Article

Plant Growth-Promoting (PGP) Traits of Endophytic Bacteria from In Vitro Cultivated *Tectona grandis* L.f

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Abstract: *Tectona grandis* L.f. (teak) is a tropical tree cultivated mainly due to its resistance, valuable wood and tolerance to biotic and abiotic factors. An abundant bacteria community exists in teak tissues and knowledge of the functional roles of teak endophytic bacteria, from in vitro tissue culture, is essential for improving micropropagation techniques. In this study, we isolated endophytic bacteria with plant growth-promoting (PGP) traits from two teak clones (Proteca® A3 and E4) in calli, leaves, and stems segments in a culture medium. We analyzed colony pigmentation, gram reaction, and evaluated PGP traits (phosphorous solubilization, nitrogen fixation and indol-acetic acid production) of 54 colonies from clone A3 and 50 colonies from clone E4. A total of 35 colonies of clone A3 and 42 colonies of clone E4 were capable of fixing nitrogen. Four isolated bacteria from clone A3 were capable of solubilizing phosphorous (P-$Ca_3(PO_4)_2$), while no strain of E4 clone showed that capacity. Furthermore, 49 endophytic bacteria from clone A3 showed capacity to synthesize indol-acetic acid, while only 4 bacteria from the E4 clone presented that characteristic. We also identified six teak endophytic bacteria, by analysis of the 16S-23S rDNA intergenic spacer region, showing high identity with the genera *Curtobacterium* sp., *Ochrobactrum* sp., and *Bacillus* spp. Therefore, we demonstrate here that the abundant bacterial community existing in teak tissues, including those of in vitro cultivated plants, have PGP traits that can be further harnessed for preparation of bioformulations, for example. Our findings open the possibility for studying these isolated endophytic bacteria more closely in order to understand their association with teak growth.

Keywords: nitrogen fixation; phosphorous solubilization; AIA production; *Bacillus*; *Ochrobactrum*; *Curtobacterium*

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1. Introduction

Teak (*Tectona grandis* L.f.) belongs to the Lamiaceae family and naturally occurs in Thailand, Myanmar, Laos, and India [1,2]. However, commercial plantations are currently found in many other countries, mainly in tropical regions, which are favorable sites for the growth of teak species [3]. Teak is cultivated commercially in the tropics mainly for its high-quality wood, especially due to the wood's high durability, dimensional stability, sapwood to heartwood proportions and resistance to the external environment [4]. Teak wood is resistant to deterioration because of anti-fungal properties assigned to the presence of quinones and other extractives, which confer longevity to the wood [5].

Teak reproduction through seeds is inefficient in most cases, as the seeds have a hard coat, low seed quality, and a poor germination rate [6]. Therefore, in vitro micropropagation is a feasible and recommended technique for commercial reproduction of teak [7]. Several micropropagation protocols for teak species have been developed and are used for the production of plantlets [8]. The use of in vitro propagated plantlets enhances multiplication rates of clonal plant material [8].

Recently, the presence of endophytic bacteria has been studied in many in vivo plant species and in different tissues [9,10]. In several species, endophytic bacteria occurrence has been reported in plant tissues in commercial laboratories and in scientific studies [11]. These studies have allowed study of the differentiation of bacterial contamination from naturally occurring endophytes [11]. Endophytic bacteria usually have positive effects on the plant propagation process; however, it was reported that the presence of these endophytic microorganisms, in some cases, may affect in vitro propagation of some species of trees [11].

Thus, understanding the influence of endophytic bacteria on plant development has become crucial. Endophytic bacteria produce metabolites that enhance plant growth, known as plant growth-promoting (PGP) substances [12]. The PGP features of some endophytic bacteria may occur by direct or indirect mechanisms [13]. The direct mechanism regards the capacity of bacteria to facilitate nutrient acquisition or modulate the plant endogenous hormonal level [13]. Some endogenous bacteria also affect plant growth indirectly by decreasing the damage caused by phytopathogen infections [13]. Endophytic bacteria promote uptake, mainly of nitrogen (N) [14] and phosphorous (P) [15], while modulating hormonal levels by the biosynthesis of one or more hormones, such as auxin and cytokinin [16].

Most studies have investigated PGP bacteria associated with crops; nevertheless, some investigations have described the presence of these endophytic bacteria in tree tissues, such as roots, leaves, and stems [17]. Endophytic bacteria have been reported to occur in teak tissues, which necessitates better understanding of their functional roles, mainly in in vitro tissue culture [18].

Here, we isolated and characterized endophytic bacteria, The endophytic bacteria were isolated from segments of stems, leaves, and calli of two teak clones cultivated in vitro. We characterized these isolates according to their PGP traits and opened the possibility of further exploration of the relationship between teak and its endophytic bacterial community. The bacterial identification was investigated by sequencing the 16S-23S rDNA intergenic spacer (IGS) region. We also evaluated in vitro the putative influence of these endophytic bacteria on plant growth.

2. Materials and Methods

2.1. Plant Material

We used *T. grandis* plantlets from clones A3 and E4, donated by Proteca Forest Biotech®, as explant sources. Nodal segments were placed in PT medium (ProTeca®) supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} BAP, and 2.3 g L^{-1} phytagel. The medium pH was adjusted to 5.8 before autoclaving. The cultures were sealed with polyvinyl chloride (PVC) plastic wrap and, then, incubated at 25 °C under a 16-h photoperiod for 60 days for the formation of plantlets.

