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RESEARCH ARTICLE

Plant–microbe–microbe interactions influence the faba bean nodule colonization by diverse endophytic bacteria

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One sentence summary: Faba bean root nodules harbor a large diversity of non-nodular endophytes that are shaped by plant–microbe–microbe interactions.

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ABSTRACT

Legume root nodules harbor rhizobia and other non-nodulating endophytes known as nodule-associated bacteria (NAB) whose role in the legume symbiosis is still unknown. We analysed the genetic diversity of 34 NAB isolates obtained from the root nodules of faba bean grown under various soil conditions in Egypt using 16S rRNA and concatenated sequences of three housekeeping genes. All isolates were identified as members of the family *Enterobacteriaceae* belonging to the genera *Klebsiella*, *Enterobacter* and *Raoultella*. We identified nine enterobacterial genospecies, most of which have not been previously reported as NAB. All isolated strains harbored *nifH* gene sequences and most of them possessed plant growth-promoting (PGP) traits. Upon co-inoculation with an N_2 fixing rhizobium (*Rlv* NGB-FR128), two strains (*Enterobacter sichanensis* NGB-FR97 and *Klebsiella variicola* NGB-FR116) significantly increased nodulation, growth and N-uptake of faba bean plants over the single treatments or the uninoculated control. The presence of these enterobacteria in nodules was significantly affected by the host plant genotype, symbiotic rhizobium genotype and endophyte genotype, indicating that the nodule colonization process is regulated by plant–microbe–microbe interactions. This study emphasizes the importance of nodule-associated enterobacteria and suggests their potential role in improving the effectiveness of rhizobial inoculants.

Keywords: nodule-endophytes; genetic diversity; colonization; co-inoculation; faba bean; enterobacteria

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INTRODUCTION

Faba bean (*Vicia faba* L.) is a major food and feed legume containing 24–30% protein and 51–68% carbohydrate (Alharbi and Adhikari 2020). It is used as a staple dietary food for human nutrition and in animal feed. It is also considered a valuable source of fiber, vitamins, minerals antioxidants and bioactive compounds (Sharan *et al.* 2021). Globally, faba bean is among the leading legumes in terms of harvested area and grain yield (Alharbi and Adhikari 2020). Worldwide, faba bean was cultivated on 2.57 million ha with a total production of 5.43 million tons during 2019 (FAO 2021). Faba bean provides several agricultural benefits, particularly when grown in crop rotations or intercropped with cereals and other cultivated crops (Karkanis *et al.* 2018).

The inclusion of faba bean in cropping systems improves soil fertility due to having high efficiency in symbiotically fixing atmospheric N_2 (Youseif *et al.* 2021). Faba bean generally forms a symbiotic relationship with *Rhizobium leguminosarum sv. viciae* (Boivin *et al.* 2019). *Rhizobium anhuiense* (Zhang *et al.* 2015), *Rhizobium fabae* (Tian *et al.* 2008), *Rhizobium laguerraeae* (Saïdi *et al.* 2014) and *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter*; Youseif *et al.* 2014) have also been described as faba bean nodulating rhizobia. Additionally, few reports have shown that faba bean also establishes effective symbiosis with *Rhizobium etli*, *Rhizobium hidalgonense*, *Rhizobium mesosinicum*, *Rhizobium sophorae* and *Rhizobium vallis* (Youseif *et al.* 2014; Xu *et al.* 2015; Chen *et al.* 2018; Efstathiadou, Savvas and Tampakaki 2020).

For decades, rhizobia have been described as the only exclusive inhabitants of legume nodules (Martínez-Hidalgo and Hirsch 2017). However, several other bacterial taxa which are not typically rhizobia are frequently found within nodules alongside symbiotic rhizobia and are suggested to affect the growth and fitness of the host plant (Ríos-Ruiz *et al.* 2019). Several studies have indicated that *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Enterobacter*, *Pantoea*, *Serratia*, *Stenotrophomonas* spp. and many other bacteria live inside legume nodules (Martínez-Hidalgo and Hirsch 2017; Ríos-Ruiz *et al.* 2019). These nodule-associated bacteria (NAB) can behave as plant growth-promoting (PGP) bacteria, enhancing plant growth by a variety of direct and indirect mechanisms such as nitrogen fixation, phosphate solubilization, production of siderophores and phytohormones, the expression of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) and/or the biocontrol of plant pathogens (Velázquez *et al.* 2017; Ríos-Ruiz *et al.* 2019). It has been shown that some NAB also act synergistically with rhizobia to improve nodulation and growth of several legumes such as alfalfa (Chinnaswamy *et al.* 2018), chickpea (Egamberdieva *et al.* 2017), common bean (Korir *et al.* 2017) and peanut (Ibañez *et al.* 2014).

Considering the potential value of employing these nodule endophytes in sustainable agriculture, there is a need to investigate their role in improving growth, nodulation and the nitrogen-fixing ability of legumes. In a previous investigation, we assessed the genetic diversity of forty-two strains belonging to traditional rhizobial genera including *R. leguminosarum*, *R. etli* and *R. radiobacter* (syn. *Agrobacterium tumefaciens*) isolated from root nodules of faba beans grown under various soil conditions (Youseif *et al.* 2014). However, the present study focuses on the non-nodulating endophytic bacteria which were recovered from the same collected root nodule samples described in (Youseif *et al.* 2014). We hypothesized that these endophytic bacteria have an ecological role in impacting the nodulation and growth of the host plant. We also raised the question to what extent do the host–symbiont–endophyte interacting partners influence the percentage of nodules occupied by NAB. To do that, the objectives of this study were as follows (1) to analyze the molecular diversity of NAB isolated from root nodules of faba bean plants, (2) to screen the bacterial strains for PGP activities and (3) to evaluate their effect on plant growth when inoculated or co-inoculated with rhizobia on faba bean. Additionally, we performed co-inoculation experiments using a set of GFP- labeled NAB (4) to investigate the significant role of plant genotype and genetic variation in the symbiotic partner during nodule colonization by diverse endophytes.

MATERIALS AND METHODS

Nodule collection and bacterial isolation

Nodule samples were recovered randomly from faba bean plants growing under different agroclimatic conditions in Egypt (Fig. 1). Isolate codes, geographical locations and soil data of the sampling sites are presented in Table S1 (Supporting Information). Bacterial isolates were obtained from surface-sterilized nodules as described by Vincent (1970). Individual root nodules from each plant sample were surface sterilized by washing for 30 s with 95% ethanol, immersed in 10% sodium hypochlorite for 90 s and finally washed six times using sterile distilled water. To confirm the efficiency of the surface sterilization procedure, 100 μL aliquot of the water from the last nodule wash was inoculated on yeast extract mannitol (YEM) agar plates. Following sterilization, nodules were crushed aseptically in 0.1 mL sterile distilled water. The nodules suspension was streaked on the surface of YEM agar plates supplemented with 0.025 g/L of Congo Red and incubated at 28°C for 3–5 days (Somasegaran and Hoben 1994). Congo Red dye is often used to distinguish rhizobia from non-rhizobial strains. *Rhizobium* colonies typically do not absorb Congo Red, while other bacteria absorb it (Soares *et al.* 2020). Representatives from colonies that have identical morphological characteristics were selected for further studies.

