Two species-specific TaqMan-based quantitative polymerase chain reaction assays for the detection in soil of *Paenibacillus polymyxa* inocula

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Abstract

Aims: The increasingly widespread use of beneficial microbial inocula in agriculture gives rise to two primary needs: i) the assessment of the environmental risk, i.e. their impact on local soil microbiome and soil properties; ii) being able to track them and monitor their persistence and fate to both optimize their formulation and application method. In previous years, PCR-based methods have detected bacterial or fungal bioinoculant at the species or strain level. However, the selective detection, quantification, and monitoring of target microbial species in a complex ecosystem such as soil require that the tests possess high specificity and sensitivity.

Methods and results: The work proposes a quantitative real-time PCR detection method using TaqMan chemistry, showing high specificity and sensitivity for the *Paenibacillus polymyxa* K16 strain. The primer and probe sets were designed using the polymyxin gene cluster targeting *pmxC* and *pmxE* sequences. Validation tests showed that these assays allowed a discriminant and specific detection of *P. polymyxa* K16 in soil.

Conclusion: The TaqMan-assay developed could thus ensure the necessary level of discrimination required by commercial and regulatory purposes to detect and monitor the bioinoculant in soil.

Significance and impact of the study:

The assay could be used to track the persistence and fate of the bacterium for regulatory and registration purposes and for gaining the knowledge needed to optimize its application to crops.

Keywords: bioinoculant, molecular markers, polymyxin gene, PGPR, TaqMan probes, qPCR, RNA

Introduction

The use of microbial-based products (hereafter bioinoculant) in agriculture has been growing in the last decades due to the increasing concerns about the environmental impact of synthetic fertilizers. Soils are becoming more vulnerable due to several factors affecting quality and fertility, including anthropogenic pressures, and climate change. Thus, there is a pressing need to develop a new strategy for introducing bioinoculants and function-specific microorganisms to improve soil fertility and plant nutrition. Understanding and predicting the consequences of the introduction of bioinoculants on natural communities of soil microorganisms require dedicated studies on their interactions and on their combined effects on the functioning of the ecosystem. However, it is becoming noticeable that the impact on the microbial native biodiversity, the environmental fate as well as the traceability of the bioinoculant are aspects that need to be better assessed to ensure the safe use of bioinoculants in agricultural productions (Trabelsi and Mhamdi 2013, Mawarda et al. 2020, Mitter et al. 2021).

Thus, the need for efficient, sensitive, and discriminant tools to track and monitor a bioinoculant in the soil is rapidly increasing (Manfredini et al. 2021). Detecting and quantifying bioinoculant persistence and fate in the soil would also contribute to the optimization of their application methods and serve regulatory or registration purposes (Malusà and Vassilev 2014, Malusà et al. 2021; Vassilev and Malusà 2021). Having a tracking protocol available for each microorganism on the market would mean understanding their interaction with the local microbial community, thus improving their application in the field (Berg et al. 2021). However, detecting soil bioinoculants requires analytical methods with high specificity and sensitivity. The accuracy of an analytical test that reports the presence or absence of a given condition is mathematically described by sensitivity and specificity (Kralik and Ricchi 2017). In the case of tracking a microbial inoculant within a complex matrix containing other microorganisms similar to the added one, the binomial "sensitivity and specificity" refers to the rate of "true positive" in the case of "sensitiv-

ity," i.e. the ability to detect the presence of that species or its strain/s even if present in minimal amounts. On the other hand, the term "specificity" refers to the ability to detect the presence of that strain, or one of its genes, even if it is mixed with other strains of the same species and therefore easily confusable.

In diagnostic and screening tests of the presence and abundance (copy number) of a gene by quantitative polymerase chain reaction (qPCR), there is usually a trade-off between sensitivity and specificity, indicating that a higher sensitivity corresponds to a lower specificity. The soil microbiome typically exhibits a considerable complexity, including different strains of the same organism or taxa so closely related that most of the current commercial tests are not specific enough to detect and track what has been added. Currently, the most widely used methods for the detection and quantification of a bioinoculant in soil are based on DNA analysis, mainly applying different PCR-based techniques (Enkerli et al. 2001, Schwarzenbach et al. 2007, Timmusk et al. 2009, Canfora et al. 2016, 2017, Tartanus et al. 2021). It follows that the design of customized species- or strain-specific markers suitable to ensure its univocal detection in soil closely depends on the proper genetic characterization of the target microorganism (Nehra and Choudhary 2015).

Paenibacillus polymyxa (formerly *Bacillus polymyxa*) is a nonpathogenic, endospore-forming bacterium, naturally occurring in soils, and frequently enriched in the rhizosphere of crop plants (Padda et al. 2017). This species' wide range of beneficial properties include nitrogen fixation, plant growth promotion, soil phosphorus solubilization, and the production of exopolysaccharides, hydrolytic enzymes, plant hormones, and antibiotics, including the antibiotic *polymyxin* (Lal and Tabacchioni 2009, Jeong et al. 2019, Langendries and Goormachtig 2021). These characteristics make *P. polymyxa* a species suitable for producing a commercial bioinoculant and the subject of dedicated studies aiming to understand better its genetic background (Eastman et al. 2014, Xie et al. 2016, Zhou et al. 2020). For this reason, a strain of this species (K16) was selected to develop a formulation for the control of two soil-borne pathogens (*Phytophthora nicotianae* and *Fusarium oxysporum*), which are the causal agents of root rot, stem base rot, and wilting of tomatoes, respectively (Gilardi et al. 2014, Cacciola and Gullino 2019).

