^{a}
B3	0.34 ± 0.04 ^{a}	0.18 ± 0.05 ^{a}	0.67 ± 0.03 ^{b}	0.04 ± 0.06 ^{a}
	p value < 0.001	p value < 0.001	p value < 0.001	p value < 0.001

Means within the same column followed by the same letter are not significantly different according to the Tukey test.

2.4.3. Detection of Genes Encoding Toxins in Antagonistic Bacteria

Molecular analyses revealed that the iturin and bacillomycin genes were detected exclusively in isolate B3 (*P. peli*), whereas the surfactin, fengycin, and ericin genes were not detected in any of the bacterial isolates studied.

3. Discussion

This study enabled the identification of four fungal species whose pathogenicity was confirmed on the cladodes of *Opuntia ficus-indica* and *O. megacantha*. These fungi were isolated from symptomatic plant material collected in the field. Among the identified pathogens, *Alternari alternata* was the most aggressive, followed by *Aspergillus tubingensis*, a fungus considered a causal agent of postharvest diseases, as it was isolated from harvested cladodes, and then *A. tenuissima* and *Colletotrichum gloeosporioides*.

The molecular identification of the fungal isolates in this study was based on *ITS* and *β-tubulin* sequence analysis. Although these genes are commonly used as markers for fungal identification [19–21], their resolution may be limited within certain species complexes, such as the *A. alternata* and *C. gloeosporioides* complexes. The use of additional loci (e.g., *TEF1α*, *GAPDH*, *ACT*) is often recommended to achieve higher species-level resolution [22–24]. However, in the present study, the combined analysis of *ITS* and *β-tubulin* provided consistent results sufficient to identify the main causal agents associated with the disease. Further multilocus analyses would be required to refine species delimitation within these complexes and represent a perspective for future research.

Biological control assays were conducted against *A. alternata* due to its high aggressiveness and prevalence, using antagonistic microorganisms isolated from the rhizospheric soil of cactus pear. The results demonstrated a significant inhibitory effect of *B. siamensis*, *B. halotolerans*, and *P. peli* under both in vitro and in vivo conditions. In contrast, although *T. harzianum* exhibited strong inhibitory activity against *A. alternata* in vitro, it induced disease symptoms on one-year-old cladodes during direct in vivo confrontation. This contrasting behavior underscores the necessity of clearly distinguishing between in vitro antagonistic potential and in vivo host safety, highlights the need for caution regarding its routine application on cactus pear, and emphasizes the importance of rigorous isolate selection prior to field application.

Investigation of the modes of action of the three selected isolates revealed the involvement of multiple indirect mechanisms in their antagonistic activity against *A. alternata*.

All strains produced volatile organic compounds (VOCs) that inhibited fungal growth without direct contact and exhibited significant lytic enzyme activities (amylase, chitinase, protease, and cellulase), suggesting a role in fungal cell wall degradation. These combined mechanisms likely explain the antagonistic effects observed in vitro and in vivo. Although lipopeptide biosynthetic genes were not detected in some isolates, this does not exclude their potential to produce antifungal metabolites, given the known genetic variability within these clusters.

The antifungal activity of *Bacillus* spp. and *Pseudomonas* spp. is mainly attributed to the production of extracellular enzymes involved in fungal cell wall degradation, including chitinases, glucanases, cellulases, amylases, and proteases [25–27]. These enzymes often act synergistically with cyclic lipopeptides and other antifungal metabolites, such as volatile organic compounds, thereby enhancing antifungal efficacy [26,27]. Although genes involved in lipopeptide biosynthesis have been reported in several *Bacillus* species, their non-detection in *B. siamensis* and *B. halotolerans* in the present study should be interpreted with caution, as primer specificity and sequence variability may limit PCR-based detection. Indeed, high variability within lipopeptide biosynthetic clusters has been reported, which may account for the difficulties in detecting these genes by PCR when only specific primer sets are used [28]. Furthermore, comparative genomic analyses have revealed substantial sequence diversity in fengycin and iturin gene clusters across *Bacillus* strains, suggesting ongoing diversification within these biosynthetic clusters [29].