2.2. Isolation of Culturable Endophytic Bacteria

Endophytic bacteria were isolated from the plantlets and from the calli formed at the plantlet base. Tissue segments from stems, leaves, and calli were triturated separately using a pestle and mortar under aseptic conditions. The plant material was homogenized in 5 mL of sterile phosphate-buffered saline (PBS: Na_2HPO_4, 1.44 g L^{-1}; KH_2PO_4, 0.24 g L^{-1}; KCl, 0.20 g L^{-1}; NaCl, 8.00 g L^{-1}; pH 7.4). After serial dilutions, 0.1 mL of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions were spread onto NDLA (Nutrient-dextrose-yeast-agar) medium (meat extract, 5 g L^{-1}; peptone, 10 g L^{-1}; yeast extract, 4 g L^{-1}; sucrose, 2.5 g L^{-1}; K_2HPO_4, 0.5 g L^{-1}; agar, 15 g L^{-1}). Each plate was considered a biological repetition. The plates were kept at room temperature and the bacterial growth was evaluated weekly. Isolated colonies were sub-cultivated, purified in NDLA medium, and characterized phenotypically. After purification, the bacterial DNA was extracted and the bacteria were stored in a 25% glycerol solution at −80 °C for further evaluation.

2.3. Bacteria Identification

For the extraction of genomic DNA, bacterial cells were harvested from cultures grown overnight in an NDLA liquid medium. After culture centrifugation (12,000 rpm, room temperature for 2 min.), the pellet was resuspended in 570 µL TE buffer and the genomic DNA was extracted using the CTAB method [19]. The final DNA concentration was adjusted to 200 ng/ul. For identification, the 16S-23S rDNA intergenic spacer (IGS) region amplification was performed using universal primers FGPS1490 5′-TGCGGCTGGATCACCTCCTT-3′ and FGPL132 5′-CCGGGTTTCCCCATTCGG-3′. The PCR products were purified (150 µL isopropanol 100% followed by 500 µL and ethanol 70%) and sequenced. Sequencing inspection was performed using MEGA v.10.1.7 [20]. The nucleotide sequences were aligned with the most similar bacteria using BLAST (NCBI). All sequences are available at the GenBank under the accession numbers: MT996003 to MT996008.

2.4. Morphological and Biochemical Characterization

The N fixation capacity was determined by using the N free semi-solid medium NFb [21]. The presence of a bacterial pellicle in the medium surface after incubation for 2 d at 28 °C was the indication of bacterial ability to fix N.

The mineral phosphate solubilization activity was assayed by inoculating bacteria on agar medium plates containing insoluble phosphate (glucose, 10 g L^{-1}; NH_4Cl, 5 g L^{-1}; NaCl, 1 g L^{-1}; $MgSO_4.7H_2O$, 1 g L^{-1}; $Ca_3(PO_4)_2$, 0,8 g L^{-1}; pH 7.2). The capacity of solubilizing mineral phosphate was confirmed by the presence of a zone of clearance around the colonies after 48 h at 30 °C [22].

The amount of indol-acetic acid produced (IAA) was determined according to the colorimetric method [23]. For estimation, bacteria were cultivated in an NDLA medium supplemented with L-Tryptophan (0.005 g mL^{-1}) at 28 °C with shaking (120 rpm) for 24 h in the dark. The IAA production per mL was estimated by collecting 1.5 mL of culture supernatant and mixing with 1 mL of Salkowski reagent ($FeCl_3 \bullet 6H_2O$, 0.5M; Perchloric acid, 35%). Absorbance was measured at 530 nm after 20 min.

Gram typing of bacteria was determined by the potassium hydroxide (KOH) method. One drop of 3% KOH solution was placed on a clean microscope slide and isolates of the endophytic bacteria were emulsified to the drop. After stirring continuously for 60 s, the reaction of gram-negative was indicated when the organisms became thick and stringy, and formed long strands within the first 30 s. Gram-positive organisms did not alter the suspension.

2.5. Evaluation of Bacterial Influence in Plant Growth

The influence of the endophytic bacteria on teak growth was evaluated by using an isothiazolinone biocide in the culture medium. Internodes with lateral shoots of both clones, A3 and E4, were grown in a multiplication culture medium without biocide (MM) and in the presence of biocide 250 µL L^{-1} (MMP). The internodes were incubated at 25 °C under a 16-h photoperiod for 30 d. At the end of the incubation time, we evaluated total height, number of leaves, and number of shoots. The commercial biocide Polybac7D® (Polyorganic Tecnologia, São Paulo, Brazil) was used for the analysis. Polybac7D® is composed of a mixture of isothiazolinones (methylchloroisothiazolinone and methylisothiazolinone) at 1.5%. We also evaluated teak in vitro growth in the media containing 0, 62.5, 125, and 250 µL L^{-1} of the biocide to analyze the influence of isothiazolinone concentrations.

