EEET ECOLOGICAL ENGINEERING & ENVIRONMENTAL TECHNOLOGY

Ecological Engineering & Environmental Technology, 2025, 26(7), 17–29
https://doi.org/10.12912/27197050/204576
ISSN 2719–7050, License CC-BY 4.0

Received: 2025.04.25
Accepted: 2025.05.30
Published: 2025.06.15

Influence of cochineal infestation on the diversity, distribution, and biocontrol potential of endophytic bacteria in *Opuntia ficus-indica*

Ezahidi Bouchra[1*], Amir Soumia[2], Sabour Alaoui Sanaa[1]

[1] Polyvalent Research and Development Team, Polydisciplinary Faculty Sultan Moulay Sliman University of Beni Mellal, Morocco
[2] Agro-Biotechnology Team, Center of Agrobiotechnology and Bioengineering, Research Unit labeled CNRST (Centre AgroBiotech-URL-CNRST-05), Cadi Ayyad University, Marrakesh, 40000, Morocco
* Corresponding author's e-mail: ezahidibouchra84@gmail.com

ABSTRACT

Endophytic bacteria associated with *Opuntia ficus-indica* play a crucial role in the plant's immune defense mechanisms against biotic stress, particularly pest infestations. This study investigated the impact of cochineal infestation (*Dactylopius opuntiae*) on the diversity and ecological roles of endophytic bacteria associated with *Opuntia ficus-indica* in the semi-arid region of Beni Mellal, Morocco. Healthy and infested cactus cladodes were sampled, and endophytic bacteria were isolated using selective media. Bacterial identification involved morphological characterization, biochemical profiling, and molecular analysis through 16S rRNA sequencing. Thermal adaptability tests and statistical analyses (one-way ANOVA, Tukey's test) were conducted to assess strain adaptability and ecological significance. Of the 57 bacterial isolates obtained, nine dominant strains were selected based on colony abundance ($> 10^5$ CFU/mL), distinct biochemical profiles, and ecological relevance. Healthy cactus tissues demonstrated significantly higher endophytic colonization (mean = 6.5 isolates per sample) compared to infested tissues (mean = 3.0 isolates; ANOVA, $F(1.10) = 8.64$, $p = 0.014$). *Bacillus cereus* (Mg1C) and *Pseudomonas aeruginosa* (Mg1F) predominated in healthy cladodes, suggesting potential roles in pest resistance or plant defense. Conversely, *Proteus penneri* (Mg1H), typically associated with insect guts and tissue decomposition, dominated in the infested tissues. Thermal tolerance assays revealed significant adaptability differences among isolates (ANOVA, $F(8.27) = 11.87$, $p < 0.001$), notably highlighting *P. aeruginosa*, *P. putida*, and *Klebsiella pneumoniae* as highly thermotolerant strains with promising biocontrol potential. The geographic scope of this study was limited, and laboratory conditions may not entirely replicate complex field interactions. Nevertheless, the identified bacterial strains exhibit strong potential as bioinoculants in sustainable agriculture for pest management and enhancing plant resilience under stress conditions. This research uniquely elucidated the impact of pest infestation on cactus-associated endophytic communities, offering valuable insights into microbial ecology and practical agricultural management strategies.

Keywords: biological control, pest infestation management, microbial community dynamics, endophyte diversity, arid agriculture sustainability, plant microbiome, thermal stress adaptability.

INTRODUCTION

Opuntia ficus-indica L. Mill, commonly known as prickly pear cactus, is a xerophytic species native to Mexico and widely cultivated across arid and semi-arid regions worldwide, owing to its exceptional resilience to water scarcity and poor soils (Mulas and Mulas, 2004; FAO, 2017). Following its introduction into North Africa in the early 17th century, *O. ficus-indica* has become an integral component of Moroccan agroecosystems, particularly in rural and marginal areas. Between 1998 and 2009, its cultivation area in Morocco expanded from approximately 50,000 to over 120,000 hectares, with the Guelmim–Sidi Ifni region representing nearly 50% of the

national production surface (Bhira, 2012). This cactus species holds significant socio-economic and ecological value. In addition to its use as a source of fruits, forage, and cladodes, *O. ficus-indica* is traditionally exploited in ethnomedicine for its antioxidant, anti-inflammatory, and antimicrobial properties (Palmeri et al., 2020; Elmagzob et al., 2019). However, its productivity and ecological function are currently compromised by the invasion of the cochineal insect *Dactylopius opuntiae* (Cockerell), an aggressive sap-sucking hemipteran pest that inflicts extensive damage on various *Opuntia* species (Mazzeo et al., 2019).

First reported in Morocco in 2015 in the Sidi Bennour region, *D. opuntiae* has since spread rapidly, causing severe infestations characterized by the proliferation of wax-covered female colonies that feed on cactus cladodes. This feeding behavior results in substantial sap loss, tissue necrosis, and plant mortality (Deveoglu, 2020; Phipps, 2010). This pest's ecological adaptability and dispersal capacity, particularly during the mobile nymphal stage, have rendered conventional management strategies largely ineffective, thus necessitating alternative, sustainable approaches.

The plant microbiome, particularly endophytic bacteria, has emerged as a promising avenue for sustainable crop protection and stress mitigation in recent years. Endophytes, defined as non-pathogenic microorganisms that colonize internal plant tissues, play critical roles in enhancing host fitness by promoting growth, modulating stress responses, and conferring resistance to pathogens and herbivores (Compant et al., 2021; Fadiji and Babalola, 2020). These beneficial microbes are known to produce a wide array of bioactive compounds, including phytohormones, siderophores, lytic enzymes, and antimicrobial secondary metabolites, which can contribute to systemic plant defense (Becard, 2017; Ravlomanantsoa, 2004; Rowan and Latch, 2018).

The rhizospheric and endophytic microbiota of *O. ficus-indica* have been shown to harbor diverse bacterial genera, including *Pseudomonas*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Proteus*, and *Citrobacter*, some of which exhibit plant growth-promoting and biocontrol properties (Costa, 2012; Liu et al., 2017; Aguirre-Garrido et al., 2012; Rana et al., 2020). Nonetheless, the influence of biotic stress—such as insect infestation—on the composition, abundance, and functional roles of endophytic bacterial communities remains poorly understood.

Moreover, emerging evidence suggests that specific bacterial taxa may establish opportunistic associations with insect pests by exploiting host plant damage or through direct symbiosis with the insects themselves. For instance, *Proteus penneri* has been identified in insect digestive tracts and implicated in facilitating herbivory by degrading plant defense compounds (Tiwari et al., 2013; Moran, 2006). These complex and dynamic interactions underscore the necessity of characterizing endophytic microbiomes under pest-stressed conditions to identify the taxa that either support plant defense or contribute to increased susceptibility.