Research on diseases associated with cactus pear remains limited, mainly due to the nature of this crop, which is predominantly cultivated in arid and semi-arid regions characterized by dry climates, where plant diseases rarely cause economically significant losses [30]. Black spot disease is the most widespread disease in cactus production areas worldwide. Numerous studies conducted in Mexico have focused on isolating and identifying the fungal pathogens responsible for this disease. Flores-Flores et al. (2013) [11] identified seven fungal isolates associated with BS, belonging to different species, identified several fungal isolates associated with black spot disease, including *C. gloeosporioides*, *A. alternata*, *Fusarium lunatum*, and *Curvularia lunata*. In the same context, Chavarría-Cervera et al. (2024) [31] identified, in addition to *A. alternata*, two other fungal pathogens responsible for black spot disease, namely *Corynespora cassiicola* and *Neoscytalidium dimidiatum*. Furthermore, Montiel-Salero et al. (2022) [32] demonstrated that *A. alternata* is the causal agent of golden spot disease on *O. matudae*.

In Brazil, the most common disease affecting cactus pear (*Nopalea cochenillifera*) is cladode brown spot disease. According to Infante et al. (2021) [33], this disease is associated with several *Alternaria* species, including *A. alternata*, *A. tenuissima*, *A. longipes*, *A. gossypina*, *A. jacinthicola*, and *A. tomato*. In addition, Oliveira et al. (2018) [34] identified several *Colletotrichum* species responsible for CBS on *N. cochenillifera*, namely *C. siamense*, *C. fructicola*, and *C. karstii*. Similar symptoms have also been reported on cladodes and fruits of *O. ficus-indica* in Egypt. The causal agents were identified as *A. alternata*, *Botryodiplodia theobromae*, and *F. solani* [14]. In Europe, scabby canker disease has been reported on *O. ficus-indica* and was associated with species belonging to the Botryosphaeriaceae family [35].

In Morocco, only one study has previously reported diseases affecting cactus pear. That study described symptoms of cladode and fruit browning, as well as dark spot formation, in production orchards located in the southern regions of the country [36]. These symptoms were associated with the genera *Alternaria*, *Fusarium*, and *Mycosphaerella*, without species-level identification based on molecular analyses. In contrast, the present study provides a detailed characterization of fungal pathogens associated with cactus diseases in Morocco through a combination of morphological observations, pathogenicity tests, and molecular identification. To the best of our knowledge, this work represents

the first report in Morocco providing species-level identification of fungal pathogens affecting cactus pear. Furthermore, it documents for the first time the association of *A. tubingensis* with postharvest soft rot of cladodes of *O. ficus-indica* and *O. megacantha* under the conditions investigated.

Studies focusing on the control of diseases affecting cactus pear remain even more limited, largely because these diseases generally do not cause economically significant losses. The application of chitosan and various fungicidal active ingredients has nevertheless been shown to significantly reduce mycelial growth and disease severity under both in vitro and in vivo conditions. Although efficacy varies depending on the pathogen and the active compound, these treatments have generally proven effective in reducing disease incidence in cactus pear production systems [11,13,15].

Several *Trichoderma* species have also been evaluated as biocontrol agents against the soilborne fungi *F. solani* and *Lasiodiplodia theobromae*, which are responsible for basal cladode rot in forage cactus (*N. cochenillifera*). In vitro confrontation assays showed that *T. aureoviride* significantly reduced the mycelial halo growth of *F. solani* and *L. theobromae* by 34% and 89%, respectively. Inoculation of the same isolate into forage cactus soils also demonstrated significant efficacy [16]. The antagonistic effect of *T. harzianum*, *T. viride*, *T. atroviride*, and *T. hamatum* was also evaluated against *Pythium* sp., *Fusarium* sp., and *Colletotrichum* sp., pathogens of *O. ficus-indica*. The results showed that *T. harzianum* was the most effective against *Fusarium* sp. and *Colletotrichum* sp., with inhibition rates of 73.7% and 68%, respectively. *T. hamatum* exhibited high efficacy against *Colletotrichum* sp. and *Pythium* sp., with inhibition rates of 71.6% and 73.8%, respectively. However, this study was limited to in vitro assays, with no evaluation on plant material [17].