Nodulation test

The ability of bacterial isolates to induce nitrogen-fixing nodules in their original host (*V. faba* L.) was tested using sterilized Leonard jar systems. Characteristics of sandy soil used in greenhouse experiments in this study were analyzed according to (Page, Miller and Keeney 1982) and presented in Table S2 (Supporting Information). Inoculation, seed sterilization and planting procedures were performed as previously described in Youseif *et al.* (2014). Each sterilized Leonard jar assembly was planted with three surface-sterilized seeds of faba bean (cv. Giza 843) and irrigated with the nitrogen-free nutrient solution (Broughton and Dilworth 1970). Each seed was inoculated with a 1 mL bacterial culture of 10^9 cells/mL. Faba bean plants inoculated with *R. leguminosarum sv. viciae* (*Rlv*) strain NGB-FR128 (Youseif *et al.* 2014), an effective faba bean-nodulating rhizobium, were used as a positive control. Plants were cultivated using a randomized complete block design with three replicates in a controlled greenhouse at 24°C for 12 h (light) and 12°C for 12 h (dark). After 5 weeks, the nodulation was visually checked.

PCR amplification and gene sequencing

Total genomic DNA of bacterial cells was isolated and purified using GeneJet Genomic DNA purification Kit (Thermo Scientific®, Waltham, Massachusetts, USA) according to the

Figure 1. GIS map of faba bean nodule sample collection sites in Egypt.

manufacturer's instructions. The 16S rRNA gene of bacterial isolates was amplified using primers fD1 and rP2 (Table S3, Supporting Information). PCR was performed using the standard reaction mixture (25 μL) containing: 1× PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 mM of each dNTPs, 15 pmol of each primer, 1 U of Taq polymerase enzyme (Promega® Corporation, Madison, WI) and 50 ng of DNA template. PCR conditions and primer sequences used for gene amplification and sequencing of housekeeping genes (*rpoB*, *pgi* and *infB*) and nitrogen fixation *nifH* gene are shown in Table S3 (Supporting Information).

Phylogenetic sequence analyses

Sequence reads were edited and assembled using DNASTAR software (Promega® Corporation, Madison, Wisconsin, USA); (Lasergene, Madison, Wisconsin, USA). The 16S rRNA genes were compared to closely related sequences in EzBioCloud database (https://www.ezbiocloud.net/identify) while, *rpoB*, *infB*, *pgi* and *nifH* genes were blasted in GenBank database (https://www.ncbi.nlm.nih.gov) to search for homologous reference strains. All sequences of the newly isolated strains were deposited in the

NCBI database. The sequences were aligned using Clustal W version 1.8 (Altschul *et al.* 1997) and subjected to phylogenetic analyses. Phylogenetic trees were constructed using the maximum likelihood (ML; Saitou and Nei 1987) in MEGA X software (Kumar *et al.* 2018) using the TamuraNei model. Bootstrap support (BT) for each node was evaluated with 1000 replicates. There was a total of 1142, 939, 815, 584 and 270 alignment sites in the final datasets for 16S rRNA, *rpoB*, *infB*, *pgi* and *nifH* based phylogenetic trees, respectively, and 2338 alignment sites for the tree obtained from concatenated sequences of the *rpoB–pgi–infB* genes. Gene alignments were calculated using MEGA X (Table S4, Supporting Information). The pairwise nucleotide similarity was calculated from the concatenated sequences between tested isolated and closely related reference strains using MEGA X software.

In-vitro screening of PGP activities

Phosphate (P) solubilization

A quantitative analysis of P-solubilization activity was done using the molybdate blue color method (Watanabe and Olsen 1965). Briefly, bacterial isolates were inoculated in a 25 mL Pikovskaya broth medium (Pikovskaya 1948) and incubated for 5–7 days at 28°C. Bacterial cultures were centrifuged at 15 000 rpm for 30 min. A total of 1 mL of supernatant was mixed with 10 mL of chloromolibidic acid and the volume was filled to 45 mL with distilled water. Cholorostannous acid (0.25 mL) was added and the volume was filled to 50 mL with distilled water. The absorbance of the developing blue color was measured by spectrophotometry (Thermo Scientific®, Waltham, Massachusetts, USA) at 600 nm. The amount of solubilized phosphate was detected using the standard curve of a pure substance of KH_2PO_4 (Sigma-Aldrich®, St. Louis, Missouri, USA).

Siderophore production

Bacterial strains were assayed for siderophore production on the Chrome azurol S agar medium (Acros Organics, Belgium) as described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into two equal sectors and spot inoculated with tested bacteria (10 μL of 10^8 CFU/mL). Plates were incubated at 28°C for 48–72 h. The development of a yellow–orange halo around the growth was considered positive for siderophore production.

NH_3 production

Bacterial strains were tested for the production of ammonia in peptone water. Freshly-grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48–72 h at 28°C with shaking at 150 rpm. Nessler's reagent (0.5 mL) was added to each tube. The development of brown to yellow color was considered positive for ammonia production (Cappuccino and Sherman 1992).

Co-inoculation plant assay

Based on the results of the nodulation assay and PGP activities, 14 endophytic strains were selected for the co-inoculation assay. Bacterial strains were inoculated singly or co-inoculated with *Rlv* strain NGB-FR128 (Youseif *et al.* 2014) to seeds of faba bean (cv. Giza 843). The experiment was carried out in sandy soil (Table S2, Supporting Information). Sterilized plastic pots (13 cm diameter) were filled with 2 kg of sterilized soil and arranged in a randomized complete block design with six replicates. All treatments received the recommended dose of phosphate and potassium as described in Youseif, Abd El-Megeed and Saleh (2017). Each seed was inoculated with 1 mL containing 10^9 CFU of an endophytic bacterial strain alone or in combination with *Rlv* strain NGB-FR128 in a ratio of 1:1 (v/v). Plants were cultivated in a controlled greenhouse following the same conditions as previously mentioned in the nodulation experiment. Chlorophyll content was measured after 45 days of sowing according to (Dere, Güneş and Sivaci 1998). Briefly, 1 g of small discs of plant leaves were placed in tubes containing 50 mL of 100% acetone (Sigma-Aldrich®) and were homogenized using a homogenizer (Thermo Scientific®) at 1000 rpm for 1 min. The tubes were incubated for 12 h in dark in the refrigerator, then the homogenate was centrifuged at 2500 rpm for 10 min. The absorbance of the supernatant was measured at 400–700 nm using a spectrophotometer (Thermo Scientific®). The amount of chlorophyll was calculated following the equation described by Lichtenthaler (1987). After 60 days of sowing, plants were uprooted and nodulation, growth parameters as well as shoot N-content of faba bean plants were determined.