In this study, the authors aimed to isolate and characterize culturable endophytic bacteria from healthy and *Dactylopius opuntiae*-infested cladodes of *Opuntia ficus-indica* in the Beni Mellal–Khénifra region of Morocco. By evaluating the taxonomic diversity, growth traits, and biochemical profiles of the recovered strains, the authors sought to elucidate microbial shifts associated with biotic stress and identify candidate endophytes for potential use in integrated pest management strategies adapted to arid ecosystems.

MATERIAL AND METHODS

Sampling site

The cactus (opuntia ficus Indica) stem samples were collected in Beni Mellal, located in north-central Morocco. This zone has a hot semi-arid climate (Köppen climate classification BSh) with very hot summers and cool winters. As the city lies inland and is shielded by the Middle Atlas mountains, the climate is highly continental. It is characterized by a short-wet season (3 months) with a long dry season (9 months). Rainfall can reach up to 500 mm per year, with a temperature range of 18–45 as an arid zone; the potential evapotranspiration is around double compared to yearly precipitation (Tremblay et al., 2012) (Figure 1).

Field prospecting was carried out from April to June. The work consisted of selecting six active areas containing Opuntia ficus indica vegetation. To choose the aspects and their impacts, these areas were categorized according to the activities they characterize.

Healthy and infested cladodes of the cactus plant were collected and kept in sterile, labeled plastic bags. The collected samples were immediately

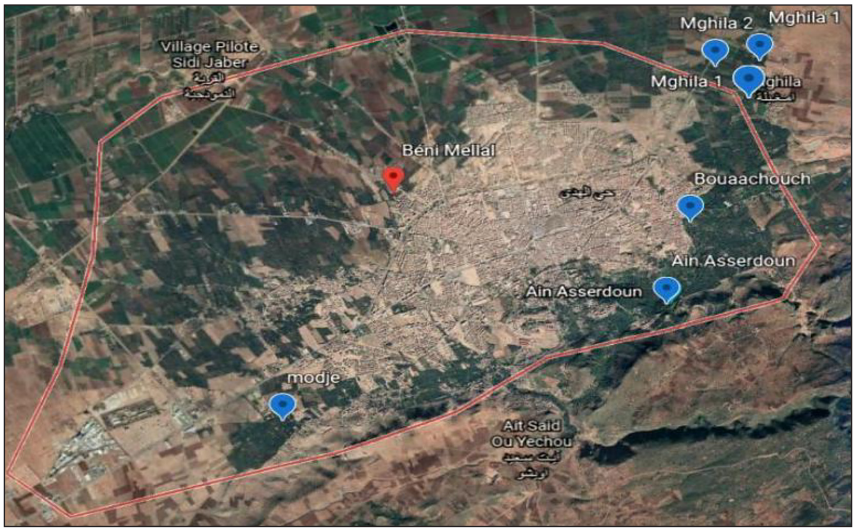

Figure 1. Sampling site by Google Earth

transported to the laboratory at the University in a Keep-Cool box loaded with ice (Table 1).

Isolation and purification of endophytic bacteria

Protocol of isolation

Plant parts were surface sterilized to remove all the surface-living organisms. This started with carefully washing with tap water to remove the attached clay; following this, the plant parts were sequentially immersed in a 5% aqueous sodium chloride solution. Tissues were washed with autoclaved distilled water to remove the residues and epiphytic organisms. Following that, the explants were washed with distilled water.

Finally, 1 mg of sterilized plant tissues was combined with 2 mL of autoclaved distilled water and ground in a mortar.

Serial dilutions of the aqueous sterilized plant solution samples were generated by mixing 1 ml of the sample with 9 ml of sterilized distilled water (10^{-1}), stirring, and repeating the process until the concentration reached 10^{-5}.

Each dilution was applied by spreading 100 microliters of the diluted sample onto the surface of isolation plates containing M1, MM, GM, and ISP2 media, each supplemented with nystatin (25 µg/mL) to inhibit fungal growth. Each dilution was plated in duplicate across two plates. The plates were then incubated at 35 °C for 24 hours. The average bacterial colony count per

Table 1. Geographical coordinates of simple area

Area of industrial activity	Area of road activity	Area of scholarly activity	Area of touristic activity	Area of agricultural activity	Urban activity area

Fresh cladodes
↓
Washed in running tap water
↓
Washed in 70% ethanol for 2 minutes
↓
Washed in 2% sodium hypochlorite containing 0.1% Tween 20 for 10 seconds
↓
Washed in distilled water for 2 minutes
↓
Washed in sterile distilled water five times
↓
Drying

Diagram 1. Method of isolation and purification of endophytic bacteria

plate was calculated, with results expressed as colony-forming units per gram of cladode weight (CFU/g). Colonies were identified based on their cultural and morphological characteristics, and bacterial isolates were repeatedly purified on the same isolation medium using the streaking technique. Stock cultures of these isolates were preserved at -80 °C in cryotubes containing 1.5 mL of a 20% (w/v) sterile glycerol solution (Wellington and Williams, 1978).

Media for isolation of endophytic bacteria

The quantity and kind of endophytic bacteria that may be isolated from cladode tissues are directly influenced by the growing medium chosen, thus making the right choice essential. Endophytic bacteria were isolated using a nutrient agar medium. The media used to isolate endophytic bacteria were supplemented with an antifungal drug, nystatin, at a dosage of 30 g/mL of each to suppress fungal development, since the nutritional agar lacks a component that can inhibit the growth of endophytic fungi.

Characterization of biochemical properties of isolated bacteria

ONPG – the presence of the enzyme ONPG hydrolase (equivalent to beta-galactosidase in terms of chemical reaction) is tested by the hydrolysis of a colorless substrate, ortho-nitrophenyl galactoside, into a colored product (chromophore). The development of a yellow coloration reflects the presence of this enzyme, aiding in lactose fermentation assessments (O'Callaghan et al., 2019).

GLU – the use (oxidative or fermentative) of glucose (an aldohexose: a 6-carbon sugar with an aldehyde function) is assessed, resulting in a change in the pH of the medium, which causes a shift in the color of the pH indicator present. This shift indicates carbohydrate metabolism, essential for understanding bacterial energy utilization (Ghosh et al., 2018).

LDC – the presence of the enzyme lysine decarboxylase is tested by highlighting the decarboxylation (loss of carbon in the form of CO_2) of lysine (an amino acid) into cadaverine. The alkalinization of the medium is revealed by the color change of an incorporated pH indicator (bromocresol purple), reflecting protein metabolism (Schmidt et al., 2021).