Although *T. harzianum* showed significant antagonistic activity against *A. alternata* in vitro in the present study, its in vivo application on detached cladodes of *O. ficus-indica* and *O. megacantha* resulted in soft rot symptoms. However, Koch's postulates were not formally fulfilled for this isolate, and therefore these observations should be interpreted with caution. The observed symptoms may reflect opportunistic colonization under artificial wound inoculation and high-humidity experimental conditions rather than true pathogenicity under natural field conditions. While pathogenic behavior of *Trichoderma* species has been reported in other crops, including maize, where several species have been associated with ear rot disease [37]. Pathogenicity of *Trichoderma* species is associated with excessive colonization of host tissues, mediated by hydrolytic enzymes and fungal metabolites that disrupt plant cell walls and suppress plant defense responses [38–40]. In our case, inoculation of *T. harzianum* on detached cladodes may have favored the expression of pathogenicity, as host control mechanisms are likely reduced under these conditions. Further studies on healthy, intact *Opuntia* plants are therefore required to confirm this behavior.

Several studies have highlighted the antagonistic potential of bacteria belonging to the genera *Bacillus* and *Pseudomonas* as biocontrol agents against *A. alternata* on various crops. including tomato, pepper, bean, and tobacco [41–44]. In Cactaceae, *Bacillus* spp. have shown high efficacy against fungal diseases affecting grafted cactus and pitaya under both in vitro and in vivo conditions [18]. However, to the best of our knowledge, no studies have been published on the use of bacteria as an alternative control strategy against diseases affecting cactus pear. The present study is therefore the first to report the effectiveness of these microorganisms against *A. alternata* on *O. ficus-indica* and *O. megacantha*.

Although these bacteria were isolated from the rhizosphere of cactus pear, their reintroduction at high inoculum densities may have ecological implications beyond their biocontrol activity, potentially disrupting the native microbial balance [45]. In addition, the efficacy observed under laboratory conditions may vary under field conditions, as the performance of bacterial biocontrol agents strongly depends on soil physicochemical

properties, environmental factors, and the structure of native microbial communities [46,47]. Regulatory and biosafety assessments will also be necessary prior to large-scale agricultural application. These considerations highlight the need to evaluate both agronomic efficacy and ecological impact under variable environmental conditions. Consequently, field trials and rhizospheric microbiome analyses will be essential to assess the long-term sustainability of these bacteria in cactus pear production systems.

4. Materials and Methods

4.1. Isolation and Purification of Pathogenic Agents

During the collection of cactus cochineal samples from hedgerows of cactus pear (*Opuntia ficus-indica* and *O. megacantha*) in various regions of Morocco (34.40809° N, 6.52863° W; 33.81479° N, 5.42496° W; 34.42545° N, 3.98964° W), symptoms of fungal diseases were observed on the cladodes. These symptoms were characterized by dry and black necrotic spots of varying sizes, which gradually developed into crusts and eventually perforations. Yellowing that progressed to necrosis and finally to complete tissue desiccation was also noted (Figure 1). Three infected cladodes were randomly collected from three different plants within each cactus plantation exhibiting disease symptoms and transported to the Phytopathology Laboratory of the National School of Agriculture in Meknes (ENAM) for the isolation, purification, and identification of the pathogenic agents. Additionally, symptoms of soft rot were observed on apparently healthy one-year-old cladodes that had been collected for research purposes and kept in the laboratory for approximately two weeks.

Pathogen isolation was performed from small fragments taken from the interface between symptomatic and asymptomatic tissues, with four fragments collected per cladode sampled in the field, using a sterile scalpel. Under a laminar flow hood, the fragments were surface disinfected in 3% sodium hypochlorite for 15 min, rinsed three times with sterile distilled water, and then left to dry on sterile absorbent paper. The disinfected fragments were subsequently placed in 9 cm diameter Petri dishes containing Potato Dextrose Agar (PDA) medium and incubated at 25 °C for one week in dark. The resulting fungal colonies were purified and preserved for further analyses and experiments. Colonies exhibiting similar macroscopic and microscopic characteristics were considered to belong to the same isolate. This approach was intended to identify the predominant fungal species associated with the disease symptoms rather than to assess intraspecific diversity. While morphologically similar colonies may potentially represent genetically distinct strains, the objective of this study was to determine the main causal agent of the disease.

4.2. Pathogenicity Test and Validation of Koch's Postulates

The pathogenicity of the purified fungal isolates was evaluated on two cactus species, *Opuntia ficus-indica* and *O. megacantha*, using a completely randomized design with five replicates per isolate and a control group for each cactus species. Each replicate consisted of one independent one-year-old cladode obtained from a different healthy plant, resulting in five plants per isolate. The cladodes were collected from the Domaine El Bassatine collection (33.89415° N, 5.49659° W), part of the Les Domaines Agricoles group. Wounds resulting from sampling were sealed with grafting mastic to prevent contamination by external fungi. After the mastic had dried, the plant material was surface-sterilized under a laminar flow hood by immersion in a 3% calcium hypochlorite solution for 15 min, followed by three rinses with sterile distilled water and drying on sterile absorbent paper. Once dry, the disinfected cladodes were placed in sterile plastic boxes containing moistened paper to maintain an environment suitable for pathogen survival and development.