Fluorescent tagging and nodule colonization analysis

Based on the results of the co-inoculation assay, three endophytic strains from diverse genera were selected to study how genetic variation of plant and symbiont rhizobia affect nodule colonization with NAB. Fluorescently tagged *Raoultella terrigena* NGB-FR77, *E. sichanensis* NGB-FR97 and *K. variicola* NGB-FR116 were co-inoculated with symbiotic *Rlv* NGB-FR128 or *R. radiobacter* NGB-FR39 (Youseif *et al.* 2014; Youseif, Abd El-Megeed and Saleh 2017). A total of two faba bean cultivars (cv. Giza 843 and Nubaria 1) from distinct genetic lineages (El-Rodeny *et al.* 2014) were included. The endophytic bacteria 'recipients' were transformed with the pHC60 plasmid encoding for the green fluorescent protein (GFP), Tetracycline^R (Cheng and Walker 1998) via a triparental mating (Sambrook and Russell 2001). Conjugation of plasmids was accomplished by mixing the donor *Escherichia coli* containing pHC60, the helper *E. coli* strain containing pRK2013 (Figurski and Helinski 1979) and the recipient strains. Following the triparental mating protocol, the transconjugants were subcultured for several generations, examined using a dark reader transilluminator (Clare Chemical Research Inc., Dolores, Colorado, USA) and confirmed for plasmid stability. The identity of transconjugants was confirmed using 16S rRNA sequencing. After 14 days of sowing, nodule colonization of faba bean plants with selected endophytes was observed using fluorescence (Olympus BX53 model, Germany) microscope using GFP filter (excitation wavelength: 488 nm, emission: 505–530 nm). After 35 days of sowing, faba bean plants were uprooted, surface-sterilized root nodules were crushed separately and tested for the presence of the GFP-labeled endophyte and/or the symbiont. The presence of an endophyte expressing GFP inside the individual nodules was confirmed by streaking the nodule extracts on YEM agar plates amended with 10 μg/mL tetracycline and screening them using the dark reader transilluminator. The identity of bacteria expressing GFP and the symbiont inside the co-infected nodules was verified using 16S rRNA gene sequencing and BOX–PCR fingerprinting (Naganandhini *et al.* 2015).

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the effect of bacterial inoculations on growth, nodulation and shoot-N content of faba bean plants. The effects of the symbiotic partner, endophyte and plant genotypes were assessed using two-way ANOVA. The data were analyzed using CoStat Version 6.45

statistics software (Cardinali and Nason 2013). The least significant difference (LSD) values were used to compare treatment means ($P \leq 0.05$).

RESULTS

Bacterial isolates and plant nodulation assay

The present study is based on the same root nodule samples which were described in (Youseif *et al.* 2014). Our previous investigation only studied the traditional rhizobia strains that could nodulate their host (Youseif *et al.* 2014). However, in the present study, we report on the non-nodulating endophytic bacteria that were isolated from the same root nodules (Table S1, Supporting Information). Out of the inspected 42 nodule samples, we found that the root nodules of 17 plants harbored both symbiotic rhizobia and non-nodulating endophytic bacteria (Table S4, Supporting Information). A total of 34 non-nodulating endophytes were recovered from surface-sterilized root nodules. All of these isolates failed to re-nodulate their original host using sterilized Leonard jar assemblies so they were determined to be non-nodulating endophytes (Table 1). We found that numerous of those nodules housed diverse NAB communities (as described later in the MLSA phylogenetic analysis). There was no direct relationship between the origin of strains and their diversity (Table S4, Supporting Information).

Bacterial identification and phylogenetic analyses

16S rRNA gene

For preliminary identification, 16S rRNA gene sequences (1325–1500 bp) from all isolates were sequenced and blasted to the EZBioCloud database (Table 1). The 16S rRNA sequence analysis showed that all isolated endophytes belonged to the family *Enterobacteriaceae,* phylum Proteobacteria. The 16S rRNA phylogeny did not reveal a clear distinction at the species level but divided the 34 isolates into three clades corresponding to the following genera; *Klebsiella* (23 isolates), *Raoultella* (five isolates) and *Enterobacter* (six isolates; Figure S1, Supporting Information). Due to the conserved nature of the 16S rRNA gene, the taxonomic positions of all isolates were further investigated by multilocus sequence analysis (MLSA) of three housekeeping genes.

MLSA phylogeny of concatenated housekeeping genes

Fragments from *rpoB* (960–1004 bp), *infB* (834–883 bp) and *pgi* (612–657 bp) genes were sequenced, aligned and deposited in the Genbank database. The genes *rpoB*, *infB* and *pgi* encode the β subunit of RNA polymerase, translation-initiation factor IF2 and the glucose phosphate isomerase protein, respectively. The pairwise nucleotide similarity among local isolates and closely related reference strains identified in the MLSA is shown in Table 2. At the genus level, the results of MLSA were generally congruent with those of the 16S rRNA gene phylogeny. However, the MLSA phylogeny (Fig. 2) provided a better resolution of the isolates, classifying the isolates into nine distinct groups 'genospecies' (I–IX).

A total of five isolates in genospecies I had 98.1–99.9% *rpoB-pgi-infB* nucleotide similarity with *K. grimontii* 06D021^T. A total of four isolates in genospecies II had nearly identical (99.5–99.7%) *rpoB–pgi–infB* nucleotide identity with *K. pasteurii* SB6412^T. A total of five isolates in genospecies III were closely related to *K. michiganensis* W14^T, exhibiting 96.1–100.0% *rpoB–pgi–infB* nucleotide identity with this type strain. Three isolates in genospecies IV had 99.7% *rpoB–pgi–infB* nucleotide identity with *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^T. Genospecies V contained six isolates that were closely affiliated (99.7% *rpoB-–pgi–infB* sequence identity) to *K. variicola* DSM 15968^T. A total of five isolates in genospecies VI had 99.4–99.6% *rpoB-–pgi-–infB* nucleotide similarity with *R. terrigena* ATCC 33257^T. Isolates NGB-FR97 and 98 represented genospecies VII formed a well-supported group (BT 100.0%) with *E. sichuanensis* WCHECL1597^T, sharing nearly identical (99.8%) *rpoB–pgi–infB* nucleotide similarity with this type strain. Whereas isolates NGB-FR109 and 110 of genospecies VIII, constituted a sister monophyletic group (BT 100.0%, 99.7% *rpoB–pgi–infB* nucleotide identity) with DSM 1364^T, the type strain of *E. kobei*. Finally, isolates NGB-FR105 and 106 of genospecies IX had 98.4% *rpoB–pgi–infB* nucleotide similarity with *E. asburiae* ATCC 35953^T.

Overall, members of three non-rhizobial genera (*Klebsiella*, *Raoultella* and *Enterobacter*) and nine species (*K. grimontii, K. pasteurii*, *K. michiganensis*, *K. quasipneumoniae*, *K. variicola*, *R. terrigena*, *E. sichuanensis*, *E. kobei* and *E. asburiae*) were identified within root nodules of faba bean plants in Egypt.