2.6. Statistical Analysis

For the analyses of height, number of leaves, and number of shoots of teak plants cultivated with the presence or absence of isothiazolinone biocide, the data analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). The means were compared with the unpaired t-test at the 1% level after the distribution analysis by the Kolmogorov-Smirnov test. The linear regression analysis was

performed with Excel software (Microsoft Corporation, Redmond, WA, USA) to investigate the effect of different concentrations of isothiazolinone biocide on teak growth.

3. Results

3.1. Bacterial Isolation and Characterization

We evaluated *T. grandis* endophytic bacteria from A3 and E4 ProTeca® clones cultivated in vitro. We selected 54 individual colonies from A3 culture, based on distinct characteristics of the colonies, and 50 distinct colonies from E4 clones. Table 1 shows the number of isolates from each tissue.

Table 1. Number of bacteria isolates from *T. grandis* tissues of clones A3 and E4.

Clones	Total Number of Isolates	Calli Isolates	Stem Isolates	Leaves Isolates	Culture Medium
T. grandis A3	54	18	14	19	3
T. grandis E4	50	19	17	13	1

All isolates of bacteria were classified according to the tissue origin, colony pigmentation, and gram type (Table 2 and Figures S1–S3). The strain names refer to the species name, *Tectona grandis* L.f. (TG), to the name of the clone (A3 or E4), and a reference number was added to each strain, respectively. According to their pigmentation, the isolates of bacteria were classified as solid light yellow, solid bright yellow, translucid light yellow, translucid bright yellow, solid yellow, solid ivory, translucid ivory, light beige, light orange, and light pink (Table 2).

Table 2. Bacteria strains, origin, color pigmentation, gram reaction from endogenous bacteria of A3 and E4 clones.

Clone A3				Clone E4			
Strain	Origin	Colony Pigmentation	Gram Reac.	Strain	Origin	Colony Pigmentation	Gram Reac.
TG A3.1	In vitro growth	solid light yellow	+	TG E4.1	in vitro growth	light pink	−
TG A3.2	In vitro growth	solid ivory	−	TG E4.2	Leaf	light pink	+
TG A3.3	In vitro growth	solid ivory	−	TG E4.3	Leaf	light orange	−
TG A3.4	Leaf	transl.light yellow	−	TG E4.4	Leaf	light pink	+
TG A3.5	Leaf	transl.bright yellow	−	TG E4.5	Leaf	light orange	+
TG A3.6	Leaf	transl.bright yellow	−	TG E4.6	Leaf	light pink	+
TG A3.7	Leaf	transl.bright yellow	−	TG E4.7	Leaf	light orange	−
TG A3.8	Leaf	transl.bright yellow	−	TG E4.8	Leaf	light orange	−
TG A3.9	Leaf	solid ivory	+	TG E4.9	Leaf	light pink	+
TG A3.10	Leaf	solid bright yellow	+	TG E4.10	Leaf	light pink	+
TG A3.11	Leaf	light beige	−	TG E4.11	Leaf	light orange	+
TG A3.12	Leaf	solid ivory	+	TG E4.12	Leaf	light pink	−
TG A3.13	Leaf	solid ivory	+	TG E4.13	Leaf	light pink	+
TG A3.14	Leaf	solid light yellow	+	TG E4.14	Leaf	light pink	+
TG A3.15	Leaf	solid light yellow	+	TG E4.15	Stem	light orange	+
TG A3.16	Leaf	light pink	−	TG E4.16	Stem	light orange	−
TG A3.17	Leaf	solid ivory	+	TG E4.17	Stem	light pink	+
TG A3.18	Leaf	transl.ivory	+	TG E4.18	Stem	light orange	+
TG A3.19	Leaf	light orange	−	TG E4.19	Stem	light pink	+
TG A3.20	Leaf	solid bright yellow	+	TG E4.20	Stem	light orange	−