Despite the interest in using *P. polymyxa* strains for commercial purposes, to our knowledge, the detection and quantification of this bacterium in the rhizosphere have been reported only once, limited to intragenera specificity and exploiting the 16S rRNA gene polymorphism (Timmusk et al. 2009).

The polymyxin antibiotics gene cluster (*pmx*) in both the core and accessory genomes of several *P.polymyxa* strains isolated from diverse environments (Zhou et al. 2020) could be considered a suitable target to design a helpful assay for species and/or strain-specific detection.

The present study reported a method based on two TaqMan-based qPCR assays for tracking and quantifying the *P. polymyxa* K16 strain. Validation tests were performed on soil artificially inoculated with *P. nicotianae* var. *nicotianae* or *F. oxysporum* and inoculated with *P. polymyxa* K16 under greenhouse conditions, also verifying the expression of the polymyxin genes.

Materials and methods

Microbial isolates for specificity assay

Paenibacillus polymyxa K16 was isolated from the roots of tomato plants grown in a trial at the experimental field "Ribickiego" in Skierniewice and introduced in the SYMBIOBANK culture collection of the National Institute of Horticultural Research in Skierniewice (PL). The strain is characterized by several functions to promote plant growth (including N-fixing capacity, production of siderophores) and others more suitable for plant protection, i.e. production of secondary metabolites and toxins inhibiting the growth of some soil-borne pathogens (e.g. *Fusarium* sp., *Verticillium dahliae*) as well as of some Gram-negative bacteria (e.g. *Pseudomonas* sp. or *Rahnella aquatilis*).

A total of 16 fungal strains and 40 bacterial strains, including 23 different *P. polymyxa* strains, were used in primers and probes specificity tests (Table 1) to assess the specificity of the detection method toward *P. polymyxa* K16. Pure cultures of all bacterial strains were prepared on LB nutrient medium (Difco™, Detroit, MI, USA), with overnight incubation at 28°C. The fungal strains were cultured on Potato Dextrose Agar (PDA) medium (Difco™, Detroit, MI, USA) for 1 week at 25°C.

Microcosm experiment in soil inoculated with *P. Polymyxa* K16

Fresh inocula of *P. polymyxa* K16 were prepared from LB broth suspensions and grown for 24–48 h. The cultures were washed three times with sterile saline solution (9% w/v NaCl). After assessing the purity on Tryptic Soy Agar (TSA) agar plates, the final concentration was adjusted to a 0.5 McFarland standard turbidity (Zapata and Ramirez-Arcos 2015).

The 3 soil samples collected in 3 different experimental sites located in Italy, Germany, and Poland were selected from the trials of the EXCALIBUR project (H2020 grant n. 817 946). The 3 soil samples showed different physico-chemical properties (Table 2) and were used to set up microcosms experiments by adding *P. polymyxa* K16 bacterial cells. These soils were used to test the primers and probes designed to detect and discriminate the *P. polymyxa* from the strains of Table 1.

A volume of 1 mL of *P. polymyxa* K16 was inoculated into 40 g of soil (not sterilized) at two concentrations (dose 'I'= 1×10^6 cells mL^{-1} and dose 'II'= 1×10^2 cells mL^{-1}) to test the sensitivity, specificity, and reproducibility of the method. The microcosms were set up in triplicates in 100 mL sterile polypropylene containers with a ventilated screw cap (the caps were pierced sterile, and a 0.22 μm filter was fitted to facilitate gas exchange) and incubated at 25°C in a ventilated heater thermostat for 28 days in dark conditions, monitoring, and maintaining constant soil moisture.

DNA and RNA isolation from soil samples

According to the manufacturers' recommendations, DNA and RNA from samples were extracted from 600 mg wet soil aliquots using the commercial kit ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, USA). The DNA was separated following the manufacturers' recommendations. The environmental DNA extract yields were determined using the Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) High Sensitivity Kit following the manufacturer's instructions. DNA quality was evaluated using Nanodrop1000™ (Invitro-

Table 1. List of bacterial and fungal strains used in microcosm experiments to evaluate the specificity of the TaqMan-based assay toward *P. polymyxa* K16*