Phylogenetic analysis of nifH

Partial sequences (290–329 bp) of *nifH* (gene coding for the iron protein of the enzyme nitrogenase) for all isolated bacteria were successfully sequenced and analyzed. Consistent with the grouping based on MLSA-phylogeny, the ML-phylogenetic tree based on *nifH* showed that strains in genospecies I and genospecies II had identical partial *nifH* sequences to *K. grimontii* 06D021^T and *K. pasteurii* SB6412^T, respectively (Fig. 3). Strains in genospecies III harbored identical *nifH* gene sequences to *K. michiganensis* E718, a clinical strain isolated in Taiwan (Liao *et al.* 2012). The *nifH* sequences from strains in genospecies IV were closely affiliated (98.5% nucleotide similarity) to *K. quasipneumoniae* 01A030^T. Strains of the genospecies V possessed *nifH* genes with high sequence similarities (99.2–99.6%) to *K. variicola* DSM 15968^T and *K. variicola* 342, the latter is an N_2-fixing endophyte with a broad host range (Martínez-Romero *et al.* 2018a; Iniguez, Dong and Triplett 2004). Strains in genospecies VI shared 98.9–100% *nifH* sequence identity to *R. terrigena* ATCC 33257^T and *R. terrigena* DR-E5, the latter is a nitrogen-fixing bacterium isolated from a bark beetle gut (Morales-Jiménez *et al.* 2013). Conversely, strains in genospecies VII, VIII and IX were classified into *Enterobacter* spp. based on 16S rRNA and MLSA phylogenies, had *nifH* nucleotide sequences closely related (<99.8% similarity) to the homologous genes of *Klebsiella* spp. and not those of *Enterobacter.*

PGP traits

NAB strains were tested for their *in vitro* ability to solubilize inorganic phosphate based on Pikovskaya medium and to produce siderophores and ammonia (Table 1). Out of the 34 endophytic strains recovered from faba bean root nodules, 27 strains exhibited the three evaluated PGP traits. All strains were able to solubilize inorganic phosphorus within the range of 10–136 μg/mL. The highest phosphate solubilization efficiency was achieved by strain NGB-FR50 (136 ± 8.7 μg/mL) followed by strain NGB-FR1 (132 ± 8.1 μg/mL). Both strains were classified into *K. pasteurii* and *K. michiganensis*, respectively. All strains were able to produce ammonia, while 79% of them indicated the production of siderophores.

Effect of NAB strains on plant growth and nodulation of faba bean

The putative PGP effects of efficient NAB strains alone and in co-inoculation with strain *Rlv* NGB-FR128 on faba bean plants were

Table 1. Taxonomic affiliation of NAB isolated from faba bean and their PGP activities.

Bacterial strain	Identity based on 16S rRNA gene sequence using EZTaxon blast				Phosphate solubilization (μg/mL)	Siderophores production	Ammonia production	Nodulation of host plant	*nifH* gene amplification
	Length (bp)	Closest species	Accession number	Identity (%)					
NGB-FR 1	1355	*K. michiganensis* W14^T	JQ070300	99.92	132 ± 8.1	+	+	–	+
NGB-FR 3	1371	*K. michiganensis* W14^T	JQ070300	100.00	90 ± 6.5	+	+	–	+
NGB-FR 19	1460	*K. michiganensis* W14^T	JQ070300	99.71	81 ± 7.1	+	+	–	+
NGB-FR 21	1462	*K. oxytoca* ATCC 13182^T	AB004754	99.65	83 ± 5.9	++	+	–	+
NGB-FR 40	1325	*K. oxytoca* ATCC 13182^T	AB004754	99.62	80 ± 7.6	++	+	–	+
NGB-FR 49	1461	*K. pasteurii* SB6412^T	MN091366	99.79	98 ± 7.7	++	+	–	+
NGB-FR 50	1500	*K. pasteurii* SB6412^T	MN091366	99.59	136 ± 8.7	–	+	–	+
NGB-FR 52	1464	*K. pasteurii* SB6412^T	MN091366	99.93	95 ± 5.4	–	+	–	+
NGB-FR 60	1369	*K. pneumoniae* subsp. *rhinoscleromatis* SB3432^T	ACZD01000038	100.00	48 ± 6.3	+++	+	–	+
NGB-FR 67	1362	*K. oxytoca* ATCC 13182^T	AB004754	99.71	55 ± 6.9	+	+	–	+
NGB-FR 72	1367	*R. terrigena* ATCC 33257^T	Y17658	99.71	17 ± 2.9	–	+	–	+
NGB-FR 73	1465	*K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^T	HG933296	99.86	93 ± 5.4	+	+	–	+
NGB-FR 74	1364	*R. terrigena* ATCC 33257^T	Y17658	99.85	38 ± 5.9	–	+	–	+
NGB-FR 75	1465	*K. quasivariicola* KPN1705^T	CP022823	99.52	81 ± 8.3	++	+	–	+
NGB-FR 77	1458	*R. terrigena* ATCC 33 257 ^{T}	Y17658	99.86	28 ± 2.6	+	+	–	+
NGB-FR 79	1460	*K. pneumoniae* subsp. *rhinoscleromatis* SB3432^T	ACZD01000038	99.66	34 ± 5.7	+	+	–	+
NGB-FR 80	1500	*K. pneumoniae* subsp. *rhinoscleromatis* SB3432^T	ACZD01000038	99.79	30 ± 3.4	+	+	–	+
NGB-FR 87	1460	*K. quasivariicola* KPN1705^T CP022823	CP022823	99.52	71 ± 4.7	–	+	–	+
NGB-FR 89	1365	*K. michiganensis* W14^T	JQ070300	99.92	86 ± 6.0	–	+	–	+
NGB-FR 96	1375	*K. quasivariicola* KPN1705^T CP022823	CP022823	99.85	122 ± 9.3	+	+	–	+
NGB-FR 97	1357	*E. roggenkampii* EN-117^T	CP017184	99.85	109 ± 8.5	++	+	–	+
NGB-FR 98	1361	*E. sichuanensis* WCHECl1597^T	POVL01000141	99.85	97 ± 5.2	++	+	–	+
NGB-FR 100	1467	*K. oxytoca* ATCC 13182^T	AB004754	99.58	84 ± 5.9	++	+	–	+
NGB-FR 105	1462	*E. sichuanensis* WCHECl1597^T	POVL01000141	99.52	94 ± 4.8	+	+	–	+
NGB-FR 106	1464	*E. sichuanensis* WCHECl1597^T	POVL01000141	99.45	91 ± 3.6	+++	+	–	+
NGB-FR 108	1464	*K. oxytoca* ATCC 13182^T	AB004754	99.51	56 ± 6.0	+++	+	–	+
NGB-FR 109	1364	*E. kobei* DSM 13645^T	CP017181	99.71	33 ± 3.9	–	+	–	+
NGB-FR 110	1372	*E. kobei* DSM 13645^T	CP017181	99.71	20 ± 3.0	+++	+	–	+
NGB-FR 111	1364	*K. oxytoca* ATCC 13182^T	AB004754	99.56	73 ± 4.8	+++	+	–	+
NGB-FR 112	1372	*R. terrigena* ATCC 33 257 ^{T}	Y17658	99.71	10 ± 2.1	++	+	–	+
NGB-FR 113	1366	*K. quasivariicola* KPN1705^T CP022823	CP022823	99.85	50 ± 6.2	+	+	–	+
NGB-FR 115	1376	*R. terrigena* ATCC 33 257 ^{T}	Y17658	99.71	64 ± 5.1	+	+	–	+
NGB-FR 116	1366	*K. quasivariicola* KPN170^T 5^T CP022823	CP022823	99.85	74 ± 4.7	+	+	–	+
NGB-FR 129	1400	*K. michiganensis* W14^T	JQ070300	99.92	61 ± 5.6	+	+	–	+

The '+' and '–' signs indicate efficiencies as follows: –, negative result; +, weakly positive; ++, moderately positive and +++, highly positive. Data are average values of three replicates ± standard deviation (SD).