A volume of 100 µL of a spore suspension at a concentration of 10^4 spores/mL [48] was inoculated into wells made on the cladodes using sterile micropipette tips (100–1000 µL). Three wells were made for each replication. An additional inoculation by injection with the same volume of spore suspension was also carried out to ensure that the symptoms observed on the cladodes were indeed caused by the inoculated fungi, as the syringe wounds were very fine and minimized the risk of external contamination. The control group was inoculated with 100 µL of sterile distilled water. The inoculated cladodes were incubated for 15 days at 25 °C in darkness to recreate conditions favorable for pathogen development. At the end of the incubation period, symptom severity was assessed by measuring lesion size using a caliper.

To validate Koch's postulates, a new isolation was performed from the observed lesions, and the resulting fungal colonies were compared with the originally isolated strains using the same isolation and purification protocol described for the initial fungal recovery. The re-isolated strains were subjected to the same cultural and morphological characterization procedures as the original isolates and were found to be identical. Furthermore, pathogenicity validation was conducted following the same experimental conditions and inoculation protocol described above, confirming that the re-isolated fungus induced identical symptoms on healthy cladodes. This sequential confirmation ensured that the observed disease symptoms were exclusively caused by the inoculated fungal species.

4.3. Molecular Identification of Pathogenic Fungal Isolates

DNA extraction from the pathogenic isolates was performed using mycelial tissue following the protocol of Doyle and Doyle (1987) [49]. Molecular identification was carried out using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). To confirm the identities obtained using the ITS gene, a fragment of the *β-tubulin* gene was amplified using the primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGACCCTTGGC-3′). PCR amplification was conducted in a total reaction volume of 25 µL, consisting of 2.5 µL of template DNA, 1.25 µL of each primer, 7.5 µL of sterile distilled water, and 12.5 µL of DreamTaq™ Green PCR Master Mix (×2) (Thermo Fisher Scientific, Waltham, MA, USA). For *ITS* amplification, the PCR cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 35 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension step was performed at 72 °C for 10 min. Purification and sequencing of the PCR products were conducted following the Applied Biosystems™ protocol. For *β-tubulin* amplification, the PCR conditions included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 54 s. A final extension step was performed at 72 °C for 5 min. Purification and sequencing of the PCR products were conducted following the Applied Biosystems™ protocol.

4.4. Biological Control of Cactus Pear Pathogens

4.4.1. Isolation and Purification of Rhizospheric Antagonists

Soil samples were collected from a depth of 30 to 40 cm in the rhizosphere of cactus plants at the El Bassatine Domaine (33.89415° N, 5.49659° W). In the laboratory, the sampled soil was sieved through a 2 mm mesh to remove coarse particles. Isolation was performed using 20 g of soil mixed with 100 mL of sterile distilled water. The resulting suspension was shaken for one hour to detach microorganisms, and then subjected to a series of serial dilutions following the protocol of Fages & Mulard (1988) [50].

A volume of 100 µL of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was spread onto Petri dishes containing Luria–Bertani (LB) medium and onto dishes containing PDA medium and

incubated in darkness at 28 °C and 25 °C, respectively, to isolate antagonistic bacteria and fungi. After two days of incubation for bacteria and five days for fungi, distinct colonies were subcultured to obtain pure cultures.

4.4.2. Preliminary Screening Assay

Direct in vitro confrontation tests were performed between the most aggressive pathogenic isolates (which exhibited the greatest lesion development on cladodes during the pathogenicity test) and the antagonists isolated from cactus rhizosphere soil as part of a preliminary screening assay. The assay was conducted using a completely randomized design with four replications. For antagonistic bacteria, the protocol consisted of placing a 5 mm mycelial plug, taken from a 7-day old pathogenic fungal culture grown on PDA medium, at the center of a 9 cm diameter Petri dish containing PDA. Around the plug, four equidistant bacterial streaks, each approximately 4 cm long, were made using a sterile inoculation loop from a 24 h old bacterial culture grown in LB medium [51,52]. The control group consisted of PDA plates containing only the central mycelial plug.