	Bacterial strains	Collection number
1.	*Agrobacterium* sp.	A3
2.	*B. cereus*	ATCC 10 876
3.	*B. firmus*	KIS
4.	*B. licheniformis*	PCM B/00 106
5.	*B. licheniformis*	INHORT BSLC
6.	*B. subtilis*	PCM B/00 105
7.	*B. subtilis*	ATCC 6633
8.	*Escherichia coli*	PGVS
9.	*P. polymyxa*	K16
10.	*Pseudomonas protegens*	Ps55BA
11.	*Pseudomonas* sp.	FC7
12.	*Pseudomonas* sp.	FC8
13.	*Pseudomonas* sp.	FC9
14.	*Rahnella aquatilis*	Pi3A
15.	*Serratia plymutica*	HRO-C48
16.	*Solibacillus* sp.	BHBSAA
17.	*Stenotrophomonas rhizophila*	DSM14405
18.	*B. polymyxa*	4P1-21
19.	*B. polymyxa*	5P1-8
20.	*B. polymyxa*	L10-1-8
21.	*B. polymyxa*	RE1-4-13
22.	*B. polymyxa*	BB4-1-18
23.	*B. polymyxa*	BB4-1-28
24.	*B. polymyxa*	RB4-3-10
25.	*B. polymyxa*	14R3-301
26.	*B. polymyxa*	14R4-401
27.	*B. polymyxa*	14R8-301
28.	*B. polymyxa*	2R5-401
29.	*B. polymyxa*	2R6-401
30.	*B. polymyxa*	2R7-401
31.	*B. polymyxa*	2R8-401
32.	*B. polymyxa*	4R17-401
33.	*B. polymyxa*	5R2-401
34.	*B. polymyxa*	5R6-401
35.	*B. polymyxa*	7R3-401
36.	*B. polymyxa*	4R6-102
37.	*B. polymyxa*	SF1-37
38.	*P. polymyxa*	PB71 = GnDBI71/1
39.	*P. polymyxa*	Wb2-3
40.	*P. polymyxa*	Mc5Re-14
	Fungal Strain/Collection number	**Collection**
41.	*Beauveria brongniartii*	INHORT
42.	*Botrytis cinerea*	BC
43.	*Clonostachys rosea*	1881
44.	*F. oxysporum*	FC29
45.	*F. oxysporum*	141/89
46.	*F. oxysporum*	233/1RB
47.	*F. oxysporum*	257/8WT
48.	*F. oxysporum*	FC21
49.	*F. oxysporum*	FC3
50.	*F. oxysporum*	MSA35
51.	*Fusarium* sp.	AF25
52.	*Fusarium* sp.	UNITO
53.	*Pythium* sp.	Pythium 1
54.	*Trichoderma virens*	FC80
55.	*Trichoderma longibrachiatum*	FC6
56.	*Trichoderma asperellum*	TW2

*The strain identifiers were provided by the owners of the strain

gen, Thermo Fisher Scientific, USA). After extraction, the total amount of eluted DNA (0.1 mL) was purified using Amicon Ultra 0.5 mL Centrifugal Filters 30 K NMWL (EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer's instructions. The purified and concentrated DNA was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) High Sensitivity Kit, diluted to 10 ng μL^{-1} and stored at $-20°C$ for downstream analyses.

The extracted RNA was reverse transcribed into cDNA using SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific Inc, USA.), following the manufacturer's instructions. The obtained cDNA was quantified using Qubit® 2.0 Fluorometer with a DNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions, diluted to 10 ng μl^{-1} and stored to $-20°C$ until used downstream analyses.

Design of primers and probes specific for the detection of *P. Polymyxa* K16

Two sequences coding for polymyxin genes sequences *pmxC* and *pmxE* (JN660148.1, NCBI), were used to design two sets of primers and their corresponding TaqMan probes (Table 3) using Primer Express 3 software (Applied Biosystems, CA, USA). The primer set specific for the *pmxE* gene amplified a region of 93 bp, while the primer set specific for the *pmxC* gene amplified a region of 66 bp.

The *in silico* specificity of primers and probes was checked using the Basic Local Alignment Search Tool (BLAST) network service of GenBank.

Primers and probe specificity were verified on the whole genome sequence of *P. polymyxa K16* (the genome has been deposited on NCBI, BioProject Id: PRJNA889341). The *in vivo* specificity of the primers and probes was tested using the 16 fungal, and 40 bacterial (of which 23 *P. polymyxa*) strains listed in Table 1. The assays (primers and probes) were synthesized by Life Technologies (Thermo Fisher Scientific, Europe BV).

TaqMan qPCR assay

The qPCR analysis of DNA or cDNA obtained from the microcosm experiment was carried out with the QuantStudio™ 5 (ThermoFisher, USA). The qPCR reaction mix was conducted with a final volume of 20 μL containing: 10 μL of TaqMan® Fast Advanced Master Mix, 1 μL TaqMan® Assay (20X), μL of Nuclease-Free Water, and 2 μL of isolated DNA or cDNA. The conditions for the qPCR analysis were as follows: 2 min for enzyme activation at $95°C$, followed by 40 cycles of denaturation at $95°C$, 1 and 20 s for annealing/extension at $60°C$.

The standard curve for DNA quantification was created (in duplicate) using a 10-fold dilution series of DNA extracted from *P. polymyxa* K16 covering a range of 10^8 to 10 fg of purified genomic DNA μL^{-1}. An internal positive control (IPC) kit (MicroGaia, ES) was used in the qPCR assays to detect PCR inhibition, preventing false-negative interpretation.