Table 2. Pairwise sequence similarities (%) between NAB isolated in this study and closely related members of *Klebsiella*, *Enterobacter* and *Raoultella* genospecies in MLSA.

	MLSA (*rpoB –pgi–infB*; 2338 bp)							
	K. grimontii 06D021^T	*K. oxytoca* ATCC 13182^T	*K. michiganensis* W14^T	*K. pasteurii* SB6412^T	*K. quasipneumoniae* 01A030^T	*K. quasipneumoniae* ATCC 700603^T	*K. variicola* DSM 15968^T	*K. quasivariicola* KPN1705^T
NGB-FR1	97.0	94.9	99.9	97.1	91.4	91.7	91.7	92.3
NGB-FR3	96.9	95.0	100.0	97.0	91.5	91.7	91.6	92.3
NGB-FR19	96.9	95.0	100.0	97.0	91.5	91.7	91.6	92.3
NGB-FR21	99.9	94.7	97.1	97.6	91.7	92.2	91.9	92.5
NGB-FR40	99.9	94.7	97.1	97.6	91.7	92.2	91.9	92.5
NGB-FR49	97.6	94.2	97.0	99.7	91.7	91.7	91.4	92.4
NGB-FR50	97.6	94.2	97.0	99.7	91.7	91.7	91.4	92.4
NGB-FR52	97.6	94.2	97.0	99.7	91.7	91.7	91.4	92.4
NGB-FR60	91.8	91.4	91.8	91.8	99.7	98.0	95.6	95.9
NGB-FR67	99.9	94.7	97.1	97.6	91.7	92.2	91.9	92.5
NGB-FR73	92.0	91.7	91.9	91.8	95.7	95.9	99.7	96.8
NGB-FR75	92.0	91.7	91.9	91.8	95.7	95.9	99.7	96.8
NGB-FR79	91.8	91.4	91.8	91.8	99.7	98.0	95.6	95.9
NGB-FR80	91.8	91.4	91.8	91.8	99.7	98.0	95.6	95.9
NGB-FR87	91.8	91.6	91.7	91.6	95.6	95.8	99.7	96.6
NGB-FR89	95.0	95.4	96.1	94.8	93.2	93.1	94.1	93.5
NGB-FR96	92.0	91.7	91.9	91.7	95.7	95.9	99.6	96.8
NGB-FR100	99.9	94.7	97.1	97.6	91.7	92.2	91.9	92.5
NGB-FR108	97.8	94.3	97.2	99.5	92.0	92.1	91.8	92.7
NGB-FR111	98.1	94.1	96.4	97.1	91.2	91.8	91.5	92.2
NGB-FR113	92.0	91.7	91.9	91.8	95.7	95.9	99.7	96.8
NGB-FR116	92.0	91.7	91.9	91.8	95.7	95.9	99.7	96.8
NGB-FR129	95.5	96.4	97.7	95.4	92.1	92.1	92.1	92.6
	E. asburiae ATCC 35953^T	*E. chengduensis* WCHECl-C4^T	*E. cloacae* ATCC 23373^T	*E. cloacae* ATCC 13047^T	*E. ludwigii* EN-119^T	*E. kobei* DSM 1364^T	*E. roggenkampii* EN-117^T	*E. sichuanensis* WCHECL1597^T
NGB-FR97	94.3	96.4	95.1	95.4	94.0	93.9	96.1	99.8
NGB-FR98	94.3	96.4	95.1	95.4	94.0	93.9	96.1	99.8
NGB-FR105	98.4	95.1	93.8	93.7	95.0	95.4	94.8	94.4
NGB-FR106	98.4	95.1	93.8	93.7	95.0	95.4	94.8	94.4
NGB-FR109	95.5	94.2	93.9	93.7	94.4	99.7	94.2	94.3
NGB-FR110	95.5	94.2	93.9	93.7	94.4	99.7	94.2	94.3
	R. electrica DSM 102253^T		*R. ornithinolytica* ATCC 31898^T		*R. planticola* ATCC 33531^T		*R. terrigena* ATCC 33257^T	
NGB-FR72	93.0		93.3		93.5		99.6	
NGB-FR74	93.0		93.4		93.5		99.5	
NGB-FR77	93.0		93.4		93.5		99.5	
NGB-FR112	92.9		93.3		93.5		99.5	
NGB-FR115	92.9		93.4		93.6		99.4	