Table 2. *Cont.*

Clone A3				Clone E4			
Strain	**Origin**	**Colony Pigmentation**	**Gram Reac.**	**Strain**	**Origin**	**Colony Pigmentation**	**Gram Reac.**
TG A3.21	Leaf	solid light yellow	+	TG E4.21	Stem	light orange	+
TG A3.22	Leaf	solid light yellow	+	TG E4.22	Stem	light orange	+
TG A3.23	Stem	transl.bright yellow	−	TG E4.23	Stem	light orange	−
TG A3.24	Stem	solid bright yellow	−	TG E4.24	Stem	light orange	+
TG A3.25	Stem	solid yellow	+	TG E4.25	Stem	light orange	−
TG A3.26	Stem	solid light yellow	−	TG E4.26	Stem	light orange	−
TG A3.27	Stem	transl.bright yellow	−	TG E4.27	Stem	light orange	+
TG A3.28	Stem	solid bright yellow	+	TG E4.28	Stem	light pink	+
TG A3.29	Stem	solid bright yellow	+	TG E4.29	Stem	light orange	+
TG A3.30	Stem	transl.bright yellow	−	TG E4.30	Stem	light orange	+
TG A3.31	Stem	transl.bright yellow	−	TG E4.31	Stem	light orange	−
TG A3.32	Stem	transl.light yellow	−	TG E4.32	Callus	light orange	−
TG A3.33	Stem	solid bright yellow	+	TG E4.33	Callus	light orange	+
TG A3.34	Stem	light orange	+	TG E4.34	Callus	light orange	−
TG A3.35	Stem	solid bright yellow	+	TG E4.35	Callus	light beige	+
TG A3.36	Stem	solid light yellow	+	TG E4.36	Callus	light orange	−
TG A3.37	Callus	transl.bright yellow	−	TG E4.37	Callus	light orange	+
TG A3.38	Callus	solid bright yellow	+	TG E4.38	Callus	light orange	+
TG A3.39	Callus	transl.light yellow	−	TG E4.39	Callus	light pink	+
TG A3.40	Callus	solid bright yellow	+	TG E4.40	Callus	light orange	+
TG A3.41	Callus	solid light yellow	+	TG E4.41	Callus	light orange	−
TG A3.42	Callus	transl.bright yellow	−	TG E4.42	Callus	light orange	−
TG A3.43	Callus	solid light yellow	−	TG E4.43	Callus	light pink	−
TG A3.44	Callus	light orange	+	TG E4.44	Callus	light orange	+
TG A3.45	Callus	transl.bright yellow	−	TG E4.45	Callus	light orange	−
TG A3.46	Callus	transl.light yellow	−	TG E4.46	Callus	light orange	−
TG A3.47	Callus	transl.light yellow	−	TG E4.47	Callus	light orange	+
TG A3.48	Callus	solid ivory	−	TG E4.48	Callus	light orange	+
TG A3.49	Callus	transl.bright yellow	−	TG E4.49	Callus	light orange	+
TG A3.50	Callus	solid ivory	+	TG E4.50	Callus	light orange	+
TG A3.51	Callus	solid bright yellow	−				
TG A3.52	Callus	transl.bright yellow	−				
TG A3.53	Callus	transl.bright yellow	−				
TG A3.54	Callus	transl.light yellow	−				

(−) Gram-negative; (+) Gram-positive.

Bacteria strains from A3 and E4 displayed differences in their colors. The colors of endogenous bacteria from A3 were predominantly between yellow and ivory. The colonies showed differences in the intensity and transparency of their colors. Conversely, endogenous bacteria of E4 clones were predominantly pink and orange without translucency. The gram typing indicated that A3 and E4 had both gram-positive and gram-negative endophytic bacteria without predominance.

3.2. PGP Traits Evaluation

We also evaluated the capacity of the bacterial strains to solubilize P, fix N, and produce IAA (Table 3).

Table 3. Characterization of endophytic bacteria according to PGP traits, phosphorous (P) solubilization, nitrogen (N) fixation, and indol-acetic acid (IAA) production.

Clone A3				Clone E4			
Strain	**P Solubilization**	**N Fixation**	**IAA mg L^{-1}**	**Strain**	**P Solubilization**	**N Fixation**	**IAA mg L^{-1}**
TG A3.1	−	+	1.69	TG E4.1	−	+	4.27
TG A3.2	−	−	4.23	TG E4.2	−	−	0.00
TG A3.3	+	−	3.32	TG E4.3	−	+	0.00
TG A3.4	−	+	5.24	TG E4.4	−	−	0.00
TG A3.5	−	−	4.07	TG E4.5	−	+	0.00
TG A3.6	−	+	5.26	TG E4.6	−	+	13.80
TG A3.7	−	−	5.25	TG E4.7	−	+	0.00
TG A3.8	−	+	3.78	TG E4.8	−	+	0.00
TG A3.9	−	+	2.50	TG E4.9	−	−	0.00
TG A3.10	−	+	10.49	TG E4.10	−	−	0.00
TG A3.11	+	−	3.58	TG E4.11	−	+	0.00
TG A3.12	−	+	2.72	TG E4.12	−	+	0.00
TG A3.13	−	−	2.34	TG E4.13	−	+	0.00
TG A3.14	−	+	5.69	TG E4.14	−	−	0.00
TG A3.15	−	−	2.26	TG E4.15	−	+	0.00
TG A3.16	−	+	0.40	TG E4.16	−	+	0.00
TG A3.17	−	−	4.69	TG E4.17	−	−	0.00
TG A3.18	+	−	1.66	TG E4.18	−	+	0.00
TG A3.19	−	+	8.36	TG E4.19	−	−	0.00
TG A3.20	−	+	1.60	TG E4.20	−	+	0.00
TG A3.21	−	−	5.03	TG E4.21	−	+	0.00
TG A3.22	−	−	4.94	TG E4.22	−	+	6.18
TG A3.23	−	−	0.00	TG E4.23	−	+	0.00
TG A3.24	−	+	4.37	TG E4.24	−	+	0.00
TG A3.25	−	+	3.94	TG E4.25	−	+	0.00
TG A3.26	−	+	2.11	TG E4.26	−	+	0.00
TG A3.27	−	+	4.10	TG E4.27	−	+	0.00
TG A3.28	−	+	2.91	TG E4.28	−	−	0.00
TG A3.29	−	−	0.00	TG E4.29	−	+	0.00
TG A3.30	−	+	2.93	TG E4.30	−	+	0.00
TG A3.31	−	+	5.43	TG E4.31	−	+	0.00
TG A3.32	−	+	3.19	TG E4.32	−	+	0.00
TG A3.33	−	+	2.76	TG E4.33	−	+	0.00
TG A3.34	−	+	7.69	TG E4.34	−	+	0.00
TG A3.35	−	+	5.76	TG E4.35	−	+	0.00
TG A3.36	−	+	4.28	TG E4.36	−	+	0.00
TG A3.37	−	+	4.54	TG E4.37	−	+	0.00
TG A3.38	−	+	3.94	TG E4.38	−	+	0.00
TG A3.39	−	−	0.00	TG E4.39	−	+	0.00
TG A3.40	−	+	0.74	TG E4.40	−	+	0.40
TG A3.41	+	+	0.36	TG E4.41	−	+	0.00
TG A3.42	−	+	3.44	TG E4.42	−	+	0.00
TG A3.43	−	−	0.00	TG E4.43	−	+	0.00
TG A3.44	−	+	1.00	TG E4.44	−	+	0.00
TG A3.45	−	+	5.85	TG E4.45	−	+	0.00
TG A3.46	−	−	4.39	TG E4.46	−	+	0.00
TG A3.47	−	−	4.00	TG E4.47	−	+	0.00
TG A3.48	−	−	0.37	TG E4.48	−	+	0.00
TG A3.49	−	+	3.77	TG E4.49	−	+	0.00
TG A3.50	−	+	0.00	TG E4.50	−	+	0.00
TG A3.51	−	+	0.05				
TG A3.52	−	+	1.81				
TG A3.53	−	+	2.36				
TG A3.54	−	−	1.08				

