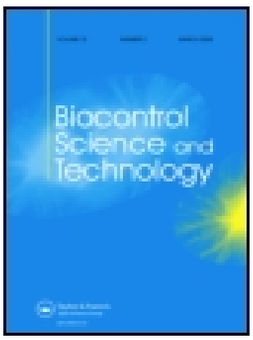

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Pseudomonas putida strain B2017 produced as technical grade active ingredient controls fungal and bacterial crop diseases

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ABSTRACT

Biological control is emerging as a feasible alternative to chemical pesticides for the protection of crop plants. *Pseudomonas putida* shows enormous potential as biological control agent (BCA) not only because it is well reported to control a number of relevant crop diseases, but also because it has a short generation time, colonises plants and their environment, exerts its BCA activity through a range of different mechanisms, and promotes plant growth. Despite of these advantages no plant protection product based on *P. putida* is available in the market so far. In the present manuscript we describe the production of *P. putida* B2017, a novel biofungicide and biobactericide strain, at increasing scale – from laboratory (Erlenmeyer flasks) to pilot scale (125-L bioreactors) – as part of development process of a new commercial plant protection product. The technical grade active ingredient (TGAI) produced, regardless of the production scale, is as effective as the chemical reference against *Fusarium oxysporum f.sp. radicis-lycopersici* in tomato, *Rhizoctonia solani* in potato, *Sclerotinia sclerotiorum* in lettuce and *Pectobacterium atrosepticum* in potato. The TGAI titration obtained in 125-L bioreactors is high enough as to warrant effective TGAI concentration in a putative end-product even after typical viability losses expected during formulation. In summary, we hereby show that *P. putida* B2017 is a promising BCA not only because its efficacy against plant pathogens, but also because it can be produce at large scale.

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Biological control; crop diseases; *Pseudomonas putida* B2017; technical grade active ingredient; scale-up

1. Introduction

Pseudomonads have great potential in agronomic applications and bioremediation since many strains are bioactive, fast-growing and prolific colonisers of plants, and are able to suppress or compete with pathogenic microorganisms (OECD, 1997). Partly due to these attributes, pseudomonads are regarded as potential biological control agents (BCA) and plant growth promoters (Gravel, Antoun, & Tweddell, 2007; O'Sullivan & O'Gara, 1992; Preston, 2004; Walsh, Morrissey, & O'Gara, 2001). The mechanisms underlying the BCA activity of pseudomonads include the ability to produce particular molecules (e.g. antibiotics, antifungal compounds, phytohormones, lytic enzymes and

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siderophores) and the ability to compete with other microorganisms (directly by hyperparasitism or indirectly by nutrient consumption or physical niche occupation) (Haas & Défago, 2005; O'Sullivan & O'Gara, 1992). Among pseudomonads, *Pseudomonas putida* is a ubiquitous and metabolically versatile microorganism, very common in soils and rhizospheres, where it exerts a stimulant effect on different plants (Çakmakçi, Dönmez, Aydın, & Şahin, 2006; Lifshitz et al., 1987; Mehnaz & Lazarovits, 2006). Different *P. putida* strains have been reported as antagonists of a number of fungal and bacterial diseases of agricultural crops such as *Fusarium oxysporum* (Bora, Özaktan, Göre, & Aslan, 2004), *Sclerotinia sclerotiorum* (Expert & Digat, 1995), *Rhizoctonia solani* (Tariq, Yasmin, & Hafeez, 2010), *Collecotrichum orbiculare* (Wei, 1991), *Botrytis cinerea* (Meziane, Van Der Sluis, Van Loon, Hofte, & Bakker, 2005), *Pythium ultimum* (Paulitz & Loper, 1991), *Phomosis viticola* (Haggag, Saleh, Mostafa, & Adel, 2013), *Botryodiplodia theobromae* (Haggag et al., 2013), *Glomerella tucumensis* (Hassan, Afghan, & Hafeez, 2011), *Phytophthora nicotiniae* (Shi, Liu, Li, & Chen, 2013), *Xanthomonas campestris* (Liao, 1989), and *Pectobacterium carotovorum* (syn. *Erwinia carotovora ssp. carotovora*) (Liao, 1989), among others.

Despite of the abundant scientific evidence of the biocontrol and plant growth promoter activity of pseudomonads, only a few *Pseudomonas* species – *P. aureofaciens*, *P. chloroaphis*, *P. fluorescens*, an unidentified *Pseudomonas* strain (*Pseudomonas sp.*), and *P. syringae* – are currently being used as active ingredients of commercial biopesticides in the USA and the EU (see the EPA Pesticide Product and Label System; EU Pesticide Database).

The translation of the promising research mentioned before to commercial plant protection products requires the production of a high quality technical grade active ingredient (TGAI) with reproducible biocontrol activity (Walsh et al., 2001). The mass production must be cost-effective, scalable to industrial level and reproducible, and yield a TGAI with high titration – at least high enough as to maintain the efficacy at the concentrations present in the end-product–, among other desirable features (Fravel, 2005; Montesinos, 2003). The first step to produce such a high quality TGAI is to maximise the amount of viable cells in the fermentation broth while minimising the loss of viability during downstream processing (Montesinos, 2003). The aim of the present study was to evaluate the mass production of a new strain –B2017– using replicable fermentation technologies, and to assess the efficacy of the strain as technical grade active ingredient (TGAI) on a range of fungal and bacterial plant diseases, when applied at market-compatible doses.

2. Materials and methods

2.1. Strain isolation and identification

The bacterial strain B2017 was isolated from a *Meloidogyne javanica* egg mass from a wasteland in Gavà (Spain) in 2012. B2017 was grown on nutritive agar (NA), and stored at −80°C in soy peptone media and glycerol (20%) until identification. After identification, strain B2017 was deposited in the Type Culture Spanish Collection with the accession number CECT8538.