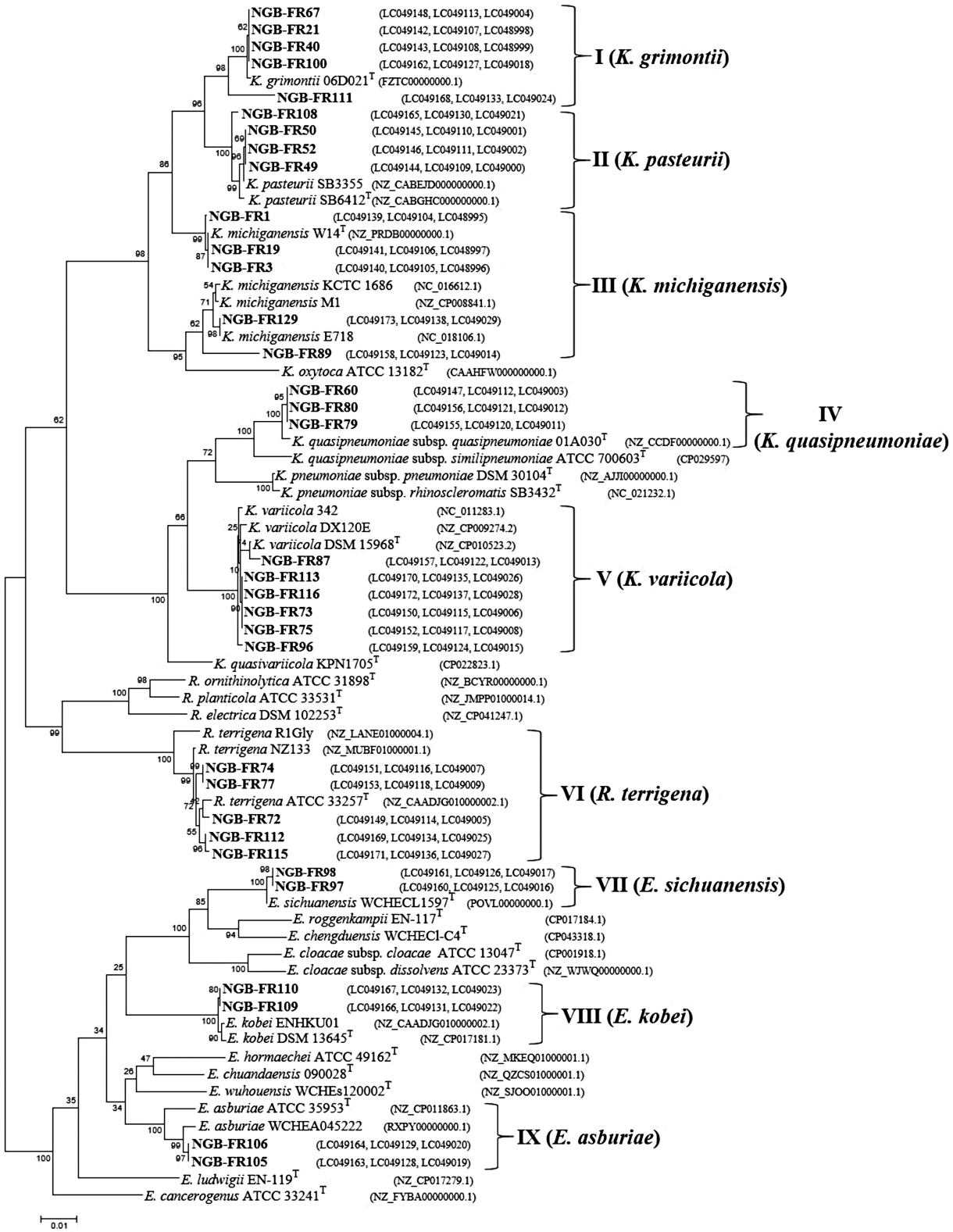

Figure 2. The phylogenetic relationships between the NAB strains (in bold) and reference strains based on multilocus sequence analysis (MLSA) of *rpoB*, *pgi* and *infB* genes. Genbank accession numbers are in parentheses. Bootstrap values are indicated for each node (1000 replicates). *E*: *Enterobacter*; K: *Klebsiella*; and *R*: *Raoultella*.

Figure 3. The phylogenetic relationships between NAB strains (in bold) and reference strains based on *nifH* gene sequences. Genbank accession numbers are in parentheses. Bootstrap values are indicated for each node (1000 replicates). *E: Enterobacter*; K: *Klebsiella* and *R: Raoultella*.

investigated in this part of the study (Table 3). The single inoculation of all NAB strains significantly ($P \leq 0.05$) increased the shoot dry weight (26–59%) and shoot N-content (27–74%) of faba bean plants compared to the non-inoculated control (Table 3). The NAB-inoculated plants also produced higher chlorophyll content (13–127%) compared to the non-inoculated control.

In the cohort of *Rlv* NGB-FR128-inoculated plants, we found that nodule dry weight in most of the co-inoculation treatments (9/14) was significantly lower ($P \leq 0.05$) than when only the Rhizobium was applied. However, only the co-inoculation with *Rlv* NGB-FR128 and either strain *E. sichanensis* NGB-FR97 or *K. variicola* NGB-FR116 were able to produce higher ($P \leq 0.05$) nodule dry biomass than that of plants inoculated with only *Rlv* NGB-FR128. These two co-inoculated treatments also showed higher ($P \leq 0.05$) shoot dry weight (15–23%) and shoot N-content (17–19%) than the single rhizobium inoculation. Co-inoculation with several NAB strains resulted in higher chlorophyll levels than when applying only the rhizobial strain.

Drivers of NAB colonization in faba bean nodules

We studied the influence of plant genotype and the genetic variation of both the rhizobial symbiont and endophyte on nodule infection. To do that, we evaluated the capacity of three GFP-endophytes (*R. terrigena* NGB-FR77, *E. sichuanensis* NGB-FR97 and *K. variicola* NGB-FR116) to colonize nodules induced by two rhizobia strains (*Rlv* NGB-FR128 or *R. radiobacter* NGB-FR39) in two faba bean cultivars (Giza 843 and Nubaria 1). We found that the endophytic strains were able to infect the developed nodules at least in one of the tested plant/symbiont combinations (Table 4). Analysis of co-inoculated plants revealed that the three tested endophytic strains could colonize both the nodules and the intercellular spaces of faba bean roots (Fig. 4). We found that the presence of endophytes in nodules is significantly influenced by the plant genotype ($P = 0.027$), rhizobium genotype ($P = 0.004$) and endophyte genotype ($P = 0.013$) as well as their combined interactions (plant genotype × rhizobium genotype × endophyte genotype; $P = 0.000$). We also detected a significant combined effect of rhizobia genotype x endophyte genotype ($P = 0.018$). However, no significant interaction was found for the plant genotype × rhizobia genotype ($P = 0.600$) nor the plant genotype × endophyte genotype interactions ($P = 0.488$).

In faba bean nodules induced by *Rlv* NGB-FR128, the three NAB strains were found in large percentages (5.6–8.3%) in the case of Giza 843-plants, while their percentages (0.0–2.8%) were decreased in the case of Nubaria 1-plants. A similar trend was observed when we changed the symbiotic partner to *R. radiobacter* NGB-FR39. The percentages of endophytes detected within nodules were greater (3.3–10.6) in Giza 843-plants compared to those of cv. Nubaria 1 (0.0–1.1%). Here we should highlight that *K. variicola* NGB-FR116 could colonize the nodules induced either by NGB-FR128 (6.9%) or NGB-FR39 (4.4%) in the case of Giza 843-plants. While it was unable to infect any of the nodules induced either by NGB-FR128 or NGB-FR39 in the case of Noubaria 1-plants. This demonstrates the role of plant genotype as a major driver influencing the co-existence of distinct endophytes within nodules.

DISCUSSION

Culturing initiatives have detected a vast diversity of non-rhizobial species housed in legume root nodules, revealing the existence of a complex root nodule microbiome that influences the behavior and fitness of the host plant (Martínez-Hidalgo and Hirsch 2017). In the present study, we found a large diversity of non-nodulated endophytic strains belong to the family *Enterobacteriaceae* recovered from faba bean root nodules. Besides other traditional rhizobia described in (Youseif *et al.* 2014), numerous inspected nodules had a population of one or more non-nodulating enterobacteria (Table S4, Supporting Information). This indicates that these enterobacteria are common endophytic bacteria of root nodules of faba bean in Egypt and suggesting that its presence is not fortuitous, but might have an ecological role in the nodulation process. Similarly, many endophytic strains classified into *Agrobacterium, Pseudomonas* and *Enterobacter* were isolated from root nodules of *V. faba* plants in Tunisia (Saïdi *et al.* 2013).

Klebsiella species have been reported to be endophytes of several agricultural crops (Martínez-Romero *et al.* 2018b; Medina-Cordoba *et al.* 2021). They were also isolated from root nodules of numerous legume plants (Ríos-Ruiz *et al.* 2019). *Klebsiella* species associated with nodules have been shown to fix nitrogen and exhibit other PGP traits (Dhole *et al.* 2016; Noori *et al.* 2018). Here, we reported the colonization of faba bean nodules with five species of the genus *Klebsiella*: *K. grimontii*, *K. michiganensis*, *K. pasteurii*, *K. quasipneumoniae* and *K. variicola*. The capacity of *K. variicola* and *K. pneumonia* to colonize root nodules has been previously described in peanut and mungbean (Pandya, Kumar and Rajkumar 2013; Dhole *et al.* 2016). However, to our knowledge, this is the first report of *K. grimontii*, *K. michiganensis*, *K. pasteurii* and *K. quasipneumoniae* which are currently recognized as clinical bacterial species, as nodule-endophytes. *Enterobacter* is also a well-known genus for having endophytic bacteria, which colonize the root nodule of a large variety of legumes (Tariq *et al.* 2014; De Meyer *et al.* 2015) and improve nodulation when co-inoculated with rhizobia (Ibañez *et al.* 2014). In the present investigation, we identified three species within the genus *Enterobacter*: *E. asburiae*, *E. kobei* and *E. sichuanensis*. The existence of *E. asburiae* and *E. kobei* within the root nodules of legumes has been infrequently reported (Benhizia *et al.* 2004; Selvakumar *et al.* 2008). Interestingly, *E. sichuanensis* found in this study, to our knowledge has not been previously reported inside legume root nodules. Unlike *Klebsiella* and *Enterobacter*, members of the genus *Raoultella* have been well documented as human pathogens. To the best of our knowledge, the authenticating of *R. terrigena* as non-nodulating endophytes in this study is reported for the first time in legume plants.