Validation of the TaqMan-based qPCR assay: detection of *P. Polymyxa* K16 inoculated into native soil

An independent experiment testing the bio-protection properties of *P. polymyxa* K16 against tomato soil-borne pathogens was conducted to validate the detection method. A sandy loam soil was artificially inoculated with either *F. oxysporum* [FOX] or *P. nicotianae* var. *nicotianae* [PNN] before planting tomato seedlings of cv. Julia. Untreated soil (receiving the same amount of sterilized water) [Ctrl] was used as a control. *P. polymyxa* K16 was applied as a liquid suspension (6.2×10^8 CFU mL^{-1}), on which the tomato roots were soaked 30 min

Table 2. Physical-chemical characteristics of the soils used in the microcosm experiment.

		IT	GE	PL
pH		8.2	7.9	7.3
Organic matter	%	2.07	3.36	1.23
Total nitrogen	g/kg	1.31	0.22	0.75
P available	mg/kg	19	1.24	0.55
K available	mg/kg	284	2.91	0.85
Calcium	mg/kg	3915	8.19	2.15
Magnesium	mg/kg	402	4.94	0.92
Sand	%	17	63	87
Silt	%	41	30	10
Clay	%	42	7	3
Texture class (USDA)		Silty clay	Sandy loam	Sand
Latitude and		44.248678	47.779958	51.95488
Longitude		12.289 961	9.534 349	20.15 831

IT = Italy; GE = Germany; PL = Poland.

Table 3. The nucleotide sequence of the primers and probes used for the detection of *P. polymyxa* K16.

Primer pair and probe	Sequence (5'-3')	Tm (°C)	Assay Annealing temperature (°C)	Amplicon size (bp)
pmxC F	GAACATGGGCCGAATGAACG	58	60	66
pmxC R	GAGCGAGATAACATGCCGGA	58		
pmxC probe	TACGCTTGGGGGTATGCC	58		
pmxE F	CGATCGGCAGCGAATCCA	58	60	93
pmxE R	CCAGAATGCGTTCATACCGGG	59		
pmxE probe	CCGTCCAAAGTGATGGCCAG	62		

F: forward; R: Reverse.

before planting (experiment here referred to as 'A'). In the second experiment, a first inoculation, performed using a microbial suspension with the same concentration, was followed by a second one after 7 days. The seedlings were then planted into the infected soil (experiment here referred to as 'B'). A completely randomized design with four repetitions of 10 plants each was used for both experiments. Soil sampling for the detection analysis was performed 4 weeks after planting. Bulk soil samples were analyzed using both TaqMan assays sets.

The cDNA obtained from reverse transcribed RNA, isolated from rhizospheric soil samples, was used to analyze the expression of the polymyxin genes.

Results

Specificity of the TaqMan assay for *P. Polymyxa* K16

The TaqMan-based qPCR assays designed on the polymyxin gene cluster did not yield any amplification products, except for the *P. polymyxa* strains (Fig. 1), from the DNA extracted from the fungi and bacteria strains (Table 1) (Gerin et al. 2018). Moreover, no inhibition of the PCR reaction was observed in any of the experimental qPCR runs (Fig. 1) when adding the IPC.

Detection of *P. Polymyxa* in soil from a microcosm experiment

The TaqMan-based method allowed detection of the strain, irrespective of the inoculum concentration used, with the same efficiency in all three soils tested; the negative control (i.e. soil added with sterile water) did not yield any detectable amplification (Fig. 1). Moreover, no inhibition of

the PCR reaction was observed in any of the experimental runs performed to verify this occurrence. This evidence confirmed the high analytical specificity and sensitivity of the method.

The application of *P. polymyxa* K16 as liquid inoculum also resulted in a good strain detection in the microcosms experiment when the three soils were not sterilized, irrespective of the inoculum concentration (Table 4). The gene copy number per gram of dry soil differed significantly between the three soils not inoculated with the strain of interest, with the one from Poland showing a higher gene copy number per gram of dry soil compared to the other two soils. This difference is not surprising, given the Polish origin of *P. polymyxa* K16. However, the gene copy number per gram of dry soil did not differ between the three soils inoculated at the two doses, even if the quantity of *pmxE* was higher than *pmxC*.

Validation of the detection method of *P. Polymyxa* K16 in native rhizospheric soil

The *P. polymyxa* K16 detection in tomato rhizospheric soil was based on the genes' transcript. used to assess the expression of the genes selected for the design of the probe/primer sets. The standard curves, from a DNA concentration of 10^2–10^8, were made for both the *pmxC* (Fig. 2) and *pmxE* (Fig. 3) TaqMan assays, starting from the DNA of a pure culture of *P. polymyxa* K16. They resulted in fitting to a linear square regression curve ($r^2 = 0.999$) with a similar slope coefficient (−3.4562 and −3.4506, respectively) and efficiency percentage (E%) of 94.7% and 94.9%, respectively.