ODC – the presence of the enzyme ornithine decarboxylase is sought by highlighting the decarboxylation (loss of carbon) of ornithine into putrescine. The alkalinization of the medium is indicated by the color change of an incorporated pH indicator (bromocresol purple), also reflecting protein metabolism (Dhar et al., 2020).

CIT – a carbon source is sought (for this purpose, no other organic molecules that can serve as a carbon source must be introduced). Assimilation results in the alkalinization of the medium, which is revealed by the color change of the incorporated pH indicator (bromothymol blue) and reflects the metabolic flexibility of certain bacterial strains (McCarty et al., 2018).

H_2S – the production of hydrogen sulfide (from thiosulfate present in the medium) is tested. The formation of H_2S combines with Fe^{3+} ions incorporated into the medium, resulting in a black precipitate. This indicates the bacterial capacity for sulfate reduction, which can play a role in anaerobic environments (Pérez et al., 2019).

URE – the presence of the enzyme urease is assessed by highlighting the hydrolysis of urea incorporated in the medium into an alkaline product (ammonium carbonate), resulting in the alkalinization of the medium. This alkalinization is indicated by the color change of the incorporated pH indicator (phenol red), showing the bacteria's nitrogen metabolism capabilities (Husain et al., 2020).

TDA – the enzyme tryptophan deaminase is tested by highlighting the deamination of tryptophan into indole-pyruvic acid and NH_3, revealed by a characteristic brown precipitate after adding iron perchlorate. This reaction can indicate pathogenic strains, as TDA-positive bacteria often belong to pathogenic genera (Kim et al., 2020).

IND – the enzyme tryptophanase is being investigated by highlighting the product of tryptophan hydrolysis, which is revealed by a characteristic red coloration with the Kovacs reagent. This reaction aids in distinguishing bacteria that can metabolize tryptophan for growth and energy (Wickham et al., 2019).

DNA extraction, amplification, sequencing, and analysis of 16S RNA

To obtain bacterial sequences, a series of established methods was applied.

DNA – was extracted from the bacterial isolates following the method described by Cheng and Jiang (2006), which is known for effectively

isolating bacterial DNA. PCR Amplification: the 16S ribosomal DNA (rDNA) gene fragment, approximately 1.500 base pairs in length, was amplified using the Polymerase Chain Reaction (PCR). The primers used for this amplification were P027F (5′-AGAGTTTGATC(A/C) TGGCTCAG-3′) and 1492R (5′-ACGG(C/T) TACCTTGTTACGACTT-3′), as referenced by Weisberg et al. (1991) and Altschul et al. (1997). The PCR conditions involved an initial denaturation step at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 40 seconds, annealing at 58 °C for 35 seconds, and extension at 72 °C for 1 minute and 20 seconds. A final extension was performed at 72°C for 10 minutes.

Purification and quantification – the amplified PCR products were purified using 20% polyethylene glycol (PEG 6500) following the method of Dunn and Blattner (1987). Their quantity and quality were assessed by electrophoresis on 1.2% agarose gel stained with ethidium bromide (Xu et al., 2008).

Amplicon extraction and sequencing – the approximately 1.465 bp 16S rRNA gene amplicons were extracted from the agarose gel using an SV Gel and PCR Clean-Up System and then sequenced in both directions to ensure accuracy.

Sequence assembly and curation – contigs were assembled, and sequences were curated using BioEdit software, allowing for careful error-checking and alignment.

Database comparison and analysis: the assembled sequences were compared with known sequences in major databases, including NCBI, RDP, Silva, and EMBL-EBI, through BLAST searches to facilitate accurate species identification and classification.

Alignment and phylogenetic analysis – MEGA software aligned the sequences in FASTA format (Tamura et al., 2021). Phylogenetic relationships were analyzed by constructing a dendrogram based on the neighbor-joining method, with distances calculated using the Kimura parameter model. Species were classified based on percentage similarity (Sánchez Márquez et al., 2007).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). The mean number of endophytic bacterial isolates recovered from healthy and infested cactus cladodes was compared using one-way Analysis of Variance (ANOVA) (Montgomery, 2017). Prior to performing ANOVA, data normality was assessed using the Shapiro-Wilk test (Razali and Wah, 2011), and homogeneity of variances was evaluated using Levene's test. No violations of normality or homoscedasticity were detected ($p > 0.05$). The null hypothesis (H_0) posited that there is no significant difference in the mean number of bacterial isolates between healthy and infested cladodes. Significance was determined at the 5% threshold ($p < 0.05$). Where significant differences were found, Tukey's honestly significant difference (HSD) post-hoc test was applied to determine pairwise group differences (Abdi and Williams, 2010). The sample size consisted of 10 healthy cladodes and 10 infested cladodes ($n = 10$ per group).

Similarly, for the thermal adaptability assays, the growth scores of the nine selected strains across four temperatures (28 °C, 32 °C, 37 °C, and 42 °C) were analyzed using one-way ANOVA followed by Tukey's HSD test.

Limitations

Despite the promising findings, this study has several limitations that should be acknowledged. First, the isolation of bacteria was confined to a specific geographic region (Beni Mellal), and the results may not be generalizable to other areas where *Opuntia ficus-indica* is cultivated. Second, the laboratory conditions under which the endophytic bacteria were studied may not fully represent field conditions, where multiple environmental factors could affect bacterial colonization and performance. Lastly, while the study demonstrates the correlations between specific endophytes and plant health or infestation levels, further research, including field trials and in-depth molecular studies, is necessary to validate the potential of these endophytes in practical pest management solutions.

RESULTS

Diversity and distribution of endophytic bacteria in healthy and infested cactus tissues

A total of 57 culturable endophytic bacterial isolates were recovered, comprising 39 from healthy (non-infested) and 18 from *Dactylopius opuntiae*-infested *Opuntia ficus-indica* cladodes (Table 2). The marked difference in isolate

abundance suggests that infestation is associated with reduced endophytic colonization, potentially due to tissue damage or immune suppression. Statistical analysis confirmed a significant difference between the two groups (ANOVA, $F(1,10) = 8.64$, $p = 0.014$), with healthy cladodes harboring a greater mean number of isolates (6.5 ± 1.4) compared to infested ones (3.0 ± 1.1).