We studied the potential importance of the interaction between NAB strains and faba bean, both in nodulated and non-nodulated plants. In total, two findings from our greenhouse experiment should be highlighted. The first one is that the single inoculation of all enterobacterial strains increased the shoot N-content compared to the non-inoculated control. Also, the plants singly-inoculated with NGB-FR77 were able to accumulate the amount of shoot-N statistically equivalent to the plants that were only inoculated with *Rlv* NGB-FR128. These results indicate that enterobacteria had a considerable role in the fixation of N_2 as a source of nitrogen for plants. The existence of N_2-fixing enterobacteria associated with root nodules of legume plants has been previously described by other researchers (Tariq *et al.* 2014; Dhole *et al.* 2016). It is now documented that, the three bacterial genera (*Enterobacter*, *Klebsiella* and *Raoultella*) that we have identified in our studies harbor *nif* genes in their genomes (Guo *et al.* 2020; Medina-Cordoba *et al.* 2021). Interestingly, all isolated enterobacterial strains in our study possessed *nifH* genes. In agreement with our results, Noori *et al.* (2018) reported that *Klebsiella* sp. A36 and *K. cowanii* A37 isolated from root nodules

Table 3. Effect of the single inoculation of NAB strains or in co-inoculation with *Rlv* NGB-FR 128 on the growth parameters, nodulation, shoot-N and chlorophyll content of faba bean plants under greenhouse conditions.

	Length (cm per plant)				Dry weight (g per plant)				Nodules dry weight (g per plant)		Shoot N content (mgN per plant)		Chlorophyll content*
Treatment	Shoot		Root		Shoot		Root						
Control	28.2	h	13.3	i	1.43	o	0.72	k	0	k	30.7	m	7.9 ± 3.3
R. leg. sv. viciae NGB-FR 128	33.2	c–e	16.0	b–h	2.47	d–g	1.11	b–h	332	b	53.6	c–e	25.6 ± 4.9
Single inoculation with NAB strains													
NGB-FR1	31.0	fg	16.2	b–g	2.14	i–l	0.72	k	0	k	45.9	h–k	13.3 ± 7.9
NGB-FR3	32.0	d–g	14.7	g–i	2.10	i–m	0.77	k	0	k	43.8	i–l	11.8 ± 7.4
NGB-FR49	31.0	fg	17.0	b	2.02	j–n	0.81	jk	0	k	42.5	j–l	8.9 ± 3.6
NGB-FR50	31.3	e–g	17.2	b	1.97	k–n	0.84	jk	0	k	44.1	i–l	15.1 ± 4.9
NGB-FR52	30.8	g	17.3	b	1.96	k–n	0.76	k	0	k	43.0	i–l	9.3 ± 2.3
NGB-FR73	31.0	fg	16.7	b–d	1.89	mn	0.72	k	0	k	38.9	l	13.0 ± 2.9
NGB-FR75	33.1	c–e	17.5	ab	2.15	i–k	1.02	g–i	0	k	48.7	e–i	15.0 ± 0.4
NGB-FR77	33.0	c–e	17.4	ab	2.28	g–i	1.21	b–d	0	k	53.4	c–f	17.9 ± 4.3
NGB-FR96	30.8	g	15.2	d–h	1.93	l–n	1.03	f–i	0	k	43.8	i–l	10.1 ± 2.0
NGB-FR97	32.7	c–f	17.2	b	2.10	i–m	1.13	b–h	0	k	47.8	f–j	15.1 ± 1.3
NGB-FR98	30.8	g	14.5	hi	1.80	n	0.80	jk	0	k	39.1	l	9.2 ± 2.3
NGB-FR105	31.3	e–g	15.3	c–h	1.89	mn	0.93	ij	0	k	41.3	kl	9.1 ± 3.5
NGB-FR106	32.8	c–f	14.8	f–i	2.07	i–m	1.04	e–i	0	k	45.9	h–k	11.0 ± 3.1
NGB-FR116	33.5	b–d	15.3	c–h	2.19	h–j	1.14	b–h	0	k	47.1	g–k	11.4 ± 1.6
Co-inoculation with *Rlv* NGB-FR 128													
NGB-FR1	33.7	b–d	16.2	b-g	2.67	b–d	1.25	ab	225	fg	55.4	cd	27.1 ± 6.6
NGB-FR3	33.8	b–d	16.8	bc	2.49	d–g	1.07	d-i	255	ef	55.7	cd	24.9 ± 2.6
NGB-FR49	33.3	cd	16.5	b-e	2.62	c–e	1.08	c–i	193	hi	54.1	c–e	26.6 ± 3.9
NGB-FR50	32.8	c–f	16.3	b-f	2.52	d–	1.20	b–e	290	cd	53.4	c–f	25.2 ± 3.3
NGB-FR52	33.7	b–d	16.7	b-d	2.56	c–f	1.21	b–d	218	gh	52.3	c–g	24.6 ± 5.8
NGB-FR73	33.3	cd	16.8	bc	2.61	c–e	1.08	c–i	247	e–g	54.1	c–e	28.2 ± 7.7
NGB-FR75	33.0	c–e	16.5	b–e	2.51	d–f	0.93	ij	312	bc	53.5	c–f	26.9 ± 3.6
NGB-FR77	35.3	ab	17.2	b	2.77	bc	1.18	b–f	323	b	57.9	bc	29.4 ± 3.8
NGB-FR96	32.8	c–f	16.2	b–g	2.52	d–f	1.14	b–h	317	bc	52.4	c-g	27.6 ± 6.4
NGB-FR97	34.3	a–c	17.3	b	2.85	ab	1.37	a	393	a	62.7	ab	29.8 ± 2.7
NGB-FR98	33.5	cd	15.3	c–h	2.44	e–g	1.07	d–i	180	i	51.1	d–h	25.1 ± 4.2
NGB-FR105	32.7	c–g	15.0	e–h	2.13	i-l	1.00	hi	132	j	45.5	h–k	22.2 ± 8.8
NGB-FR106	33.7	b–d	16.0	b–h	2.59	c–f	1.17	b–g	263	de	53.1	c–f	26.8 ± 3.9
NGB-FR116	36.0	a	19.0	a	3.04	a	1.23	a–c	367	a	64.0	a	31.0 ± 3.5

*Chlorophyll content (chlorophyll a + chlorophyll b) was measured as (mg/g leaf fresh weight), data are average values of six replicates, ± standard deviation (SD). Means followed with the same letter within each column are not significantly different at $P \leq 0.05$.