The total RNA yields ranged between 3.14 and 8.72 ng μL^{-1} (Table 5). The amount of the genes tran-
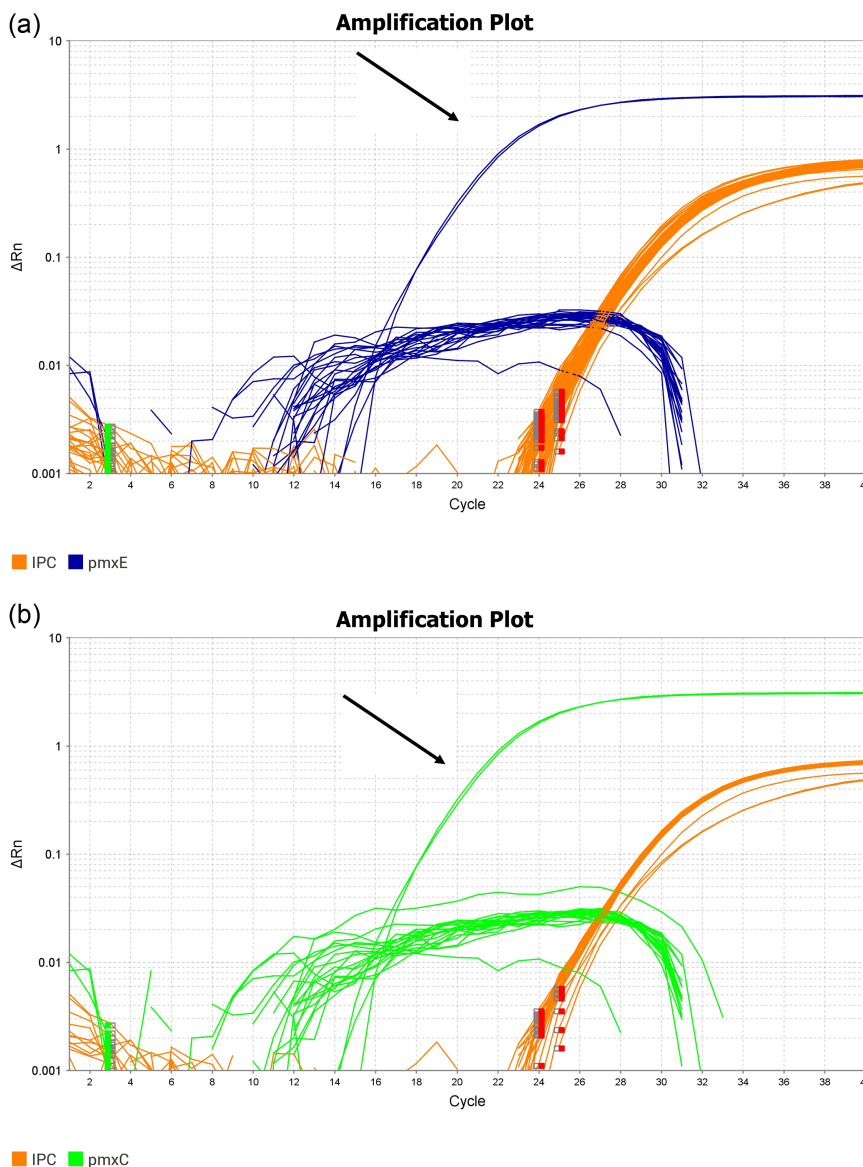
Figure 1. Specificity test. Amplification plot of nontarget microorganisms (blue and green lines) and evaluation of inhibition (IPC). The arrow shows the positive control (P. polymyxa K16). Plot A: pmxE assay; plot B: pmxC assay; IPC internal positive control.

Table 4. The mean gene copy number per gram of dry soil of the *P. polymyxa* K16 detected in microcosms soils, determined by the two TaqMan-based assays (*pmxC* and *pmxE*).

	pmxC				pmxE			
	Mean gene copy number per g of dry soil	ST.Dev	N	SEM	Mean gene copy number per g of dry soil	ST.Dev	N	SEM
G I	8.01E+07	4.03E+07	6	1.64E+07	1.67E+08	8.37E+07	6	3.42E+07
G II	7.50E+07	2.05E+07	6	8.36E+06	1.58E+08	4.36E+07	6	1.78E+07
IT I	9.37E+07	7.64E+07	6	3.12E+07	1.99E+08	1.63E+08	6	6.66E+07
IT II	4.86E+07	1.22E+07	6	4.97E+06	1.01E+08	2.57E+07	6	1.05E+07
P I	1.59E+06	6.30E+05	6	2.57E+05	3.36E+06	1.33E+06	6	5.41E+05
P II	1.03E+06	2.26E+05	6	9.24E+04	2.14E+06	4.61E+05	6	1.88E+05
CRTL_P	1.50E+06	1.57E+03	6	6.40E+02	1.50E+06	1.57E+03	6	6.40E+02
CTRL_I	1.17E+04	1.68E+03	6	6.85E+02	1.17E+04	1.68E+03	6	6.85E+02
CTRL_G	1.78E+05	2.47E+03	6	1.01E+03	1.78E+05	2.47E+03	6	1.01E+03