+ capable of P solubilization or N fixation. − Uncapable of P solubilization or N fixation.

Regarding P solubilization, few A3 endophytic bacteria (TG A3.3, TG A3.11, TG A3.18, TG A3.41) were capable of solubilizing P. On the other hand, none of the isolates were capable of solubilizing P among the E4 endophytic bacteria.

The ability to fix N was evaluated by the capacity of growing in a N-free medium [21]. Among the 54 A3 bacterial strains isolated, 35 were capable of growing in a N-free medium,

while, among the 50 E4 bacterial strains, 42 grew in a N-free medium. Most bacterial strains isolated were potentially N producers.

Many bacterial strains isolated from A3 showed the capacity to synthesize IAA in the presence of L-Tryptophan. From the 54 bacterial strains isolated from teak A3 clone, only five were not capable of synthesizing IAA. All bacterial strains isolated from the culture media and leaves produced IAA. From the strains unable to synthesize IAA, two were isolated from the stems and three from the calli. Among the isolates of bacteria from the teak E4 clone, only four strains were capable of producing auxin: TG E4.1, TG E4.6, TG E4.22, and TG E4.40. All four strains capable of synthesizing IAA were isolated from different E4 teak tissues: one from the in vitro culture medium (TG E4.1), one from the leaves (TG E4.6), one from the stems (TG E4.22), and one from calli (TG E4.22). The strain with the highest IAA production was isolated from E4 leaves, namely, the strain TG E4.6, which produced 13.8 mg L^{-1} AIA. All four isolates of bacteria from the in vitro culture medium were capable of producing IAA, despite their different characteristics of color, P solubilization, and N fixation.

We selected six bacteria among the strains isolated from A3 and E4 teak plants to identify their genera by the comparative sequence analysis (Figure S4). They were selected according to their characteristics of ability to solubilize phosphate, to fix nitrogen and to produce IAA. We used the 16S-23S rDNA intergenic spacer (IGS) region amplification to compare the sequence of the bacteria isolated from teak and the NCBI genbank using the BLAST analysis. The analysis identified similarities in the sequences with the genera *Bacillus*, *Ochrobactrum*, and *Curtobacterium* (Table 4).

Table 4. Identification of *T. grandis* endophytic bacteria based on the BLAST analysis of the 16S-23S rDNA intergenic spacer (IGS) region.

Strain	Best Alignment with Type Strains on NCBI	Max Score	Total Score	Query Cover	E-Value	Per. Ident
TG A3.1	*Curtobacterium flaccumfaciens* pv. Basellae	843	843	81%	0	98.53%
TG A3.3	*Ochrobactrum cytisi* strain ESC5 16S-23S ribosomal RNA intergenic spacer, partial sequence Accession: EF059910.1	867	867	98%	0	88.08%
TG A3.18	*Bacillus oceanisediminis* 2691 chromosome, complete genome. Accession: CP015506.1	318	1867	99%	8×10^{-83}	87.26%
TG A3.41	*Bacillus altitudinis* strain 11-1-1 chromosome, complete genome. Accession: CP054136.1	529	3973	94%	4×10^{-146}	100%
TG A3.44	*Bacillus altitudinis* strain SCU11 chromosome, complete genome. Accession: CP038517.1	510	3840	89%	1×10^{-140}	99.64%
TG E4.49	*Bacillus altitudinis* strain SCU11 chromosome, complete genome. Accession: CP038517.1	507	3803	91%	2×10^{-139}	98.25%

To assess the influence of teak endogenous bacteria on plant growth, we analyzed plant height, number of leaves, and number of shoots of A3 and E4 teak plants cultivated in the presence or absence of an isothiazolone biocide (Figure 1 and Figure S5).