Initially, B2017 was identified by 16S rDNA sequencing. For this, 16S rDNA was amplified using the bacterial forward primer 8f (5′-AGTTTGATCCTGGCTCAG-3′)

and the universal reverse-complementary primer 1492r (5′-ACGGTTACCTTGTTAC-GACTT-3′) by colony PCR (Phire Green Hot Start II PCR Master Mix; ThermoFisher, Waltham, MA). The amplicon was purified with the RealClean PCR clean-up kit (Durviz, Paterna, Spain) and sequenced using the 8f primer and BigDye® Terminator v3.1 technology (Secugen, Madrid, Spain). The sequence was then submitted to BLASTn homology search (Zhang, Schwartz, Wagner, & Miller, 2000).

For an accurate classification of B2017, the whole genome shotgun (WGS) sequence was obtained using an Illumina HiSeq500 on a Nextera DNA library (Genomix4life, Baronissi, Italy). Based on this WGS (DDBJ/ENA/GenBank accession QWEF00000000, version QWEF01000000) the sequences of B2017 *gyrB* and *rpoD*, two protein-coding genes with better discriminating capacity for *Pseudomonas* species as compared to the 16S rDNA (Mulet, Lalucat, & García-Valdés, 2010), were retrieved. A multi-locus sequence analysis (MLSA) was performed as described previously (Mulet et al., 2010) using the concatenated 16S, *gyrB* and *rpoD* sequences of 27 *Pseudomonas* type strains (Supplemental Tables 1 and 2). Phylogenetic trees were built from the MUSCLE alignment using MEGA (v.7.0.26) (Kumar, Stecher, & Tamura, 2016; Nei & Kumar, 2000; Rzhetsky & Nei, 1992; Saitou & Nei, 1987; Tamura, Nei, & Kumar, 2004). In addition, the average nucleotide identity (ANI) was calculated among B2017 and all *P. monteilii* genome sequences available (13), and 12 of the most closely related *P. putida* strains (deduced from the 16S rDNA BLAST hits), including the type strains of both species (Table 1). The ANIs were calculated using JSpeciesWS (Richter, Rossello-Mora, Oliver Glockner, & Peplies, 2016) using BLAST-based ANI (ANIb) calculator.

Table 1. Average nucleotide identity (ANI) among B2017 and selected *P. putida* and *P. monteilii* strains.

Species	Strain	Accession	ANI[a]
P. putida	NBRC 14164^T	NC_021505.1	89.18
	KT2440	**NC_002947.4**	**96.05**
	W619	NC_010501.1	84.11
	GB-1	NC_010322.1	90.00
	S16	NC_015733.1	88.20
	HB3267	CP003738.1	88.60
	BIRD-1	**NC_017530.1**	**95.85**
	B6-2	**NZ_CP015202.1**	**97.33**
	ND6	**NC_017986.1**	**97.36**
	F1	**NC_009512.1**	**97.36**
	LS46	**ALPV02**	**97.68**
	CSV86	AMWJ01	79.37
P. monteilii	DSM 14164^T	BBIS01	88.86
	B5	NZ_CP022562.1	88.57
	IOFA19	JENF01	94.21
	QM	AHGZ01	88.60
	GTC 10899	BCAP01	82.85
	GTC 10897	BCAO01	82.79
	CY06	PJCG01	94.37
	CD10_2	LSTU01	88.99
	BCN3	QWLL01	88.62
	MO2	JFBC01	88.37
	UBA3487	DFQO01	78.02
	SB3078	NC_023075.1	88.44
	SB3101	NC_023076.1	88.46

[a]Uni-directional ANI values, using B2017 as reference, are shown; reciprocal values did not differ significantly. Strains with ANI >95% are highlighted in bold.

2.2. Mass production process of B2017 TGAI

2.2.1. B2017 media optimisation

The production process of viable cells was based on liquid fermentation. First, a screening of raw materials for the multiplication broth was performed. The following media were tested: CN (beef extract, 3 g L^{-1}; soy peptone, 5 g L^{-1}); LB (yeast extract, 5 g L^{-1}; tryptone, 10 g L^{-1}, NaCl, 10 g L^{-1}); TSB (tryptone, 17 g L^{-1}; soy peptone, 3 g L^{-1}; NaCl, 5 g L^{-1}; K_2HPO_4, 2.5 g L^{-1}; glucose, 2.5 g L^{-1}); and MD op (glucose 5, g L^{-1}; $(NH_4)_2SO_4$, 5.66 g L^{-1}; Na_2HPO_4, 0.27 g L^{-1}; K_2HPO_4, 0.59 g L^{-1}; $CaCl_2 \times 2H_2O$, 0.2 g L^{-1}; $MgSO_4 \times 7H_2O$, 0.51 g L^{-1}; $CuSO_4 \times 5H_2O$, 3.93 mg L^{-1}; $MnSO_4 \times 4H_2O$, 2.03 mg L^{-1}). All media were adjusted to pH 7.0 (HCl 5N or NaOH 3N). A hundred mL of sterile media was inoculated with 500 µL of cryo-preserved inoculum in 250-mL flasks, and the mixture was incubated in an orbital shaker at 180 rpm during 24 h at 28°C. Viable counts (CFU·mL^{-1}) were measured at time 0 and 24 h, in technical duplicates.