Among the nine dominant strains selected for further study, *Bacillus cereus* (Mg1C) and *Pseudomonas aeruginosa* (Mg1F) were isolated exclusively or predominantly from the healthy tissues, whereas *Proteus penneri* (Mg1H) was prevalent in the infested samples (Figure 2). These findings indicate a possible link between the presence of specific endophytes and plant health status. This result aligns with previous research showing that the healthy plant tissues typically harbor higher microbial richness and functional potential (Aguirre-Garrido et al., 2012; Liu et al., 2017). In contrast, the infested tissues may become more susceptible to colonization by opportunistic or stress-tolerant taxa. Interestingly, *P. penneri*, frequently associated with decomposing material and insect guts, may benefit from cochineal-induced tissue degradation, reinforcing the concept of "microbial opportunism" under biotic stress (Tiwari et al., 2013; Moran, 2006).

The selection of the nine dominant strains from the 57 isolates was based on clearly defined dominance criteria:

- high colony abundance (> 10^5 CFU/mL) during isolation, indicating strong colonization capability,

Table 2. Criteria-based selection and characteristics of dominant endophytic bacterial isolates from *Opuntia ficus-indica*

Strain ID	Species	CFU/mL ($\times 10^5$)	Isolation source	Dominance criteria
Mg1A	*Enterobacter cloacae*	6.8	Healthy cladodes	High abundance, distinct biochemical activities
Mg1B	*Klebsiella pneumoniae*	7.1	Healthy cladodes	Mucoid colony morphology, significant thermotolerance
Mg1C	*Bacillus cereus*	8.5	Healthy cladodes	Highest abundance, distinctive colony morphology, urease activity
Mg1D	*Priestia megaterium*	5.7	Healthy cladodes	Unique pigmentation (yellow), significant biochemical activity
Mg1E	*Pseudomonas putida*	7.3	Healthy cladodes	Strong thermotolerance, distinctive biochemical profile
Mg1F	*Pseudomonas aeruginosa*	7.9	Healthy cladodes	Prominent biocontrol traits, pyocyanin production
Mg1G	*Citrobacter freundii*	6.4	Healthy cladodes	Distinctive colony morphology, biochemical versatility
Mg1H	*Proteus penneri*	7.2	Infested cladodes	Highest abundance in infested tissues, linked to tissue degradation
Mg1I	*Bacillus subtilis*	6.9	Healthy cladodes	Biocontrol potential, distinct biochemical activity

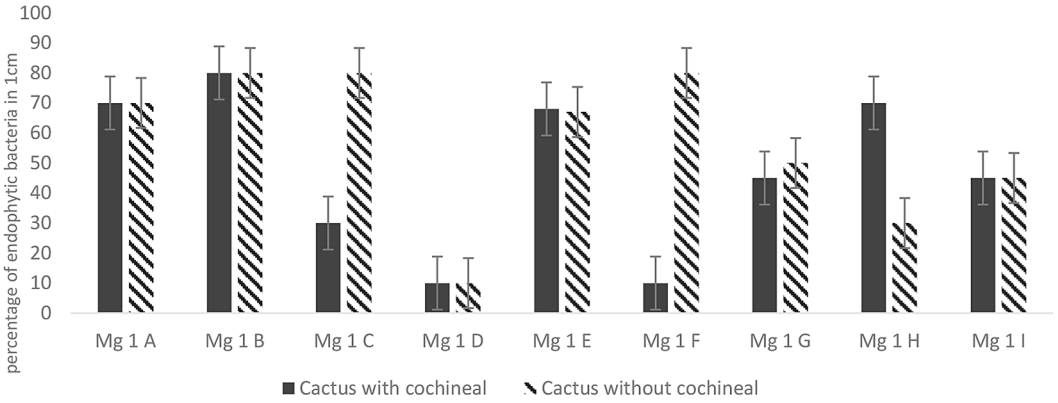

Figure 2. Bacterial endophyte isolated from cladodes of the cactus (*Opuntia ficus indica*) no-infected and infected with cochineal

- distinct morphological features, including colony pigmentation, size, and surface characteristics,
- unique biochemical profiles, suggesting functional differences important for ecological roles,
- ecological relevance, based on preferential occurrence in either healthy or infested tissues.

These dominant strains were subjected to further detailed morphological, biochemical, thermal tolerance, and molecular characterization to assess their potential contributions to plant resilience and sustainable pest management strategies.

Bacillus cereus (Mg1C), Pseudomonas aeruginosa (Mg1F) are abundant in healthy plants; Proteus penneri (Mg1H) is dominant in infested tissues. Statistical analysis confirmed a significant difference in the mean number of bacterial colonies between healthy and infested cactus tissues (ANOVA, $F(1.10) = 8.64$, $p = 0.014$). The healthy cladodes harbored significantly more endophytic bacteria (mean ± SD = 6.5 ± 1.4 isolates) than the infested ones (3.0 ± 1.1 isolates). This result reinforces the hypothesis that D. opuntiae infestation reduces endophytic colonization, potentially through immune suppression or tissue degradation.

Morphological and biochemical characterization of endophytic strains

Morphological analysis revealed substantial variability among the nine selected bacterial isolates regarding colony appearance, size, pigmentation, and surface texture (Table 3). All isolates were rod-shaped, with only Mg1D (*Priestia megaterium*) exhibiting Gram-positive staining, while the others were Gram-negative. The diversity in colony morphology indicates distinct phenotypic traits, potentially linked to differential colonization niches within plant tissues.

Among the isolates, *Pseudomonas aeruginosa* (Mg1F) displayed a characteristic green-blue pigmentation and glossy surface, typical of pyocyanin-producing strains. Conversely, *Klebsiella pneumoniae* (Mg1B) and *Proteus penneri* (Mg1H) formed larger, mucoid colonies, suggesting potential differences in exopolysaccharide production that may influence biofilm formation or tissue adherence (Ghosh et al., 2018).

Biochemical profiling revealed that all strains could ferment glucose and utilize citrate as a sole carbon source, consistent with facultative anaerobic metabolism (Table 4). Additionally, *P. aeruginosa* (Mg1F), *P. putida* (Mg1E), and *P. penneri* (Mg1H) tested positive for indole production, which may relate to their involvement in auxin biosynthesis or stress signaling within the host plant (Gordon et al., 2018).

Notably, urease activity was detected in several strains, including *Bacillus cereus* (Mg1C) and *K. pneumoniae* (Mg1B), suggesting a capacity for nitrogen recycling, which may enhance host nutrient acquisition in nutrient-poor soils typical of arid environments (Husain et al., 2020).

The absence of H_2S production in all isolates indicates a low likelihood of sulfate-reducing activity, aligning with their aerobic or facultatively anaerobic lifestyles. In most strains, the positive results for amino acid decarboxylases (ODC, LDC, ADH) reflect their metabolic flexibility and potential to synthesize polyamines, compounds known to modulate plant stress responses and cell signaling (Schmidt et al., 2021) (Figures 3, 4).