Table 4. Effect of plant and symbiotic rhizobium genotypes on colonization (%) of faba bean nodules by diverse NAB strains.

Inoculum Symbiont	Endophyte	Plant genotype	Number of tested plants	Endophyte colonization ratio colonized nodules/total nodules (%)	Frequency of plants with at least one nodule infected by the endophyte
R. leg sv. viciae NGB-FR 128	*R. terrigena* NGB-FR77	Giza 843	12	30/360 (8.3%)	10 (83.3%)
		Nubaria 1	12	4/360 (1.1%)	4 (33.3%)
	E. sichuanensis NGB-FR97	Giza 843	12	20/360 (5.6%)	6 (50.0%)
		Nubaria 1	12	10/360 (2.8%)	8 (66.6%)
	K. variicola NGB-FR116	Giza 843	12	25/360 (6.9%)	8 (66.6%)
		Nubaria 1	12	0/360 (0.0%)	0 (0.0%)
A. tumefaciens NGB-FR 48	*R. terrigena* NGB-FR77	Giza 843	12	12/360 (3.3%)	4 (33.3%)
		Nubaria 1	12	4/360 (1.1%)	4 (33.3%)
	E. sichuanensis NGB-FR97	Giza 843	12	38/360 (10.6%)	8 (66.6%)
		Nubaria 1	12	2/360 (0.6%)	2 (16.6%)
	K. variicola NGB-FR116	Giza 843	12	16/360 (4.4%)	10 (83.3%)
		Nubaria 1	12	0/360 (0.0%)	0 (0.0%)

Data are average values of six replicates.

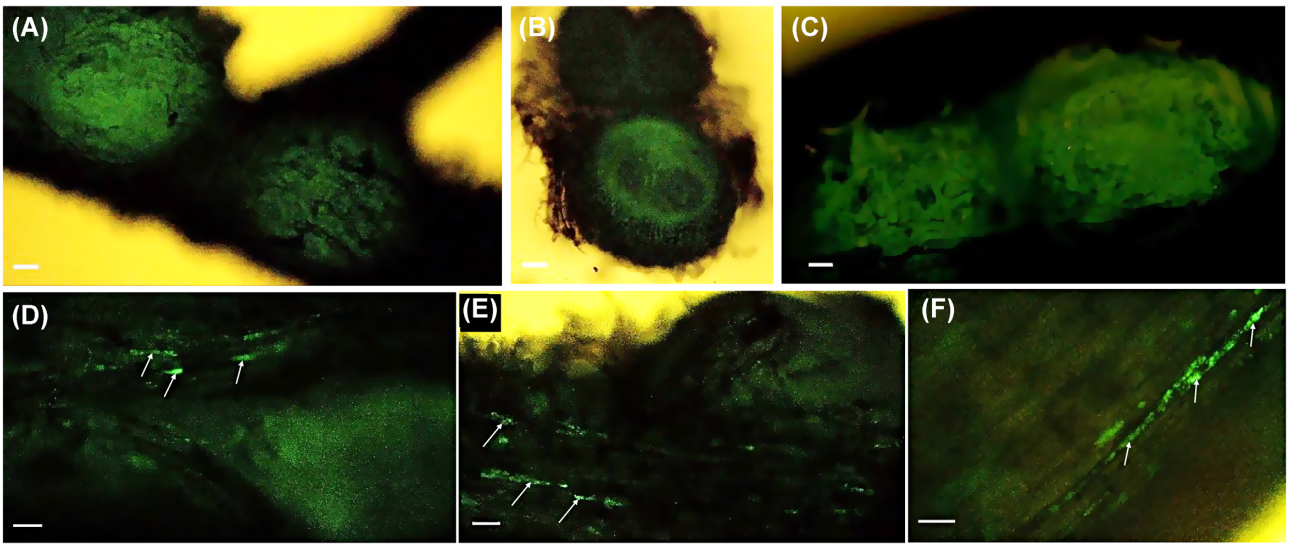

Figure 4. Colonization and infection of faba bean (A–C) nodules and (D–F) roots co-inoculated with *Rlv* NGB-FR128 and endophytic bacteria-GFP visualized on a fluorescence microscope with GFP filter. (A) and (D) *R. terrigena* NGB-FR77-GFP. (B) and (E) *E. sichuanensis* NGB-FR97-GFP. (C) and (F) *K. variicola* NGB-FR116-GFP. The white arrows (D–F) show the location of different endophytic bacteria–GFP in roots. Scale bars: (A–C) 200 μm and (B–C) 100 μm.

of alfalfa could provide plant N in the absence of symbiotic rhizobia and nitrogen.

Second, although the nodulation status (nodule dry weight) was negatively affected when several enterobacteria co-inoculated with *Rlv* NGB-FR128, a trend towards the improvement of shoot dry biomass and chlorophyll content was generally observed. This improvement could be largely due to the capability of enterobacterial strains to produce other PGP activities, such as phosphate solubilization or N_2 fixation. In this context, there are numerous studies have reported that non-nodulating endophytes have beneficial effects on the host plant (Ríos-Ruiz *et al.* 2019). The reduction of the fitness of nodulating rhizobia in presence of several enterobacteria here studied may be due to competitive exclusion at the root surface (Gano-Cohen *et al.* 2016). Similar to our finding, the effect of different non-nodulating bacteria to reduce the nodulation of legume plants when co-inoculated with rhizobia was reported in previous publications (Gano-Cohen *et al.* 2016; Korir *et al.* 2017).

Using a set of fluorescently-labeled endophytic bacteria, we found out that the accommodation of distinct endophytes within nodules is influenced by plant–microbe–microbe interactions, driven by significant effects of the plant genotype, rhizobium genotype and endophyte genotype. Our results showed a substantial difference in the percentages of endophytic colonization between genotypes Giza 843 and Nubaria 1 (Table 4). This demonstrates that the coexistence of distinct endophytes within nodules is selected or 'promoted' by the host genotype.

This finding is in line with previous studies that have reported that non-rhizobial bacterial communities in nodules are structured by plant genotype (Leite *et al.* 2017; Brown *et al.* 2020).

Changing the symbiotic partner also led to a considerable effect on the percentages of colonized nodules within the same genotype × endophyte combination. This highlights the influence of symbiotic rhizobia in regulating the presence of compatible endophytes within nodules. In agreement with our data, Zgadzaj *et al.* (2015) reported that the endophyte's capacity to colonize nodules of *Lotus japonicus* required fully-compatible Nod factors and exopolysaccharides produced by the symbiotic rhizobium (*Mesorhizobium loti*).

CONCLUSION

In this study, we demonstrated a large diversity of endophytic enterobacteria associated with the root nodules of faba bean grown in Egypt, many of which have not been previously described as nodule-endophytes. Co-inoculation of rhizobia with some of the isolated strains (*E. sichanensis* NGB-FR97 and *K. variicola* NGB-FR116) significantly enhanced the nodulation, growth and N-uptake of faba bean. We found that the colonization of faba bean nodules by endophytic enterobacteria is significantly affected by plant–microbe–microbe interactions. As a consequence of this study, some of the isolated strains (*E. sichanensis* NGB-FR97 and *K. variicola* NGB-FR116) are promising candidates to test in combination with rhizobia under field conditions for faba bean production.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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