G = Germany; IT = Italy; P = Poland. SEM = Standard Error of the Mean; I = Concentration I; II = Concentration II; CTRL = untreated soil.
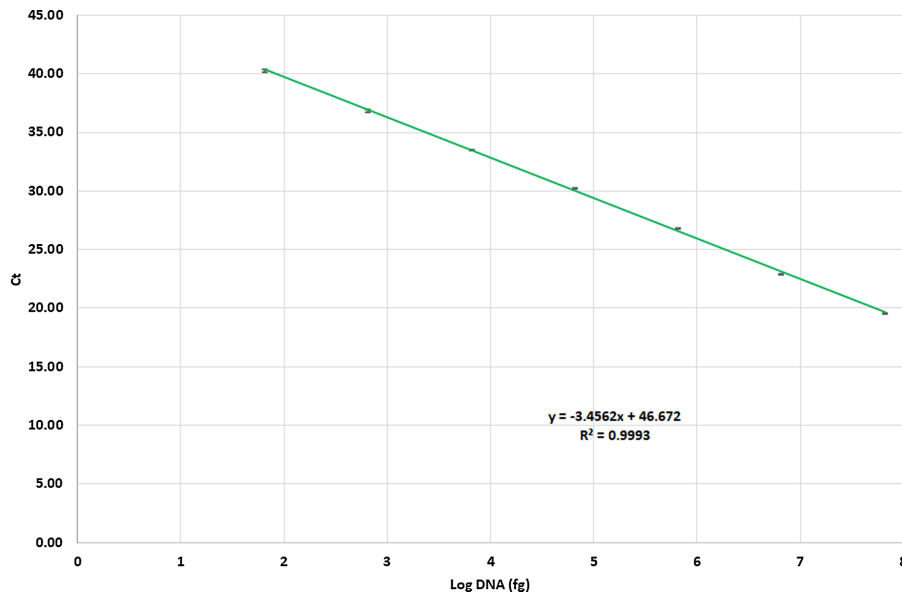
Figure 2. Calibration curve pmxC assay. Fluorescent intensity as a function of concentration of template. For each assay, a series of 10-fold dilution of DNA was used as a template for PCR (108–101 serial diluition).
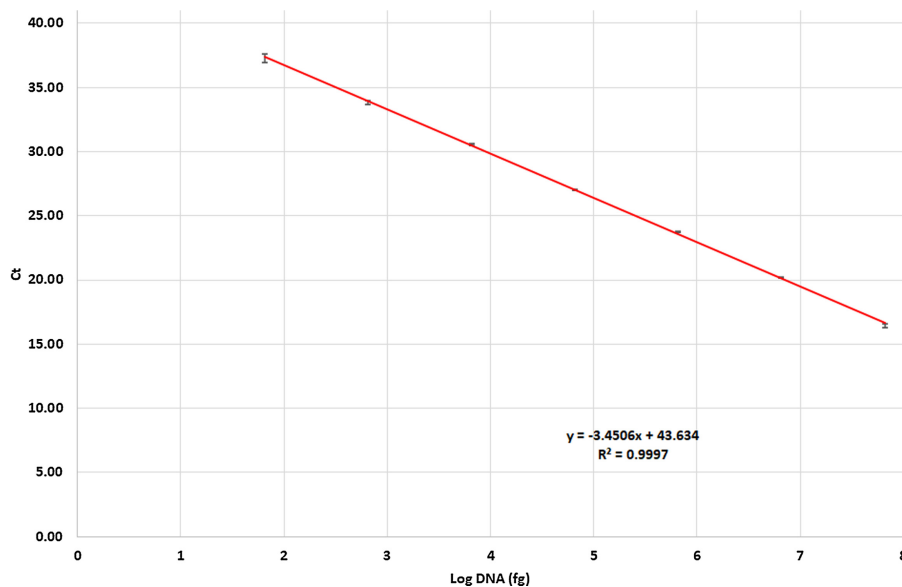
Figure 3. Calibration curve pmxE assay. Fluorescent intensity as a function of concentration of template. For each assay, a series of 10-fold dilution of DNA was used as a template for PCR (108–101 serial diluition).

script number from the *P. polymyxa* mixed with the fungal pathogens (K16+FOX or K16+PNN treatments) was 1.17 or 2.73 times higher compared to the control (calculated as $2^{-\Delta Ct}$; $\Delta Ct = Ct_{\text{treat}} - Ct_{\text{ctrl}}$), respectively (Table 6), showing a higher activity of *P. polymyxa* K16 compared to the control. Lower net values, but still appreciable, were obtained from the samples of the second tomato experiment, except for the K16+PNN_A treatment, which yielded an amount of the genes transcript not statistically different to the untreated control. Both *pmxC* and *pmxE* genes were expressed, showing that *P. polymyxa* K16 synthesized polymyxin when challenged by *F. oxysporum* or *P. nicotianae* var *nicotianae*. A different trend was observed looking at the

target gene transcript number, hereafter as gene copy number per g of dry soil. The *pmxC* transcript copy number was significantly higher than the *pmxE* one, showing a higher secretion of the polymyxin gene triggered by the presence of the pathogen (Table 6). High values were observed in the untreated soils, but this was not surprising considering that *P. polymyx*a K16 is widely distributed in the Polish soils.

Discussion

The present study used a specific trait of *P. polymyxa*, namely the *pmx* gene cluster coding for the polymyxin antibiotic, to

Table 5. Nucleic acid yields from rhizospheric tomato soil inoculated with *P. polymyxa* K16 alone or in combination with two soil-borne pathogens.