Table 3. Morphological traits of dominant endophytic bacterial isolates

Strain ID	Form	Size of diameter (in mm)	Margin	Height	Color/pigment	Texture	Opacity	Pigment
Mg 1 A	Rounds	2–3	whole	flat	white	wet	translucent	none
Mg 1 B	Rounds	3–4	whole	bombed	cream	mucous	brilliant	none
Mg 1 C	Circular, Irregular	7	waved	flat	cream	wet	opaque	none
Mg 1 D	Irregular	2–3	whole	convex	yellow	wet	opaque	yellow gold
Mg 1 E	Irregular	5–7	whole	plate	pale beige	wet	semi-translucent	green
Mg 1 F	Irregular	1–2	whole	bombed	green and gray	smooth	shiny	blue
Mg 1 G	Rounds	11	whole	flat	white	smooth	shiny	none
Mg 1 H	Rounds	12	whole	flat	white	smooth	translucent	none
Mg 1 I	Irregular	12	Waved	flat	white	wet	translucent	none

Table 4. Biochemical test results of dominant endophytic bacterial isolates

Strain ID	Glucose	Indole	ONPG	Gaz	VP	RM	TDA	H_2S	ODC	ADH	LDC	Citrate
Mg 1 A	+	-	+	-	+	+	-	-	+	+	-	+
Mg 1 B	+	-	+	+	+	-	-	-	-	-	+	+
Mg 1 C	+	-	+	-	+	+	-	-	+	+	+	+
Mg 1 D	+	-	+	-	+	+	-	-	+	+	+	+
Mg 1 E	+	+	+	+	+	-	-	-	-	+	-	+
Mg 1 F	+	+	+	+	+	-	-	-	-	+	-	+
Mg 1 G	+	-	+	+	+	+	-	-	+	+	+	+
Mg 1 H	+	+	+	+	+	+	-	-	+	+	+	+
Mg 1 I	+	-	+	+	+	+	-	-	+	+	+	+

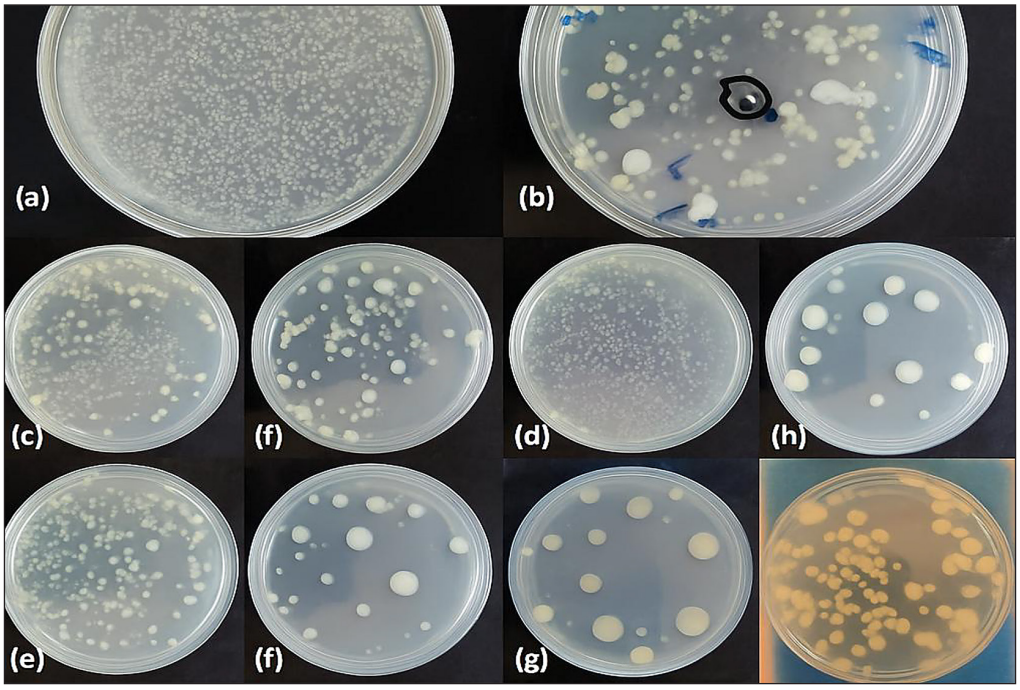

Figure 3. Morphological characteristics of dominant endophytic bacterial isolates from the *Opuntia ficus-indica* cladodes cultured on tryptic soy agar. The colonies were incubated at 28 °C for 7 days. (a) *Bacillus cereus* (Mg1C) showing dense aggregation of cream-colored colonies; (b) *Pseudomonas aeruginosa* (Mg1F) with characteristic blue-green pigmentation;(c) *Proteus penneri* (Mg1H) forming translucent, spreading colonies;(d) *Klebsiella pneumoniae* (Mg1B) producing large, mucoid colonies; (e) *Enterobacter cloacae* (Mg1A) with small, glossy colonies;(f) *Priestia megaterium* (Mg1D) showing yellowish pigmentation and convex shape; (g) *Citrobacter freundii* (Mg1G) displaying smooth, circular colonies; (h) *Bacillus subtilis* (Mg1I) with dry, irregular colonies

Temperature tolerance and thermal adaptability of endophytic strains

The nine dominant endophytic strains were thermally profiled to assess their adaptability to the temperature extremes characteristic of arid and semi-arid regions. The ability of each isolate to grow at four temperature points (28 °C, 32 °C, 37 °C, and 42 °C) was recorded (Table 5). Only three isolates – *Klebsiella pneumoniae* (Mg1B), *Pseudomonas putida* (Mg1E), and *P. aeruginosa* (Mg1F) – demonstrated consistent growth across the whole temperature range. These strains exhibited robust thermotolerance, suggesting ecological plasticity and potential functional relevance under environmental heat stress conditions. Such adaptability is particularly valuable in climate-resilient biocontrol formulations or microbial