2.2.2. Bench top process in 2-L and 14-L bioreactors

The results of the initial screening were validated in 2-L (Minifors®, Infors AG, Bottmingen, Switzerland) and 14-L biofermenters (BioFlo® 320, Eppendorf, Hamburg, Germany). The inoculum was prepared in 250-mL flasks with 100 mL of growth medium inoculated with 500 µL from mother stock vials and incubated for 24 h at 200 rpm and 28°C to reach a minimum concentration of $1{\cdot}10^8$ CFU mL^{-1}. The 2-L and 14-L fermenters were loaded with 1.2 and 10 L LB medium, respectively, sterilised and inoculated with 60 and 100 mL pre-culture, respectively, and operated as batch fermentation. The process parameters were set to 300–600 rpm, 28 ± 0.5°C, pH 7.0 (HCl 5N or NaOH 3N), air flow 1.2 L min^{-1} and minimum dissolved oxygen (DO) 50%, for 2-L fermenter and 200–450 rpm, 28 ± 0.5°C, pH 7.0 (HCl 5N or NaOH 3N), air flow of 8–9.5 L mL^{-1}, minimum dissolved oxygen (DO) 50%, maximum antifoam flow 1 mL min^{-1} for 10-L fermenter. The cascade control was set to keep a minimum concentration of DO of 50% by varying the stirrer speed and air flow. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) at different times and CFU·mL^{-1} at initial and final time. The fermentations were stopped at the highest concentration of biomass (end of exponential phase growth) which was typically between 20 and 24 h or 6 and 8 h in the 2-L and 14-L fermenters respectively.

2.2.3. Downstream processing bench top productions

The active cells were recovered by centrifugation at 7000 rpm ($5280 \times g$) for 10 min and 15°C. The supernatant was carefully discarded keeping a small volume to facilitate pellet re-suspension. The pellet was then re-suspended with 1:1 volume of cryo-protectant solution consisting of 10% (w:w) sucrose. The suspension was distributed in trays, frozen at −80°C for at least 24 h, and freeze-dried under vacuum (Heto PowerDry LL3000 Freeze Dryer; Thermo Fisher Scientific) for 72–96 h until humidity content was <5% (w:w). The lyophilised material was homogenised to a powder texture under sterile conditions. A sample was taken for quality control and the remaining material, the TGAI, was packaged in aluminium bags under vacuum, labelled appropriately and preserved at 4°C. The quality control of the TGAI batches consisted on determining the B2017 viable cell count, confirming the strain identity by 16S rDNA sequencing (see section 2.1) and verifying the

absence of microbial contaminations by checking the morphology of colonies in serial dilutions of the culture sowed in NA plates.

2.2.4. Pilot plant productions

The process took place in a 125-L fermenter (BiosaC 125, Andel Equipment PVT Ltp, Mohali, Punjab, India). The inoculum was prepared in 2-L flasks containing 500 mL LB medium inoculated with 2.5 mL from mother stock cryo-vials and incubated for 24 h at 200 rpm and 28°C. The fermenter was loaded with 100 L LB medium, sterilised *in situ* and parameters were set as follows: 100–300 rpm, 28 ± 0.5°C, pH 7.0 (HCl 5N or NaOH 3N), air flow of 100 L mL^{-1}, 50% DO. The cascade control was set to keep a minimum concentration of DO of 50% by varying the stirrer speed. The process was monitored by measuring OD_{600} and CFU·mL^{-1} at initial and final time. Fermentation was stopped at maximum concentration of cells (end of exponential phase) after 6–8 h.

2.2.5. Downstream processing pilot plant productions

The viable cells were recovered from the fermenter by centrifugation using a refrigerated continuous equipment (J-1250, Hani Scientific, Gimpo Korea) set at 7600 × g (10400 rpm) and 5°C and pump speed at 60 rpm. The pellet was re-suspended in 8 L of cryo-protectant solution consisting of 10% (w:w) sucrose and then lyophilised in an automatic freeze-drier (Scanvac Superior, Touch 95–80, Labogene Aps, Lynge, Denmark) during 120 h. After freeze-drying, the material was processed as described for bench top productions.

2.3. Efficacy assessment on fungal and bacterial pathosystems

Three bioassays, with 10–12 plants per experimental condition each, were performed to assess the biological control activity of B2017 TGAI in each of the 4 pathosystems described in the next sections. All efficacy trials were performed on 28 days-old plants in a walk-in growth chamber (Fitoclima 20000®, ARALAB) set at 20/18°C (day/night), 60–70% relative humidity, and a photoperiod of 13/11 h (day/night) with ca. 300 µmol m^{-2} s^{-1} of photosynthetically-active radiation. All trials were performed using different batches of the B2017 TGAI, produced indistinctly in 2-, 14- or 125-L bioreactors. The plants were obtained by different procedures depending on the pathosystems, and the pathogen inoculation and B2017 TGAI application methods and schedules varied slightly depending on the pathosystem according to previous experience of the authors in screening trials for the selection of BCA strains (see next sections and Table 2 for summary). By the end of the bioassays, which lasted 22–24 days after transplantation (Table 2), the disease severities were determined according to different scales for each pathosystem.

Table 2. Summary of the experimental settings of the efficacy trials.

			Time (days after transplantation)									
Pathogen	Crop	Plant source	0	1	2	7	9	14	15	21	22	23
FORL	Tomato	Seeds	*P*	@			@				X	
R. solani	Potato	Tubers	*P*		@		@		@			X
Sclerotinia sclerotiorum	Lettuce	Seeds	@		*P*	@				X		
P. atrosepticum	Potato	Tubers	@	*P*		@		@		X		

FORL, *Fusarium oxysporum f. sp. radicis-licopersici*. @, treatment with B2017 technical grade active ingredient; *P*, pathogen challenge; X, end of bioassays (disease assessment).

The efficacy of treatments over the disease severity (herein 'efficacy over severity') was calculated as $100 \times [1 - (St/Sc)]$, where St is the percent severity of the treated plants and Sc, the percent severity of control –not treated– plants (Abbott, 1925). Next, the particularities of each pathosystem are detailed.