#	ID	DNA (ng μL^{-1})	RNA (ng μL^{-1})
1	K16+FOX	33	8.72
2	K16+PNN	13.3	3.83
3	K16+FOX_A	26	7.57
4	K16+FOX_B	32.7	7.89
5	K16+PNN_A	27.1	8.08
6	K16+PNN_B	29.2	6.56
7	K16+FOX	32.1	9.07
8	K16+PNN	12.9	3.14
9	K16+FOX_A	20.8	5.34

K16 = *P. polymyxa* K16; FOX = *F. oxysporum*; PNN = *P. nicotianae* var. *nicotianae*; A = first experiment (see Section 6); B = second experiment (see Section 6).

Table 6. The mean Ct end-point fluorescence and mean gene copy number per g of dry soil of *P. polymyxa* K16 inoculated in soil alone or combined with two soil-borne pathogens detected by the two TaqMan-based assays (*pmxC* and *pmxE*).

	pmxC	pmxE
		Mean Ct
Ctrl	31.88	33.06
K16+FOX	32.10	33.28
K16+PNN	32.38	32.79
Ctrl_A	31.63	33.32
K16+FOX_A	31.37	33.09
K16+PNN_A	32.03	33.43
Ctrl_B	31.59	32.37
K16+FOX_B	31.75	32.72
K16+PNN_B	32.46	33.99
	pmxC	pmxE
	gene copy number per g of dry soil	
Ctrl	5.40E+00	3.64E−01
K16+FOX	6.72E+00	4.46E−01
K16+PNN	8.47E+00	7.90E−01
Ctrl_A	1.25E+01	6.91E−01
K16+FOX_A	1.44E+01	6.29E−01
K16+PNN_A	1.02E+01	5.86E−01
Ctrl_B	1.39E+01	6.26E−01
K16+FOX_B	1.34E+01	8.32E−01
K16+PNN_B	7.18E+00	3.91E−01

K16 = *P. polymyxa* K16; FOX = *F. oxysporum*; PNN = *P. nicotianae* var. *nicotianae*; A = First experiment (see Section 6); B = second experiment (see Section 6).

develop a TaqMan-based PCR assay that specifically detects and quantifies the *P. polymyxa* K16 strain. A PCR-based assay should discriminate phylogenetically similar microbial populations (Chakraborty et al. 2014, Kress et al. 2015, Manfredini et al. 2021). Numerous methods have been published on the application of PCR for detecting and quantifying the fate or persistence of microorganisms in different environments (Nesme et al. 1995; Barns et al. 2005), resulting in new detection protocols suitable also for the soil (Degrange and Bardin 1995, Canfora et al. 2016). The success in detecting a bioinoculant depends on the specific design and optimization of the primers and probes used in the PCR. Nevertheless, the markers used for the detection may not be sufficiently strain-specific and can detect other strains of the same species or distant organisms. The tests performed with the different arrays of bacterial and fungal species provided good evidence of the method's validity for the specific de-

tection of *P. polymyxa* (Fig. 4). Although fungi are eukaryotes, they are usually used in the bioinoculant formulation or mixed with bacteria to test biological control activity. We preferred to include also some fungi species in the specificity test.

Paenibacillus polymyxa can support plant growth and can suppress soil-borne pathogens (Langendries and Goormachtig 2021). The secretion of the antibiotic polymyxin, exhibiting both antibacterial (Gram-negative) and antifungal activity (Yu et al. 2015; Shi et al. 2017, Jeong et al. 2019), is assumed to be one of the mechanisms for the pathogens' suppression. The two primer pairs and TaqMan-based probes were designed based on the nucleotide sequences of two genes encoding for this antibiotic. The ORF designated as *pmxC* encodes a transporter-like protein, and its amino acid TycD is a member of the ABC transporter, potentially involved in the secretion of polymyxin; *pmxE* encodes polymyxin A synthetase (Jeong et al. 2019).

The application of *P. polymyxa* K16 as liquid inoculum also resulted in good strain detection in the microcosms experiment. The outcome confirmed the autochthonous nature of the strain isolated from soil of the same location. The different amount of *P. polymyxa* K16 observed between soils confirms the impact that the soil characteristics, even in the absence of the plant, can have on bioinoculant persistence (Bashan et al. 1995). The detection was also verified after 1 month from soil inoculation, as this time frame is normally used in studies for soil microbial respiration and emerged as a suitable timing in previous reports (Comeau et al. 2018). Moreover, considering the complexity and chemical-physical heterogeneity of any soil system, the DNA polymerase activity can be inhibited, producing a false-negative, or amplifying contaminating sequences, resulting in a false-positive (Schrader et al. 2012, Trombley Hall et al. 2013). The absence of such cases for all three soils tested supports the method's suitability for soil analysis.