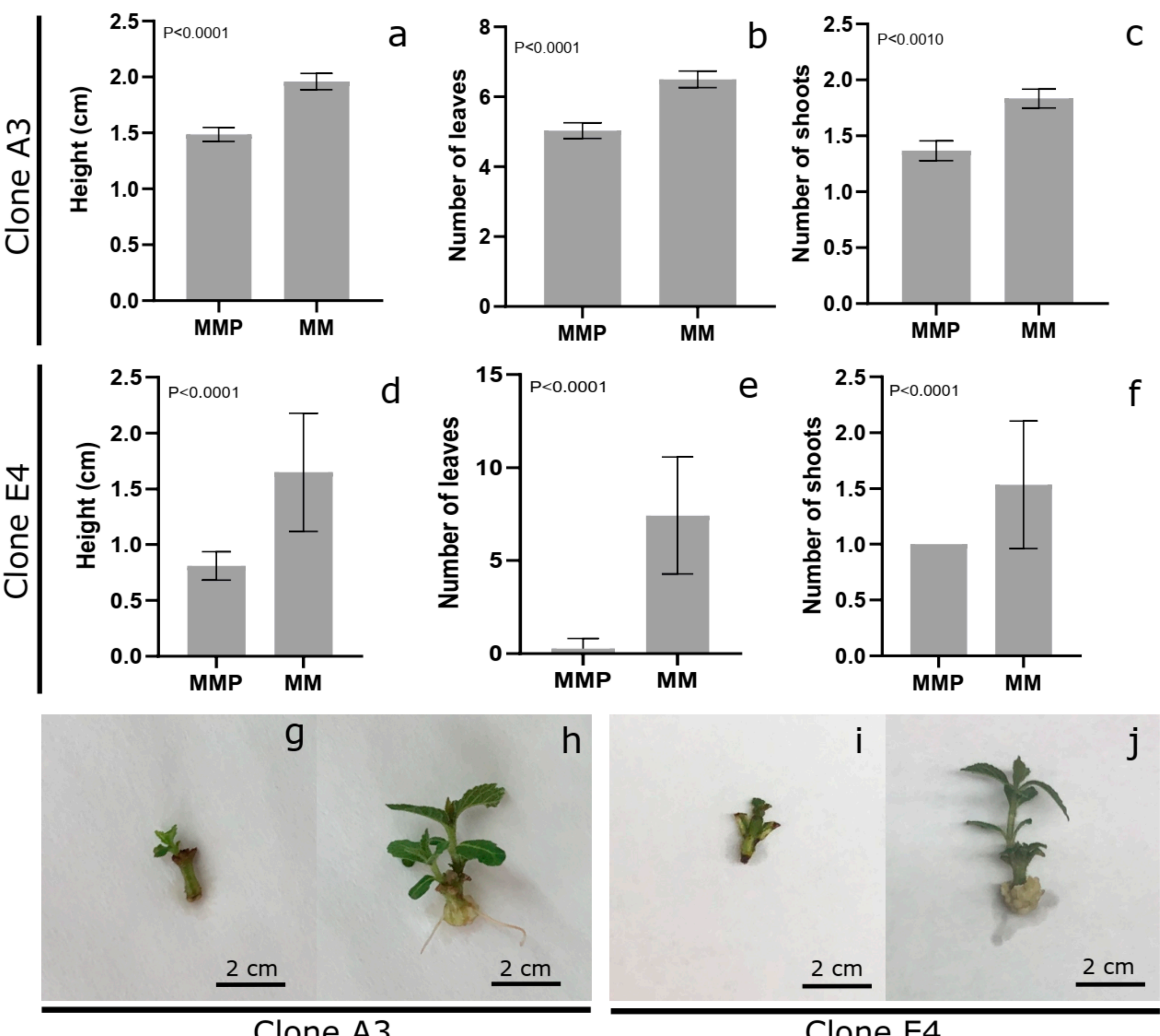

Figure 1. Growth of teak A3 and E4 shoots in PT medium in the presence (MMP) and absence (MM) of isothiazolinones biocide (Polibac). The growth parameters (plant height, number of leaves, and number of shoots) were evaluated 30 days after inoculation. Figures (**a**–**c**) show the height, number of leaves, and number of shoots of clone A3, respectively. Figures (**d**–**f**) show the height, number of leaves, and number of shoots of clone E4, respectively. Figures (**g**,**h**) show the phenotype of shoots cultivated in the presence or absence of isothiazolinones in clone A3, respectively. Figures (**i**,**j**) show the phenotype of shoots cultivated in the presence or absence of isothiazolinones in clone E4, respectively. For all the parameters analyzed, 30 clones (n = 30) were used.

The isothiazolinones in the medium (250 μL L^{-1}) were capable of controlling endophytic bacterial growth; nevertheless, the presence of the biocide affected the growth of both A3 and E4 teak plants (Figure 1, Figures S5 and S6). The height of teak plants and the number of leaves and shoots were smaller in plants cultivated in the medium containing the biocide. The average height was smaller when the plants were cultivated in the presence of 250 μL L^{-1} isothiazolinone. For A3 plants, the average height was 1.96 cm in the absence of biocide and 1.48 in its presence and, for E4, the average height was 1.55 in the absence of biocide and 0.80 in its presence (Figure 1a,d). The number of leaves and shoots decreased

in both A3 and E4 when the biocide was added to the growth medium (Figure 1b,c,e,f). The plant phenotypes reflect the great difference in their growth (Figure 1g–j).