2.3.1. *Tomato-Fusarium oxysporum f.sp. radicis-lycopersici (FORL)*

Tomato (*Solanum lycopersicum*) cv. Marmande Cuarenteno seeds were surface-sterilised (2% commercial bleach for 10 min, and three times rinsed for 1 min each in sterile distilled water), sown in sterile substrate (peat:vermiculite, 1:1, v:v), and let grow in the growth chamber. After 28 days, the plantlets were transplanted to 1-L pots filled with sterile sand:peat:perlite (2:1:0.5; v:v:v). During transplantation, the plants were inoculated with the pathogen (FORL strain H828; isolated from an infected tomato plant from Sevilla, Spain) by immersing the roots for 30 min in a conidia suspension ($5{\cdot}10^6$ conidia mL^{-1}) obtained from a 48-h culture in potato dextrose broth (PDB) at 26°C. The B2017 TGAI was applied twice in each bioassay: 1 and 9 days after transplantation (Table 2). The applications consisted of 20 mL·B2017 TGAI in aqueous suspension per plant, at average concentrations of 5.5, 1.5 and $1.9{\cdot}10^7$ CFU mL^{-1} (bioassays 1, 2 and 3, respectively; concentrations determined by dilution plating in NA plates), applied at the shoot base. The chemical control treatment was Previcur®-N [active ingredient propamocarb chlorhydrate 60.5% (w:v)] applied according to manufacturer's instructions (Bayer Crop Science, Monheim am Rhein, Germany). The disease severity was scored 23 days after transplantation according to the reference scale described by Jarvis and Thorpe (1976).

2.3.2. *Potato*-Rhizoctonia solani

Potato (*Solanum tuberosum*) tubers cv. Kennebec were forced to sprout for a period of 2–4 weeks in the dark and then disinfected in a solution of 2% commercial bleach for 10 min, washed with 70% ethanol, and rinsed with sterile tap water. After wiping, potatoes were planted in 1-L containers with sterile substrate (sand:peat:perlite; 4:2:1; v:v:v) and let grow in the growth chamber for 4 weeks. Then, plants were inoculated with the pathogen (*R. solani* strain DSM 63010; purchased from the DSMZ) by applying 30 mL $plant^{-1}$ to the substrate at the shoot base. The pathogen solution (0.5 L) was prepared from 4 plates sown with *R. solani* in potato dextrose agar (PDA; VWR, cat. nr. 846.510.500) and grown for 2 weeks at 26°C. The B2017 TGAI was applied three times throughout each bioassay: 2, 9 and 15 days after transplantation, in applications that consisted on 20 mL $plant^{-1}$, applied to the substrate at the base of the shoot, at 8.9, 1.0 and $3.3{\cdot}10^8$ CFU mL^{-1} (bioassays 1, 2 and 3, respectively; concentrations determined by dilution plating in NA plates). The chemical control treatment was Trotis® 25 SC [active ingredient pencicuron 25% (w:v)] applied following manufacturer's instructions (Bayer Crop Science). Twenty-three days after transplantation the plants were harvested, rinsed with tap water and scored for disease severity [percentage of stem canker (Weinhold, Bowman, & Hall, 1982)].

2.3.3. *Lettuce*-Sclerotinia sclerotiorum

Lettuce (*Lactuca sativa*) cv. Trocadero seeds were disinfected as described in the tomato-FORL pathosystem, and sowed on peat:vermiculite (1:1; v:v). Four weeks-old plantlets were transplanted to 1-L pots with sterile soil:peat:perlite (2:1:0.5; v:v:v). The B2017 TGAI was applied twice in each bioassay by spraying 10 mL of the BCA solution (at concentrations of

$1.3{\cdot}10^7$, $9.8{\cdot}10^7$ and $7.1{\cdot}10^6$ CFU mL^{-1}, determined by dilution plating in NA plates, in bioassays 1, 2 and 3, respectively) to leaves. The first application was performed on the day of transplantation and the second, 7 days later (Table 2). Two days after transplantation, i.e. between the two BCA applications, the plants were inoculated with the pathogen (*S. sclerotiorum* strain H886, isolated from a lettuce crop from Valencia, Spain) by placing a plug obtained from a 2-weeks culture in PDA at 26°C, at the crown of each plant. The chemical control was Teldor® [active ingredient fenhexamide 50% (w:v)] applied according to manufacturer's instructions (Bayer Crop Science). Twenty-one days after transplantation the disease incidence and white mould severity were scored based on a custom scale as follows: 0, no disease; 1, leaf chlorosis; 2, soft rot affecting <25% of leaves; 3, 25–75% leaves showing soft rot; 4, >75% of leaves showing leaf soft rot; and 5, dead plant.

2.3.4. *Potato*-P. atrosepticum

Potato tubers cv. Kennebec were induced to sprout and disinfected as described for the potato-*R. solani* pathosystem. The most healthy sprouts were planted in peat:vermiculite (1:1; v:v) and let grow for 4 weeks in the growth chamber. Then, the plantlets were transplanted to 1–L pots with soil:peat:perlite (2:1:0.5 v:v:v). The B2017 TGAI treatment was carried out by applying 20 mL $plant^{-1}$ of a B2017 TGAI solution at concentrations of $5.9{\cdot}10^6$, $3.4{\cdot}10^8$ and $5.0{\cdot}10^7$ CFU mL^{-1} (bioasays 1, 2 and 3, respectively; concentrations determined by dilution plating in NA plates) at the base of the shoot three times: during transplantation, and 7 and 14 days after transplantation (Table 2). The plants were challenged with the pathogen (*P. atrosepticum* strain DSM 30184, purchased from the DSMZ) one day after transplantation, i.e. one day after the first BCA application, as follows: a wound was made at the stem base with a sterile needle, 20 µL of pathogen solution at 10^8 CFU mL^{-1} from an overnight culture in LB medium at 28°C were placed over the wound, and the wound with the inoculum was covered with parafilm. Since no effective chemical control treatment is available for this bacterial disease, no chemical treatment was included in this pathosystem. Twenty-two days after transplantation plants were scored for bacterial soft rot disease severity (expressed as percentage of necrotic vascular lesion).

2.4. Statistical analyses

After verifying data homocedasticity and normality, ANOVA and LSD posthoc tests were performed. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed with *R*.