For antagonistic fungi, a 5 mm mycelial plug, taken from a 7-day old culture grown on PDA, was placed at one edge of a PDA plate, while an equal-sized plug of the pathogenic fungus was placed at the opposite edge, symmetrically aligned along the diameter of the plate. The control consisted of PDA plates containing only a single pathogenic mycelial plug placed at one edge. All plates were incubated at 25 °C in the dark until complete colonization of the control plates by the pathogenic fungi, which occurred after 7 days of incubation. The antagonistic activity of bacteria and fungi was assessed by calculating the percentage inhibition of mycelial growth:

$$\mathbf{I}(\%) = \left(\frac{\mathbf{Dc} - \mathbf{Dt}}{\mathbf{Dc}}\right) \times 100$$

where:

Dc: average colony diameter in the control (mm);
Dt: average colony diameter in the presence of the antagonist (mm).

4.4.3. In Vivo Direct Confrontation Assay

To validate the efficacy of the antagonistic isolates selected during the preliminary in vitro direct confrontation assay, a preventive in vivo confrontation experiment was performed on cactus cladodes using a completely randomized design. Each treatment included four independent biological replicates, with one cladode considered as a single experimental unit. The protocol followed was the one previously described in the section dedicated to the pathogenicity test. On each cladode, four wells (technical replicates) were made on the surface. A volume of 100 µL of the antagonist suspensions, prepared at a concentration of 10^8 CFU/mL (corresponding to an optical density of OD = 2) for bacteria and 10^6 CFU/mL for fungi, was applied into each well. After 24 h of incubation, the same volume of the pathogenic fungal suspension, prepared at a concentration of 10^4 spores/mL, was inoculated into each well. Positive and negative controls were established by depositing 100 µL of the pathogenic fungal suspension and sterile distilled water, respectively. Cladodes were incubated in a growth chamber at 25 °C under controlled conditions. Treatments were randomly assigned to cladodes, and their position in the growth chamber was randomized to minimize positional effects. After 15 days of incubation, disease development was assessed by measuring the radius of lesions formed around each well using a digital caliper. The mean lesion radius per cladode was calculated and used for statistical analysis.

4.4.4. Molecular Identification of Antagonistic Isolates

DNA extraction and amplification of fungal isolates that exhibited significant antagonistic activity were performed using the same protocols described previously. Genomic DNA extraction from bacterial isolates was carried out following the protocol of Queipo-Ortuño et al. (2008) [53]. Amplification targeted the *16S ribosomal RNA* gene using the universal primers: forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-ACGGCTACCTTGTTACGACTT-3′. PCR amplification was conducted in a total reaction volume of 25 µL, consisting of 2.5 µL of template DNA, 1.25 µL of each primer, 7.5 µL of sterile distilled water, and 12.5 µL of DreamTaq™ Green PCR Master Mix (×2). The PCR cycling conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 90 s. A final extension step was performed at 72 °C for 7 min. Purification and sequencing of the PCR products were conducted following the Applied Biosystems™ protocol.

Although *16S rRNA* gene sequencing is widely used for bacterial identification, its conserved nature limits accurate discrimination among closely related species. Therefore, species-level identification in this study should be considered provisional. Improved resolution could be achieved using additional housekeeping genes such as *gyrB* or through multilocus or whole-genome sequencing approaches.

4.5. Modes of Action of Antagonistic Microorganisms

4.5.1. Indirect Confrontation Assay

To gain a better understanding of the modes of action of the antagonistic bacteria that showed significant efficacy in the direct confrontation tests, an indirect confrontation assay was performed to evaluate the production of volatile organic compounds. The test was conducted using a completely randomized design with four replicates.

Following the protocol proposed by Ghorri et al. (2014) [54], a 5 mm mycelial plug of the pathogen was placed at the center of a Petri dish containing PDA medium, while three equidistant bacterial streaks were drawn on another Petri dish containing LB medium. The two dishes, without their lids, were then placed face to face and sealed together with Parafilm. The control consisted of Petri dishes containing only the pathogenic fungus. Incubation was carried out at 25 °C until the pathogen completely colonized the control plates.