4. Discussion

4.1. Teak Harbors Endophytic Bacteria with PGP Traits

The endophytic bacteria were isolated from healthy teak plants cultivated in vitro. During cultivation, the plant was grown in aseptic conditions, indicating that the isolates were indeed endophytic bacteria. Most studies on endophytic bacteria in woody species have been reported in the genera *Pinus*, *Picea*, and *Populus* [14]. In shoots from the tissue culture, endophytic bacteria were detected in *Pinus* [24,25] and *Populus* [11].

This study showed that teak harbors many culturable endophytic bacteria isolated from callus, stems, leaves, and the culture medium, with a total number of isolates of 54 and 50 A3 and E4 clones, respectively. Many studies on bacterial characterization use isolates of bacteria from the rhizosphere. However, in this study, bacteria were isolated from in vitro plants without a well-formed root system, which hampered the study on endophytic bacteria from teak roots. The analyses of P solubilization, N fixation, and IAA production demonstrated that most isolates of bacteria had different features, which enabled their potential use to promote plant growth.

N-fixing bacteria are well known in legumes, which obtain a significant amount of their N through a symbiotic relationship with N2-fixing microorganisms in root nodules [26]. However, other non-leguminous species can also successfully grow in low-N2 environments [26], where endophytic microorganisms can fix atmospheric nitrogen into usable forms [26].

The idea that plants without root nodules were capable of N2 fixation was controversial for a long time; nevertheless, the occurrence of N-fixing diazotrophic bacteria has been reported for different species, including trees [17,27,28]. Many isolates of bacteria from teak A3 and E4 clones were capable of fixing N, which was measured by the bacterial capacity to grow in an N-free medium. N2-fixing endophytic bacteria have been isolated from a wide variety of species, such as maize [29], sugarcane [30], coffee [31], and conifers [28,32].

Bacteria capable of converting insoluble P forms to a ready-to-use form for the plant are known as P-solubilizing bacteria [15]. The capacity of P solubilization is one of the main mechanisms of PGP associated with bacteria. Qualitative P solubilization activity was verified in some isolates of bacteria from A3; however, none of the bacteria from E4 teak clones had this PGP trait (Table 3). The capacity of P solubilization was also less frequent in an endophytic bacteria community isolated from *Populus tomentosa*, only 25.5% of bacterial isolates exhibited high phosphate solubilization [33].

In general, endophytic bacteria associated with plants produce growth hormones. Roots associated with bacteria commonly produce gibberellin, while bacteria isolated from leaf tissues may produce cytokinin. Auxin-producing bacteria, on the other hand, may occur in all plant tissues [25]. In teak, many bacteria isolated from the plant tissue medium, mainly from the A3 clone, were capable of synthesizing IAA in the presence of L-Tryptophan at different amounts. However, the highest production of IAA was observed in an isolated bacterium from an E4 clone leaf (TG E4.6), that produced 18.30 mg L^{-1} IAA. High amounts of IAA produced by endophytic bacteria from trees has already been described, as in the ornamental tree *Handroanthus impetiginosus*, wherein 41.6 μg mL^{-1} IAA was observed in a strain isolated from roots [34]. Although IAA is a hormone related to plant growth, its effects depend on its concentration. At small quantities, IAA produced by endophytic bacteria stimulates plant growth. However, IAA may inhibit plant growth at higher concentrations [35]. Only three endophytic bacteria isolated from the E4 clone were capable of synthesizing IAA; nevertheless, they produced high amounts of IAA (Table 3).

4.2. Curtubacterium, Ochrobactrum, and Bacillus genera Are Teak Endophytic Bacteria

The analyses of the 16S-23S rDNA intergenic spacer (IGS) regions of some isolated bacteria identified six endophytic bacteria from teak. We identified bacteria from three

phyla: *Actinobacteria*, *Proteobacteria*, and *Firmicutes*. These phyla have been identified as the most abundant among the teak endophytic bacteria [18].

The first bacteria identified showed 98.53% identity with *Curtobacterium flaccumfaciens* pv. Basellae (phylum *Actinobacteria*) by the BLAST analysis. Several studies on endophytic bacteria in woody plants identified *Curtobacterium* strains, such as in the species poplar [11], citrus [36], coffee [37], and grapevine [38]. The *Curtubacterium* genus is frequently associated with plants and is found mainly in the phyllosphere [39], corroborating our findings, since the plantlets from which endophytic bacteria were isolated originated from vegetative propagation using nodal segments. *Curdobacterium* is described as a yellow-pigmented bacterium [40], as observed in our study (Table 2).

The species *C. flaccumfaciens* is associated with plant pathogenesis in rgw common bean [41]; however, its role in plant defense responses against pathogen attacks has been reported in other species, such as citrus [42], cucumber [43], and grapevine [44]. *C. flaccumfaciens* was also considered a plant growth promotor and a stress alleviator in barley [45]. A *Curtobacterium* strain isolated from the olive had the ability to grow under severe stress [46].