3. Results

3.1. Taxonomy of strain B2017

The bacterial isolate B2017 was identified by the 16S rDNA sequence as a *Pseudomonas* sp. strain closely related to species from the so-called *P. putida* group (Mulet et al., 2010). Multi-locus sequence analysis showed that B2017 is closely related to *P. putida* and *P. monteilii* type strains (Supplemental Figure 1). Whole genome comparisons show that the B2017 genome shares less than 90% identity with either *P. monteilii* or *P. putida* type strains (Table 1). In addition, B2017 showed ANI values with other *P. monteilii* strains

Table 3. Screening of fermentation growth media.

	$Log_{10}(CFU{\cdot}mL^{-1})$	
Medium	Initial (0 h)	Final (24 h)[a]
LB	6.6 ± 6.38	9.41 ± 7.81a
CN	6.26 ± 4.34	9.53 ± 8.95ab
TSB	6.75 ± 5.78	9.26 ± 8.45ab
MD op	6.65 ± 6	9.08 ± 7.95b

Letters indicate homogeneous groups (no statistically significant differences were found in the initial inoculum). See the text for details about growth media.

ranging from 78.0 to 94.4 (Table 1). In contrast, the ANI of B2017 with other *P. putida* strains ranged from 79.4 to 97.7 (Table 1).

3.2. Mass production of B2017 TGAI

The screening of media showed the highest final concentration of B2017 in CN, followed by that in LB, TSB and MD op (Table 3). Despite of the slightly (not significant) higher concentration in CN medium, the LB medium was chosen for subsequent productions due to the lack of availability at industrial level of beef extract, incompatibility with lists of allowed inputs in organic agriculture, and hurdles for commercialisation of products with ingredients of animal origin in Muslim countries.

A total of 4 batches were produced in 2-L bioreactors, 9 in 14-L bioreactors and 2 in the 125-L bioreactor (Table 4). The average increment in viable numbers achieved was 14-, 129- and 685-fold in 2, 14 and 125-L bioreactors, respectively, which posed final viable concentrations of $8.83{\cdot}10^{8}$, $4.99{\cdot}10^{9}$ and $6.00{\cdot}10^{9}$ CFU mL^{-1}, respectively (Table 4). After lyophilisation, the TGAI obtained from 2-, 14- and 125-L bioreactors was $5.10{\cdot}10^{8}$, $1.29{\cdot}10^{11}$ and $1.16{\cdot}10^{11}$ CFU g^{-1}, respectively (Table 4). Dilution plating, showed no colonies with morphology different form the mother culture or fungal growth.

3.3. Efficacy assessment of strain B2017 on the 4 pathosystems

3.3.1. Tomato-FORL.

In all three bioassays, plants treated with either B2017 TGAI or the chemical control showed significantly lower severity than the untreated plants (Figure 1). In the first bioassay, the disease severity dropped from 43% in the untreated control to 11 and 7% in the B2017 and chemical treatments, respectively, which poses 74 and 85% efficacy over severity, respectively (Supplemental Figure 2). In the second bioassay, the severity dropped from 49% in the untreated control to 15 and 12% in the B2017 and chemical treatments,

Table 4. Scalability of the production of B2017 technical grade active ingredient from laboratory to pilot scale.

Production scale (L)	2	14	125
Number of batches	4	9	2
Initial viable cell concentration [$log_{10}(CFU{\cdot}mL^{-1})$]	7.8 ± 7.56	7.59 ± 6.35	6.94 ± 6.48
Final viable cell concentration [$log_{10}(CFU{\cdot}mL^{-1})$]	8.95 ± 8.16	9.7 ± 9.1	9.78 ± 9.04
Total TGAI[a] obtained (g)	13.0 ± 2.19	74.5 ± 6.2	902.93 ± 7.01
TGAI yield (g TGAI/L fermentation)	10.8 ± 0.55	7.45 ± 0.69	9.03 ± 3.51
TGAI quality [$log_{10}(CFU/g)$]	8.71 ± 8.45	11.11 ± 10.51	11.06 ± 10.93

TGAI, technical grade active ingredient. Results are mean of batches ± standard error of the mean.

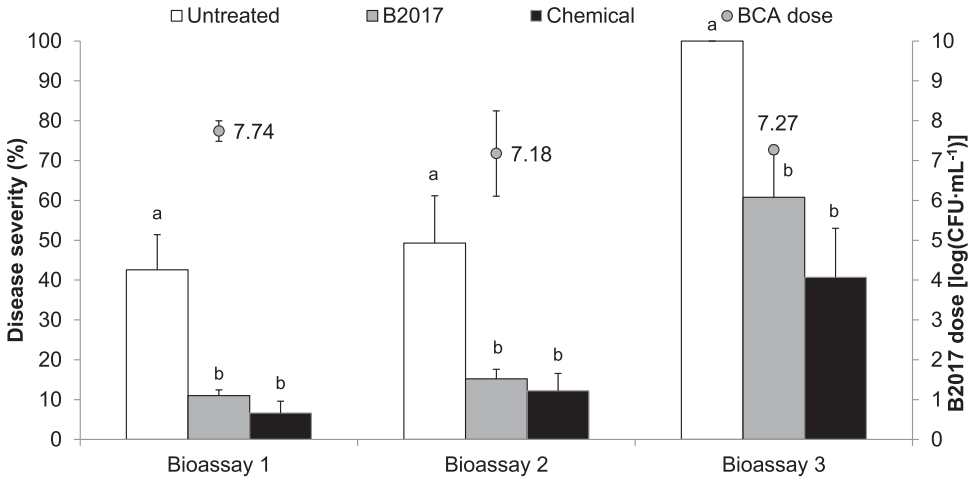

Figure 1. Disease severity induced by *Fusarium oxysporum f. sp. radicis-lycopersici* (FORL) on tomato plants in three independent bioassays (bars), and the biocontrol agent doses applied in each bioassay (dots). Untreated, plants inoculated with FORL; Chemical, plants inoculated with with FORL and treated with a reference chemical (propamocarb and fosetil); B2017, plants inoculated with FORL and treated with *P. putida* B2017 technical grade active ingredient. Letters indicate statistically significant differences among treatments within each bioassay ($p < 0.05$; ANOVA).