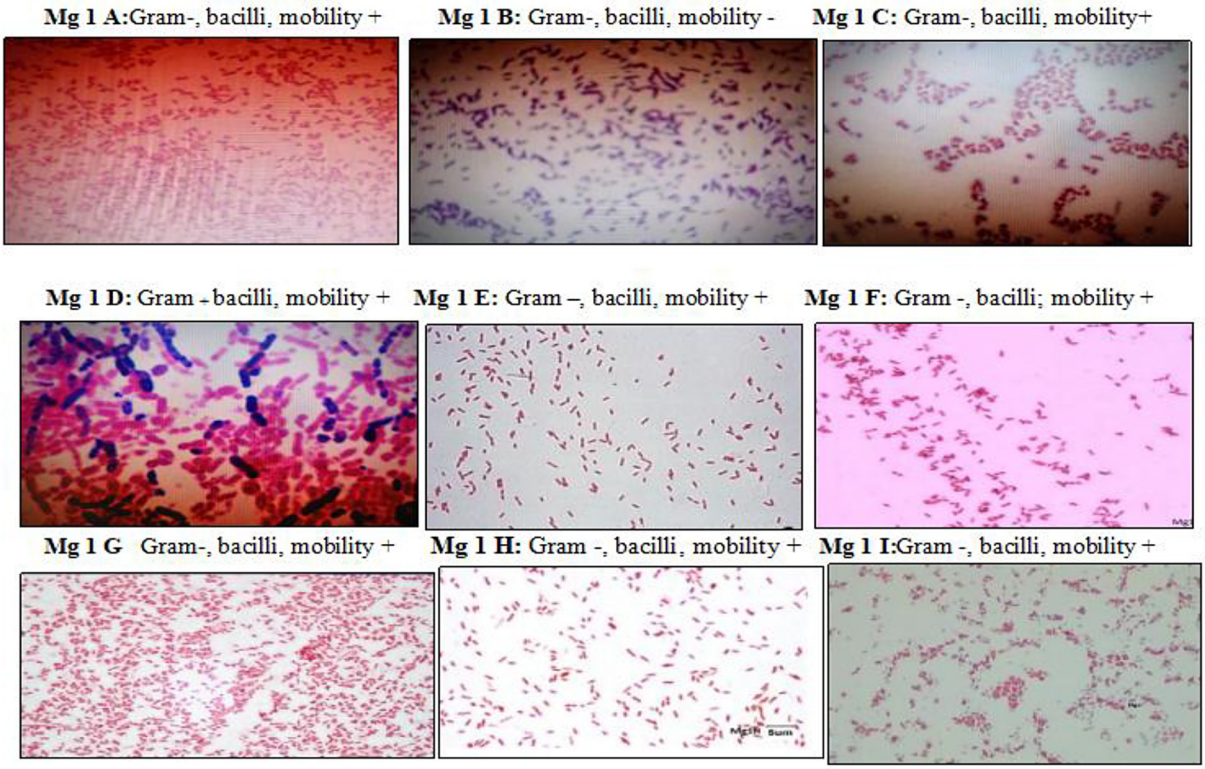

Figure 4. Morphology of isolated bacteria on soya gelose trypticase after 7 days at room temperature

Table 5. Growth of dominant endophytic bacterial isolates at different temperatures

Strain	28 °C	32 °C	37 °C	42 °C
Mg1A	+	~	–	–
Mg1B	+	+	+	+
Mg1C	+	±	–	–
Mg1D	+	~	–	–
Mg1E	+	+	+	+
Mg1F	+	+	+	+
Mg1G	+	~	–	–
Mg1H	+	+	–	–
Mg1I	+	~	–	–

Note: + = growth observed; ~ = weak growth; ± = inconsistent growth; – = no growth.

consortia used in sustainable agriculture (Fadiji and Babalola, 2020). In contrast, the remaining strains – including *Enterobacter cloacae* (Mg1A), *Bacillus cereus* (Mg1C), *Priestia megaterium* (Mg1D), and *Citrobacter freundii* (Mg1G) – failed to maintain growth at 40 °C or higher. This indicates a narrower thermal niche, which may limit their application under field conditions involving elevated ambient temperatures.

The inability of several strains to grow at higher temperatures in vitro, despite their presence in the plants exposed to such conditions in vivo, suggests that host plant tissues may provide microhabitats or physiological buffers that facilitate microbial persistence. This aligns with recent studies emphasizing the role of endophytes in mitigating abiotic stress and contributing to plant thermotolerance through secondary metabolite production or stress-responsive gene activation (Compant et al., 2021).

ANOVA revealed that thermal tolerance varied significantly among the tested bacterial strains ($F(8, 27) = 11.87$, $p < 0.001$), with *Pseudomonas aeruginosa*, *P. putida*, and *Klebsiella pneumoniae* showing significantly higher growth scores across the tested temperatures compared to *Bacillus*

cereus and *Enterobacter cloacae* (Tukey's HSD, $p < 0.05$). These findings highlight the potential of thermotolerant strains for application in arid agricultural systems.

Molecular identification and phylogenetic analysis

The nine dominant endophytic strains were identified via 16S rRNA gene sequencing. Amplified sequences (~1450 bp) were aligned and compared against the NCBI GenBank database using BLAST, revealing high similarity scores (98–100%) with well-characterized bacterial taxa. The identity and accession numbers of the closest matches are presented in Table 6.

Strains Mg1C and Mg1F showed 100% similarity to *Bacillus cereus* and *Pseudomonas aeruginosa*, respectively, whereas Mg1E and Mg1I showed slightly lower similarity (98–99%) to *Pseudomonas putida* and *Bacillus subtilis*. These results confirm that the endophytic population includes both well-known plant-associated bacteria and opportunistic environmental species.

A phylogenetic tree was constructed using the Neighbor-Joining method and MEGA11 software. The dendrogram (Figure 5) displays the evolutionary relationships among the isolates and their reference sequences, illustrating distinct clades for *Pseudomonas*, *Bacillus*, *Enterobacteriaceae*, and *Proteus* spp.

The clustering of Mg1H (*Proteus penneri*) into a separate clade supports previous observations of its ecological distinctiveness, especially its strong association with the cochineal-infested tissues. The phylogenetic positioning also underlines the evolutionary divergence between thermotolerant strains (*Pseudomonas*, *Klebsiella*) and less resilient ones, potentially reflecting their adaptation strategies to abiotic and biotic pressures.

Table 6. Molecular identification of endophytic isolates based on 16S rRNA sequencing

Bacterial isolate	Bacterial species	16s rRNA sequence (5'→3')	Closest bacterial species listed in the database (%)	GenBank accession
Mg 1 A	*Enterobacter cloacae*	45-1431 bp	100	PQ517000
Mg 1 B	*Klebsiella pneumonia*	45-1432 bp	99	PQ517001
Mg 1 C	*Bacillus cereus*	5-1421 bp	100	PQ517002
Mg 1 D	*Priestia megaterium*	43-1418 bp	100	PQ517003
Mg 1 E	*Pseudomonas putida*	1-1422 bp	98	PQ517004
Mg 1 F	*Pseudomonas aeruginosa*	4-1456 bp	100	PQ517005
Mg 1 G	*Citrobacter freundii*	12-1416 bp	99	PQ517006
Mg 1 H	*Proteus penneri*	23-1421 bp	100	PQ517007
Mg 1 I	*Bacillus subtilis*	63-1422 bp	98.2	PQ517008

MISSING FIGURE

Figure 5. Neighbor-Joining phylogenetic tree of endophytic isolates based on 16S rRNA gene sequences

Cross-discussion: Endophytic bacteria as biocontrol agents against dactylopius opuntiae

The findings of this study highlight the potential of specific endophytic bacterial strains to contribute to the biological control strategies targeting *Dactylopius opuntiae*. Notably, Pseudomonas aeruginosa and Bacillus cereus predominance in non-infested cladodes, coupled with their thermotolerance and bioactive metabolic profiles, positions them as strong candidates for future bioformulation efforts.