The *P. polymyxa* K16 detection in tomato rhizospheric soil was based on RNA reverse transcription. Besides the importance of detection, quantifying the bioinoculant population size, and activity could provide additional and essential information on the action of the inoculated strain. Combining qPCR with a reverse transcription of RNA isolated from rhizospheric soil enabled us to estimate the target bioinoculant's presence, its gene quantity and activity. The qPCR analysis of the RNA reverse transcript allowed for the detection of the microbial community's active cells (Anderson and Cairney 2004, Girvan et al. 2004); it has been shown that rRNA decay can proceed rapidly in some bacteria species after nutrient starvation (Kerkhof and Kemp 1999). Both precursor rRNA synthesis and post-transcriptional processing rates are likely to vary among environmental conditions (Blazewicz et al. 2013), further affecting a possible correlation with the cell population size.

The assay detected and quantified the *P. polymyxa* K16 strain from different soils, discriminating it from several other bacterial and fungal strains. The assay could be used to track the persistence and fate of the bacterium for regulatory and registration purposes and for gaining the knowledge needed to optimize its application to crops. This study represents an essential step in developing a qPCR protocol for the quantitative analysis of bacterial gene transcripts in soil. The use of the *pmxC* and *pmxE* generated specific and reproducible results for the *P. polymyxa* K16 and could be applied to other

(a)

(b)
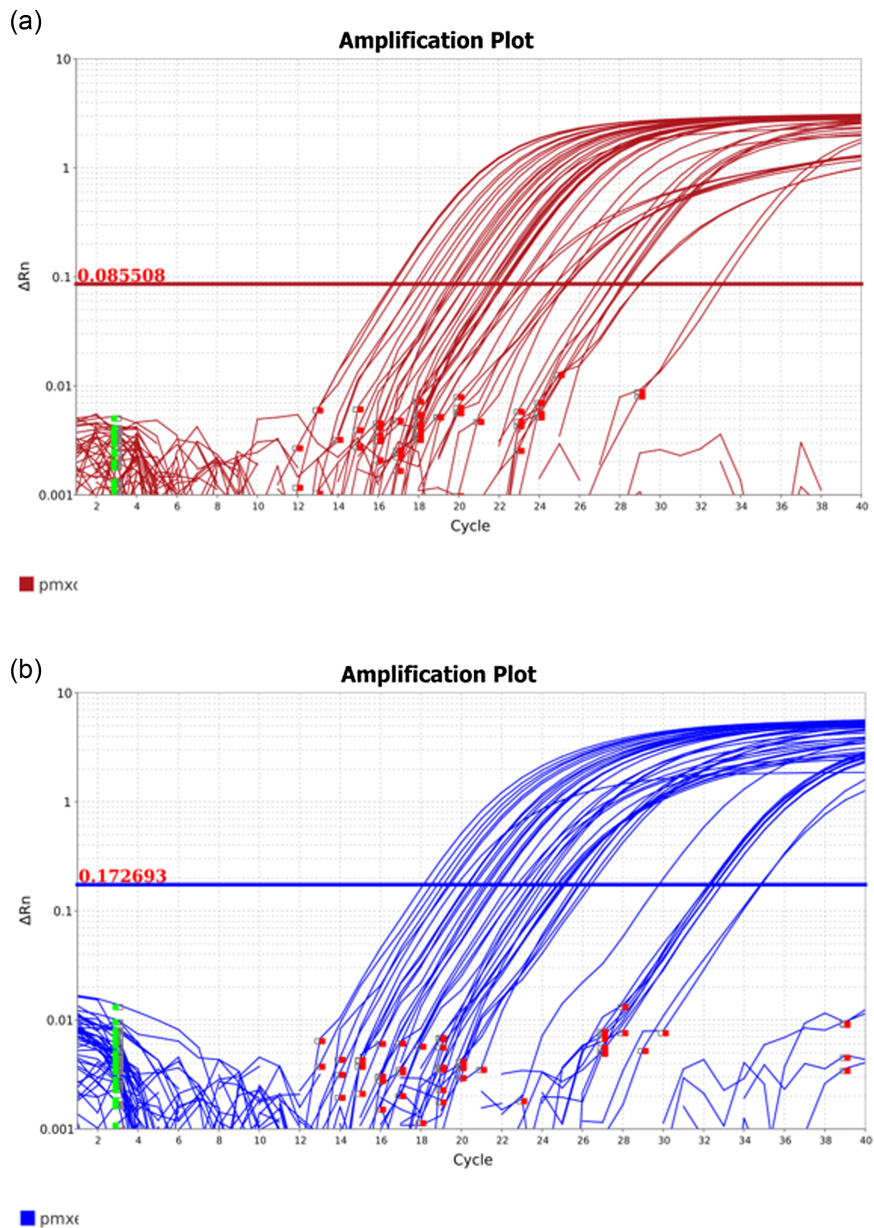
Figure 4. Amplification plot of 23 different *P. polymyxa* strains used in the experiment to assess the specificity of two MGB assay. (a): pmxC assay; (b): pmxE assay.

species/strains with the necessary adaptations and knowledge about the species/strains genome.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Author contribution

Conceptualization, design of the lab experiment, and data analysis: A.M. and L.C. Field methodology, the design of the field study, and data analysis: P.T., M.P., E.M., and L.S. Drafting, reviewing; and editing all authors. All authors have read and agreed to the published version of the manuscript.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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