respectively (i.e. 69 and 75% efficacy over severity in the B2017 and chemical treatments, respectively; Figure 1 and Supplemental Figure 2). In the third bioassay, the severity was 100% in the untreated control and the B2017 and chemical treatments decreased it to 61 and 41% in, respectively (i.e. 39 and 59% efficacy over severity in the B2017 and chemical treatments, respectively; Figure 1 and Supplemental Figure 2). Although all trials were challenged with the same amount of pathogen inoculum, the severity level in untreated control plants in the third bioassay was significantly higher than in the first two (Figure 1). Still, the disease severity was significantly reduced by the B2017 treatment to the same extent as with the chemical control (Figure 1; Supplemental Figure 2).

3.3.2. Potato-R. solani

Untreated plants challenged with the pathogen developed disease severities of 54–73% (Figure 2). In bioassays 1 and 2, plants treated with either the B2017 TGAI or the chemical showed similar, significant, reductions in the disease severities (to 28 and 26% with B2017, and to 31 and 24% with the chemical, respectively; Figure 2). These severity reductions correspond to efficacies over severity of 48 and 62% in the case of the B2017 TGAI, and of 42 and 65% in the case of the chemical (bioassays 1 and 2, respectively; Supplemental Figure 3). In bioassay 3, where the pathogen pressure (i.e. the disease severity in the untreated plants) was highest among the three bioassays (Supplemental Figure 3), the B2017 treatment reduced slightly (not significant) the disease severity from 73% to 58% (i.e. a 21% efficacy over the severity; Figure 2; Supplemental Figure 3). The chemical reduced significantly the severity, to 44% (i.e. showed a 40% efficacy over severity; Supplemental Figure 3), although neither the severity nor the efficacy over severity were significantly different from those in B2017-treated plants (Figure 2; Supplemental Figure 3).

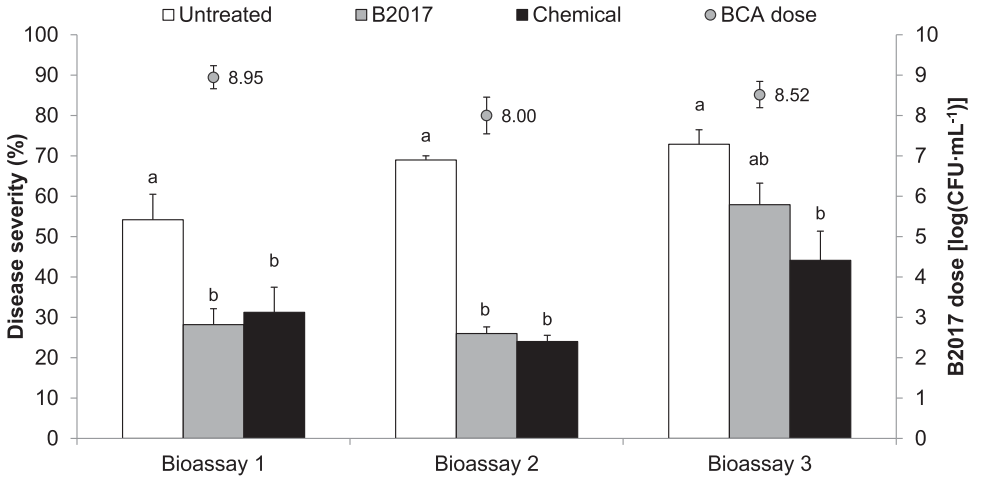

Figure 2. Disease severity induced by *R. solani* on potato plants in three independent bioassays (bars), and the biocontrol agent doses applied in each bioassay (dots). Untreated, plants inoculated with *R. solani*; Chemical, plants inoculated with with the pathogen and treated with a reference chemical (penicuron); B2017, plants inoculated with *R. solani* and treated with *P. putida* B2017 technical grade active ingredient. Letters indicate statistically significant differences among treatments within each bioassay ($p < 0.05$; ANOVA).

3.3.3. Lettuce-S. sclerotiorum

In the bioassays corresponding to this pathosystems the severities ranged from 2.7 (bioassay 1) to 3.3 (bioassay 3), in a scale from 0 to 4 (Figure 3). In the first bioassay, both B2017 and the chemical led to significant reductions of the disease severity, from 2.7 in the