TG A3.3 showed 88.08% identity with *Ochrobactrum cytisi* (phylum *Proteobacteria*). This endophytic bacterium was first isolated from *Cystisus scorparius* nodules [47]. The endophytic bacteria of the genus *Ochrobactrum* are well known as nodule colonizers of legume roots [48]. However, *Ochrobactrum* was also found in the shoots and was isolated from a broad range of wild and cultivated plant hosts [49]. Many strains of *O. cytisi* have PGP activity and showed capacity to fix N2 and solubilize P. Although TG A3.3 was capable of solubilizing P, it did not fix N2. Moreover, many strains of *O. cytisi* are capable of synthesizing IAA from 2 to 8 mg L^{-1} [50–52], and our observations showed that TG A3.3 produced 3.32 mg L^{-1}. Although *O. cytisi* strains have high PGP activity, the inoculation of this endophytic bacterium in the first days of shoot regeneration from lateral buds inhibited plant growth in potato. However, in plants inoculated 15 days later, *O. cytisi* significantly promoted plant growth [50]. In *Malus hupehensis* seedlings a strain of *Ochrobactrum* (*O. haematophilum*) could improve biotic stress resistance and promote plant growth [53].

The genus *Bacillus* (phylum *Firmicutes*) is well studied due to its presence in various environments, from soils to marine sediments [54]. Therefore, *Bacillus* strains were isolated as endophytes in various plant species and presented several PGP traits [55–57].

The occurrence of *Bacillus* has also been documented in seeds, such as maize, indicating that this endophyte can be transmitted from one plant generation to the next and highlights the importance of these bacteria for the host survival [58].

Four of the isolates of bacteria from the teak species belonged to the *Bacillus* genus. Three isolates presented sequences highly similar to the species *B. altitudidnis* (TG A3.41, TG A3.44, TG E4.49) and one was similar to the specie *B. oceanisediminis* (TG A3.18). Many bacilli are capable of producing plant hormones, such as IAA [59]. In bacterial endophytes isolated in the vegetative stage of the passionflower (*Passiflora incarnata*), the genus *Bacillus* was the most dominant, and the genus with the highest number of strains that exhibited the IAA production [60]. Among the endophytic bacteria isolated from the teak species identified as members of the *Bacillus* gender, only TG E4.49 was not able to produce IAA.

4.3. Isothiazolinones Biocide Inhibit Teak Endophytic Bacteria Growth In Vitro

Isothiazolinones have been used as biocides in tissue cultures for decades. In general, these biocides are effective to control bacteria and fungi in plant tissue cultures and show phytotoxicity at levels recommended by manufacturers [61].

In in vitro shoot cultures of teak, the occurrence of endophytic bacteria does not have a negative effect in the initial steps of the culture. In fact, endophytic bacteria seem to be beneficial for the initial shoot development. However, the uncontrolled growth of endophytic bacteria in the culture medium is a problem in the long-term and can, ultimately, cause the death of explant cultures. Thus, the use of biocides may help to control endophytic bacterial growth in teak cultivated in vitro.

Nevertheless, the results showed that the presence of isothiazolinones in the culture medium affected the development of A3 and E4 shoots (Figure 1). The effect of isothiazolinones on shoot development seems to be species-dependent and must be evaluated individually prior their use [62]. In some cases, biocides composed of isothiazolinones have no effect on the shoot growth of tree species [63]. In others, isothiazolinones are slightly toxic, reducing shoot growth, without causing death [64,65].

The reduction in the number of leaves, number of shoots, and height of shoots in teak grown in the presence of biocide might be a symptom of phytotoxicity. Studies on the beneficial effects of endophytic bacteria on plant growth indicate the importance of reaching a balance in the microbial community during the plant tissue culture, considering all PGP traits of these endophytes [66].

5. Conclusions

This study demonstrated that a diverse endophytic bacterial population exists within individual teak sections. These teak bacterial strains are capable of solubilizing P, fixing N, and synthesizing IAA.

The use of isothiazolinone biocides is effective to control endophytic bacterial growth in teak tissue cultures. However, they reduce shoot development, even at low doses, possibly due to isothiazolinone phytotoxicity. Our results showed that the presence of endophytic bacteria may be beneficial for shoot growth, considering their PGP traits. These characteristics open the possibility of further exploration of these isolated bacteria that can be further harnessed for preparation of bioformulations for agricultural systems.

The data presented here allows for comprehensive studies on isolated endophytic bacterial strains and their relation to teak growth and development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13101539/s1, Figure S1: Endophytic bacteria in calli (A-B) and stems (D-C) from A3 teak plants cultivated in vitro; Figure S2: Endophytic bacteria in calli (A-B) and stems (D-C) from E4 teak plants cultivated in vitro; Figure S3: Endophytic bacteria in explants of leaves from teak plants cultivated ex vitro; Figure S4: Samples of the teak endophytic bacteria identified by 16S-23S rDNA intergenic spacer (IGS) region amplification and sequencing; Figure S5: Development of teak E4 shoots and presence of endophytic bacteria in PT medium in the presence of different concentrations of isothiazolinones biocide (Polibac): 0, 62.5, 125 and 250 μL L^{-1}. Figure S6: Differences of phenotype and presence of endophytic bacteria in the medium in the absence (A) or presence (B) of 250 μL L^{-1} of isothiazolinones biocide (Polibac).

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