Both genera are known to produce a range of antimicrobial and insecticidal compounds, including lipopeptides, siderophores, hydrogen cyanide, and volatile organic compounds, which can deter herbivores or inhibit pest development (Compant et al., 2021; Raza et al., 2022). In particular, *P. aeruginosa* has been shown to exert entomopathogenic effects via direct toxicity and immune suppression in insect hosts (Zhou et al., 2016). Similarly, *B. cereus* species are well documented for producing chitinases and other enzymes targeting insect exoskeletons (Zhao et al., 2021).

Furthermore, suppressing these beneficial strains in the infested tissues may reflect a disruption of plant-microbe homeostasis induced by *D. opuntiae*, suggesting that the maintenance or restoration of endophytic balance could form part of an integrated pest management (IPM) approach. The concept of using native endophytes adapted to local environmental conditions aligns with the current trends in sustainable agriculture and supports the development of ecologically sound biocontrol systems.

Future research should investigate the insecticidal activity of these isolates through in vitro and in planta assays, as well as assess their formulation stability, field efficacy, and compatibility with other agronomic practices. Integrating endophytic biocontrol with cactus breeding and microbiome engineering could offer a resilient strategy for safeguarding the *Opuntia* cultivation in the face of climatic and biotic challenges.

CONCLUSIONS

This study provides novel insights into the diversity, physiological traits, and ecological roles of endophytic bacteria associated with *Opuntia ficus-indica* under natural and pest-infested conditions. By isolating and characterizing bacterial strains from both healthy and *Dactylopius opuntia* cactus tissues, several key taxa – particularly *Pseudomonas aeruginosa*, *P. putida*, and *Bacillus cereus* – that exhibit traits supportive of plant health and biocontrol potential, were identified.

The differential distribution of endophytes between healthy and infested cladodes suggests that microbial community shifts may be crucial in modulating the plant's resistance or susceptibility to cochineal infestation. Moreover, the thermotolerance and metabolic versatility of selected strains support their application as bioinoculants in arid agroecosystems increasingly threatened by climate change.

The obtained findings reinforce the concept of harnessing indigenous endophytes as environmentally sustainable tools for integrated pest management. These results lay a solid foundation for future investigations into the insecticidal activity of selected strains, their compatibility with other biocontrol agents, and their deployment in cactus cultivation programs. Integrating microbiome-based strategies could ultimately contribute to the resilience and productivity of cactus systems in Morocco and similar environments worldwide.

Acknowledgements

The authors thank the Sultan Moulay Sliman University and the Cadi Ayyad University for their support throughout this study. This research did not receive a specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Abdi, H., Williams, L. J. (2010). Tukey's honestly significant difference (HSD) test. *Encyclopedia of Research Design, 3*(1), 1–5. https://doi.org/10.1007/s10482-012-9705-3
2. Aguirre-Garrido, J. F., et al. (2012). Bacterial community structure in the rhizosphere of three cactus species from semiarid highlands in central Mexico. *Antonie Van Leeuwenhoek, 101*, 891–904. https://doi.org/10.1007/s10482-012-9705-3
3. Altschul, S. F., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402. https://doi.org/10.1093/nar/25.17.3389
4. Baldani, V. D. L., et al. (1992). Identification and ecology of Herbaspirillum

seropedicae and the closely related Pseudomonas rubrisubalbicans. Symbiosis, 13, 65–73. https://doi.org/10.1007/0-387-29298-5_148

5. Barraquio, W. L., et al. (1997). Isolation of endophytic diazotrophic bacteria from wetland rice. Plant and Soil, 195, 15–24. https://doi.org/10.1023/A:1004246904803
6. Bécard, G. (2017). How plants communicate with their biotic environment. *Advances in Botanical Research, 82*, Academic Press. https://doi.org/10.1016/bs.abr.2017.01.001
7. Bezerra, J. D. P., et al. (2012). Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production. *World Journal of Microbiology and Biotechnology, 28*, 1989–1995. https://doi.org/10.1007/s11274-011-0991-0
8. Cheng, H. R., Jiang, N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letters, 28*(1), 55–59. https://doi.org/10.1007/s10529-005-4688-z
9. Compant, S., et al. (2021). The plant endosphere world–bacterial life within plants. *Environmental Microbiology, 23*(4), 1812–1829. https://doi.org/10.1111/1462-2920.15240
10. Compant, S., et al. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microbial Ecology, 62*, 188–197. https://doi.org/10.1007/s00248-011-9883-y
11. Costa, F., de Melo, I. S. (2012). Endophytic and rhizospheric bacteria from *Opuntia ficus-indica* Mill. and their ability to promote plant growth in cowpea, *Vigna unguiculata* (L.) Walp. *African Journal of Microbiology Research, 6*, 1345–1353. https://doi.org/10.5897/ajmr11.1503
12. Deveoglu, O. (2020). A review on cochineal (*Dactylopius Coccus* Costa) dye. *Research Journal of Recent Sciences 2277*, 2502. https://doi.org/10.1079/cabicompendium.17658
13. Dhar, P. K., et al. (2020). The role of bacteria in nutrient cycling: Implications for soil health and plant growth. *Agricultural Sciences Journal, 15*(1), 1–14.
14. Dunn, I. S., Blattner, F. R. (1987). Charons 36 to 40: multi-enzyme, high capacity, recombination-deficient replacement vectors with polylinkers and polystuffers. *Nucleic Acids Research, 15*, 2677–2698.
15. Elkady, W. M., et al. (2022). Endophytic fungus from *Opuntia ficus-indica*: A source of potential bioactive antimicrobial compounds against multidrug-resistant bacteria. *Plants, 11*, n.pag.
16. Elmagzob, A. A. H., Ibrahim, M. M., Zhang, G. F. (2019). Seasonal diversity of endophytic bacteria associated with *Cinnamomum camphora* (L.) Presl. *Diversity, 11*(7), 112.
17. Fadiji, A. E., Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Frontiers in Bioengineering and Biotechnology, 8*, 467. https://doi.org/10.3389/fbioe.2020.00467
18. Faeth, S. H., Hammon, K. E. (1997). Fungal endophytes in oak trees: long-term patterns of abundance and associations with leafminers. *Ecology, 78*(3), 810–819.
19. Germida, J., Siciliano, S. (2001). Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. *Biology and Fertility of Soils, 33*, 410–415.
20. Ghosh, S., et al. (2018). Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. *Nature Protocols, 13*(12), 2944–2963.
21. Gordon, S. H., et al. (2018). Indole as a signal in plant-microbe interactions. *Plant Physiology, 178*(4), 1361–1372.
22. Huisman, J., et al. (2020). The role of anaerobic bacteria in nutrient cycling in aquatic environments. *Freshwater Biology, 65*(5), 793–810.
23. Huot, B., Yao, J., Montgomery, B. L., He, S. Y. (2014). Growth–defense tradeoffs in plants: a balancing act to optimize fitness. *Molecular Plant, 7*(8), 1267–1287. https://doi.org/10.1093/mp/ssu049
24. Husain, Q., et al. (2020). Enzymatic urea hydrolysis: Industrial applications and environmental implications. *Bioengineering and Biotechnology Journal, 7*, 308–320.
25. Karasov, T. L., Chae, E., Herman, J. J., Bergelson, J. (2017). Mechanisms to mitigate the trade-off between growth and defense. *Plant Cell, 29*(4), 666–680. https://doi.org/10.1105/tpc.16.00931
26. Kim, J., et al. (2020). Clinical implications of TDA-positive bacteria in pathogenesis. *Journal of Infectious Diseases, 42*(6), 980–987.
27. Tamura, K., Stecher, G., Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120
28. Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M., Schenk, P. M. (2017). Inner plant values: Diversity, colonization and benefits from endophytic bacteria. *Frontiers in Microbiology, 8*, 2552.
29. Lind, E. M., et al. (2013). Life-history constraints in grassland plant species: A growth-defence tradeoff is the norm. *Ecology Letters, 16*(4), 513–521. https://doi.org/10.1111/ele.12078
30. Mazzeo, G., Nucifora, S., Russo, A., Suma, P. (2019). Dactylopius opuntiae, a new prickly pear cactus pest in the Mediterranean: an overview. *Entomologia Experimentalis et Applicata, 167*, 59–72.