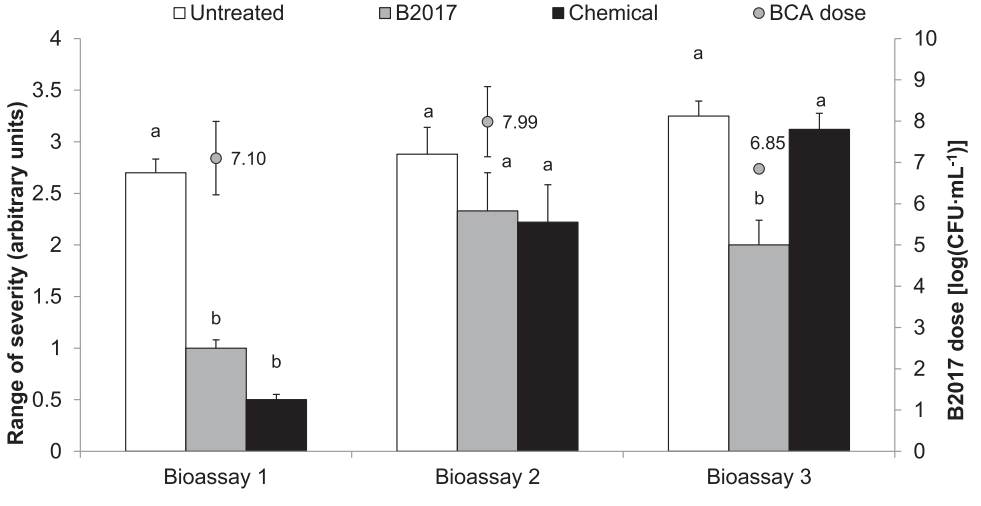

Figure 3. Range of disease severity, according to a custom-made scale, induced by *S. sclerotiorum* on lettuce plants in three independent bioassays (bars), and the biocontrol agent doses applied in each bioassay (dots). Untreated, plants inoculated with *S. sclerotiorum*; Chemical, plants inoculated with *S. sclerotiorum* and treated with a reference chemical (fenhexamide and tebuconazol); B2017, plants inoculated with *S. sclerotiorum* and treated with *P. putida* B2017 technical grade active ingredient. Letters indicate statistically significant differences among treatments within each bioassay ($p < 0.05$; ANOVA).

untreated control to 1.0 and 0.5 in B2017 and the chemical treatments, respectively (Figure 3), which corresponds to efficacies over severity of 63% and 81%, respectively (Supplemental Figure 4). In the second bioassay, none of the treatments led to significant reductions of the severity, although both treatments led to slight reductions (from 2.9 in the untreated control to 2.3 in the B2017 TGAI treatment and 2.2 in the chemical treatment; Figure 3). In the third bioassay, the pathogen pressure was highest (Supplemental Figure 4) and the B2017 treatment led to significant reductions of the severity, from 3.3 in the untreated control to 2.0 (Figure 3), which corresponds to an efficacy of 38% over the severity (Supplemental Figure 4). In this bioassay, the chemical treatment did not reduce disease severity significantly (Figure 3).

3.3.4. *Potato*-P. atrosepticum

In this pathosystem disease severity in untreated plants reached 41–57% (Figure 4). The B2017 TGAI treatment reduced the disease severity in all three bioassays (to 31%, 47% and 31%; Figure 4), although this reduction was statistically significant only in the third bioassay (from 50% to 31%, which corresponds to a 70% efficacy over severity) (Figure 4; Supplemental Figure 5).

4. Discussion

Based on the 16S sequence, MLSA and ANI analyses (Supplemental Figure 1 and Table 1), it is deduced that B2017 is a *Pseudomonas* strain of the *P. putida* complex. B2017 is closer to the *P. monteilii* type strain than to the *P. putida* type strain, but ANI values below 95% indicate that B2017 does not belong to the species of either type strains (Table 1) (Richter & Rossello-Mora, 2009). On the other hand, B2017 shares high ANI (>95%) with some strains widely reported as *P. putida*, such as KT-2440 (Belda et al., 2016) (Table 1). It

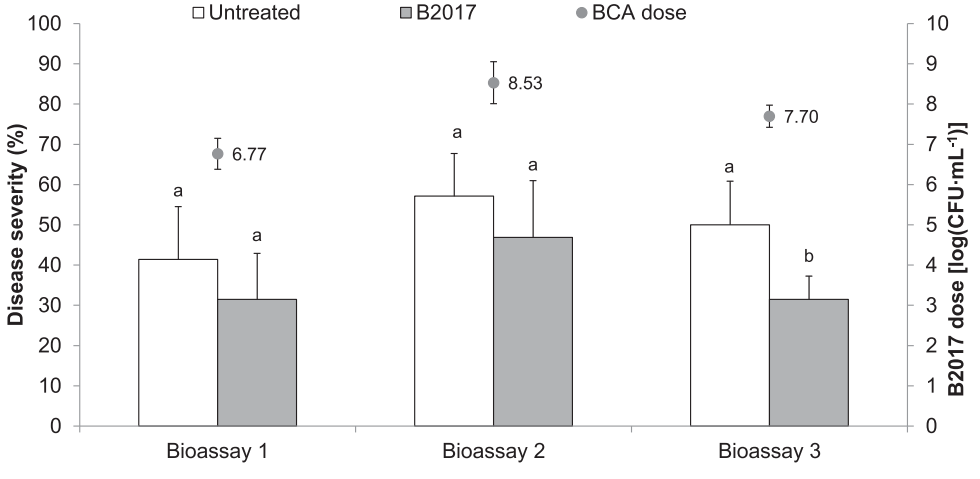

Figure 4. Disease severity induced by *Pectobacterium atrosepticum* on potato plants in three independent bioassays (bars), and the biocontrol agent doses applied in each bioassay (dots). Untreated, plants inoculated with *P. atrosepticum*; B2017, plants inoculated with *P. atrosepticum* and treated with *P. putida* B2017 technical grade active ingredient. Letters indicate statistically significant differences among treatments within each bioassay ($p < 0.05$; ANOVA).

has been previously reported that the strains included within the *P. putida* species are heterogeneous enough as to be split into one or more new species (Gomila, Peña, Mulet, Lalucat, & García-Valdés, 2015; Mulet, García-Valdés, & Lalucat, 2013; Ohji et al., 2014). The ANI matrix among selected *P. putida* strains shown in Supplemental Table 3 supports this split. Furthermore, this genomic heterogeneity is also observed among *P. monteilii* strains (Supplemental Table 4). According to the MLSA-based phylogeny of *P. putida* built by Mulet et al. (2013) B2017 would fall within the proposed 'putative new species I', together with KT-2440, F1, B6-2, BIRD-1, ND6, and LS46 (Table 1). Thus, B2017 is considered *P. putida* herein, although a revision of the taxonomy of the *P. putida* complex is needed based on the existing evidence.

A number of *P. putida* strains have been reported to exert biological control against crop pathogens (Bora et al., 2004; Expert & Digat, 1995; Haggag et al., 2013; Hassan et al., 2011; Liao, 1989; Meziane et al., 2005; Paulitz & Loper, 1991; Shi et al., 2013; Tariq et al., 2010; Wei, 1991). Still, no product with *P. putida* as active ingredient is commercially available so far, certainly not in the EU or the USA, and neither in any other country to the best of the authors' knowledge.