4.5.2. Evaluation of the Enzymatic Activity of Antagonistic Bacteria

Amylolytic Activity

The amylolytic activity of the antagonistic bacteria was assessed on a solid medium supplemented with 1% soluble starch, following the method described by Dinesh et al. (2015) [55]. Two 20 µL spots of each bacterial suspension (10^8 CFU/mL) were deposited onto the surface of the medium. After incubation at 28 °C for 72 h, 3 mL of an iodine solution were poured onto the plates to visualize starch hydrolysis. The appearance of a clear halo around the inoculation sites indicated positive amylolytic activity. Quantification was performed using the amylolytic index, calculated according to the formula proposed by Kumar et al. (2015) [56].

Chitinolytic Activity

Chitinolytic activity was evaluated on a medium containing 0.2% colloidal chitin [57]. From a bacterial suspension adjusted to an OD of 0.1, two 20 µL spots were deposited on each plate, followed by incubation at 28 °C for 5 to 7 days. Chitin degradation was evidenced by the formation of a clear halo surrounding the colonies. The chitinolytic index was calculated using the same method as for amylolytic activity.

Cellulolytic Activity

Cellulolytic activity was assessed on a solid medium supplemented with carboxymethylcellulose (CMC), following the protocol of Cattelan et al. (1999) [58]. Two 20 µL spots of each bacterial suspension were applied and incubated at 25 °C for 3 days. After incubation, a 1% Congo red solution was added for 15 min, followed by three rinses with 1 M NaCl. The presence of a clear halo around the inoculation sites indicated cellulolytic activity. The corresponding index was calculated in the same way as for the other enzymatic activities.

Proteolytic Activity

Proteolytic activity was evaluated on a solid medium containing skim milk, according to Syed-Ab-Rahman et al. (2018) [59]. Two 20 µL spots of each bacterial suspension (10^8 CFU/mL) were deposited on each plate and incubated at 28 °C for 48 h. The formation of a clear halo around the colonies indicated the hydrolysis of milk proteins, reflecting proteolytic activity [16–19]. The proteolytic index was calculated using the same formula as for the other enzymatic activities.

4.5.3. Detection of Toxin Encoding Genes in Antagonistic Bacteria

The detection of genes encoding lipopeptide toxins in bacteria exhibiting significant antagonistic activity was performed using PCR with specific primers. The targeted genes included bacillomycin (BACC1F/BACC1R), fengycin (FEND1F/FEND1R), iturin (ITUP1F/ITUP2R), surfactin (P17/P18), and ericin (Eric-F/Eric-R) [60–62]. The presence or absence of these genes was determined by analyzing the migration patterns of PCR products on an agarose gel following electrophoresis. After assessing the quality and concentration of the extracted DNA, PCR amplifications were carried out in a final reaction volume of 10 µL containing 1 µL of template DNA, 0.5 µL of each primer (forward and reverse), 3 µL of sterile distilled water, and 5 µL of DreamTaq™ Green PCR Master Mix (×2). The sequences and thermal cycling conditions of each primer are presented in Table S1 of the Supplementary Materials.

4.6. Statistical Analyses

The analysis of the different factors studied, including the mean radius of disease lesions for the pathogenicity test and the in vivo test, the inhibition rate for direct and indirect confrontation assays, and the enzymatic activity indices, was performed using one-way ANOVA after checking data normality with the Shapiro–Wilk test and homogeneity of variances with Bartlett's test. A post hoc analysis using the Tukey test was conducted after confirming the significance of the ANOVA ($p < 0.05$). All analyses were performed using SPSS software version 27. Means and standard deviations of these factors were also calculated using the same software.

5. Conclusions

In conclusion, this study reports for the first time in Morocco the identification of four fungal pathogens affecting cactus pear, with *A. alternata* identified as the most aggressive species. Biological control assays demonstrated the strong antagonistic potential of *B. siamensis*, *B. halotolerans*, and *P. peli* under both in vitro and in vivo conditions, whereas *T. harzianum*, despite its high in vitro efficacy, exhibited pathogenic behavior in vivo. These findings highlight the potential of bacterial biocontrol agents as sustainable alternatives for managing cactus pear diseases while emphasizing the importance of rigorous in vivo evaluation. Future field trials are required to validate these results under natural conditions.

In addition, further epidemiological surveys in orchards and nurseries will be essential to better assess disease prevalence and current management practices.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants15050733/s1, Table S1. Primers used for the identification of secondary metabolites; Table S2. Molecular identification of antagonistic and pathogenic microorganisms isolated in this study. Figure S1 Presentation of bacterial cultures showing the absence and presence of the four tested enzymatic activities.

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