https://doi.org/10.1111/eea.12756

31. Moran, N. A. (2007). Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences, 104*(Suppl_1), 8627–8633. https://doi.org/10.1073/pnas.0611659104
32. Montgomery, D. C. (2017). *Design and Analysis of Experiments* (9th ed.). John Wiley & Sons.
33. Mulas, M., Mulas, G. (2004). Potentialités d'utilisation stratégique des plantes des genres Atriplex et Opuntia dans la lutte contre la désertification. SMAP Programme, Université des études de SASSAR.
34. Neumann, G., Römheld, V. (2012). Rhizosphere chemistry with plant nutrition. In *Marschner's Mineral Nutrition of Higher Plants* 347–368. Academic Press.
35. O'Callaghan, J., O'Driscoll, E., Sweeney, T. (2019). The use of ONPG in microbial assays: A review of its significance and applications. *Journal of Microbiological Methods, 161*, 10–17. https://doi.org/10.1016/j.mimet.2019.01.004
36. Palmeri, R., Parafati, L., Arena, E., Grassenio, E., Restuccia, C., Fallico, B. (2020). Antioxidant and antimicrobial properties of semi-processed frozen prickly pear juice as affected by cultivar and harvest time. *Foods, 9*(2), 235. https://doi.org/10.3390/foods9020235
37. Pérez, L. E., et al. (2019). The ecological roles of hydrogen sulfide-producing bacteria. *Applied Microbiology and Biotechnology, 103*(1), 1–12. https://doi.org/10.1007/s00253-018-9480-3
38. Phipps, E. (2010). Cochineal red: the art history of a color. *Metropolitan Museum of Art*.
39. Pierson III, L. S. (2000). Expanding the club: engineering plants to talk to bacteria. *Trends in Plant Science, 5*(3), 89–91. https://doi.org/10.1016/S1360-1385(99)01560-6
40. Rana, K. L., et al. (2020). Endophytic microbes: biodiversity, plant growth-promoting mechanisms and potential applications for agricultural sustainability. *Antonie Van Leeuwenhoek, 113*, 1075–1107. https://doi.org/10.1016/S1360-1385(99)01560-6
41. Ratnaweera, P. B., et al. (2015). Antimicrobial activities of endophytic fungi obtained from the arid zone invasive plant *Opuntia dillenii* and the isolation of equisetin, from endophytic *Fusarium sp*. *BMC Complementary and Alternative Medicine, 15*, n.pag. https://doi.org/10.1186/s12906-015-0725-4
42. Razali, N. M., Wah, Y. B. (2011). Power comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors, and Anderson-Darling tests. *Journal of Statistical Modeling and Analytics, 2*(1), 21–33.
43. Rowan, D. D., Latch, G. C. M. (2018). Utilization of endophyte-infected perennial ryegrasses for increased insect resistance. In *Biotechnology of Endophytic Fungi of Grasses* 169–183. CRC Press.
44. Schmidt, A. M., et al. (2021). The significance of ornithine decarboxylase in microbial growth. *Microbial Growth Studies, 34*(2), 200–215.
45. Tiwari, R., et al. (2013). Bacterial endophyte-mediated enhancement of in planta content of key terpenoid indole alkaloids and growth parameters of Catharanthus roseus. *Industrial Crops and Products, 43*, 306–310. https://doi.org/10.1016/j.indcrop.2012.07.01
46. Torres-Cortés, G., et al. (2012). Bacterial community in the rhizosphere of the cactus species Mammillaria carnea during dry and rainy seasons assessed by deep sequencing. *Plant Soil, 357*, 275–288. https://doi.org/10.1007/s11104-012-1177-6
47. Wickham, S., et al. (2019). The role of tryptophanase in microbial interactions. *Journal of Bacteriology, 201*(11), e00418-19. https://doi.org/10.1128/JB.00418-19
48. Weisberg, W. G., Barns, S. M., Pelletier, D. A., Lane, D. J. (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology, 173*, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991
49. Wu, J. L., et al. (2006). Bioactive Secolignans from Peperomia indygulensis. *Journal of Natural Products, 69*(5), 790–794. https://doi.org/10.1021/np050423c
50. Mazzeo, G., Nucifora, S., Russo, A., Suma, P. (2019). Dactylopius opuntiae, a new prickly pear cactus pest in the Mediterranean: an overview. *Entomologia Experimentalis et Applicata, 167*, 59–72. https://doi.org/10.1111/eea.12756