The initial screening for growth media showed that B2017 could be produced with equivalent efficiency in LB and CN media (Table 3). It would be interesting to determine whether cheaper raw materials such as molasses or other sub-products could be used as fermentation media with yields comparable to LB and CN in order to restrain production costs. In this line, although more focused on the production of a particular metabolite, it has been shown that growth media based on corn flour and soybean meal as C and N source lead to high *P. putida* yields (Peng, He, Wu, Lu, & Li, 2014).

The highest final concentration in the batch fermentation and the highest viable cell increment were reached in the 125-L bioreactor production, along with the higher quantity of total TGAI produced (Table 4). Fed-batch fermentations usually improve the yield in bacterial cultures (e.g. Davis et al., 2015; Ghomi, Fazaelipoor, Jafari, & Ataei, 2012). However, the benefit in terms of bacterial biomass may not compensate the operational inconvenience. A systematic study is needed in this regard but our initial tests showed that producing B2017 by batch-feeding the C source did not significantly improve the yield.

The lowest viability losses during downstream processing were attained when the B2017 TGAI was produced either in 14-L and 125-L bioreactors (Table 4). Whilst the highest yield of TGAI grams per litre of fermentation was achieved in 2-L bioreactors, this TGAI concentration is 2.5 logs lower than those produced in 14-L and 125-L bioreactors (Table 4). Specific studies would be required to unravel the factors determining the better performance of the process at pilot scale, but nevertheless the work presented hereby evidences the feasibility of producing industrial quantities of viable cells of *P. putida* strain B2017 (Table 4). In addition, as dissolved oxygen is an important nutrient in most of the aerobic industrial microbial bioprocess (García-Ochoa, Castro, & Santos, 2000) the positive scalability results indicate low oxygen transport limitations in 125-L bioreactor.

In the three fungal pathosystems assayed B2017 reduced the severity of the disease to an extent similar to the chemical control. These bioassays were performed under a broad range of pathogen pressures, thus proving that B2017 is effective against these fungal pathogens under varying conditions. Furthermore, B2017 was effective when applied as foliar spray (Figure 3) or directly to soil (Figures 1, 2 and 4). The efficacy of B2017 was in all cases equal to that of the chemical control with the exception of the pathosystem

lettuce-*S. sclerotiorum*, in which B2017 outperforms the chemical control at highest pathogen pressures (Figure 3; Supplemental Figure 4). It is worth to note that although Previcur® is recommended to control diseases caused by Oomycetes fungi, it was used as chemical control in the tomato-FORL bioassays since it clearly outperformed Terrazole®, a fungicide indicated against *Fusarium* wilts (EPA Pesticide Product and Label System; EU Pesticide Database), in our experimental settings. In summary, B2017 is equally or more effective than the chemical in reducing the disease severity caused by the pathogens tested hereby.

For the use of a BCA as TGAI in a plant protection product a minimum concentration of viable cells must be warrantied in the application broth. This concentration depends on many factors such as the concentration at which the BCA is effective, the amount of water, if any, used as carrier, the mode of application, etc. In addition, other complex factors such as the weather, the cultivar or the pathogen genotype may influence BCA efficacy (reviewed by Bardin et al., 2015). In agreement, despite of assessing B2017 efficacy under controlled conditions, the data shown in Figures 1–4 and Supplemental Figures 2–5 evidence that the success of the BCA does not only depend on the BCA dose, but also on other factors, including disease severity in the control plant (a proxy for pathogen pressure). In addition, the factors influencing the success of a BCA are expected to be even more complex and variable in the field than in growth chambers (Bardin et al., 2015; Fravel, 2005). According to the B2017 production performance described in Table 4 a putative product with 10% B2017 TGAI in its formula applied as a 1% dilution would contain slightly over 10^8 CFU mL^{-1}. Still, additional viable losses during formulation should be taken into account (Rhodes, 1993). A B2017 concentration of 10^8 CFU mL^{-1} showed significant disease severity reductions in all pathosystems (Figures 1–4) regardless of other factors. Doses around 10^7 CFU mL^{-1} were also effective in the pathosystems tomato-FORL, lettuce-*S. sclerotiurum* and potato-*P. atrosepticum*, but these doses were not tested hereby in the pathosystem potato-*R. solani*. These data indicate that the production of B2017 method at pilot scale meets the requirements for further up-scaling provided that viability loss during formulation is kept restrained.

Another important aspect in the production of BCAs is the shelf-life of both the TGAI and the end-product. The TGAI produced in the experiments described hereby are produced using sucrose as cryo-protectant. This choice was made based on preliminary studies showing that sucrose prevented any loss of viability during cold (4°C) storage for 2 months, while other cryo-protectants (semi-skimmed milk and a mixture of sucrose and peptone) allowed 90–99% viability loss in the same period (Supplemental Figure 6). In addition, sucrose is cheap and compatible with vegan products, as compared to other cryo-protectants such as milk. Although sucrose keeps B2017 TGAI viable for 60 days, which would allow a 2 month time lapse from TGAI manufacture to formulation, further studies are required to define a formula that guarantees a market-compatible shelf-life of the end-product.

Overall, it is hereby shown that B2017, a bacterial strain from the *P. putida* complex closely related to some *P. putida* strains, can be produced as a high quality, stable, TGAI using standard fermentation technologies commonly available at semi-industrial scale. This TGAI is as effective as the reference chemical in the fungal and bacterial pathosystems tested hereby (tomato-FORL, potato-*R. solani*, Lettuce-*S. sclerotiorum* and Potato-*P. atrosepticum*), when applied either by foliar spray or directly to the soil.

Disclosure statement

No potential conflict of interest was reported by the authors.

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