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Selection of plant growth promoting rhizobacteria sharing suitable features to be commercially developed as biostimulant products

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ABSTRACT

Plant biostimulants (PBs) are an eco-friendly alternative to chemical fertilisers because of their minimal or null impact on human health and environment, while ensuring optimal nutrient uptake and increase of crop yield, quality and tolerance to abiotic stress. Although there is an increasing interest on microbial biostimulants, the optimal procedure to select and develop them as commercial products is still not well defined. This work proposes and validates a procedure to select the best plant growth promoting rhizobacteria (PGPR) as potential active ingredients of commercial PBs. The stepwise screening strategy was designed based on literature analysis and consists of six steps: (i) determination of the target crop and commercial strategy, (ii) selection of growth media for the isolation of microbial candidates, (iii) screening for traits giving major agronomical advantages, (iv) screening for traits related to product development, (v) characterisation of the mode of action of PGPR and (vi) assessment of plant growth efficacy. The strategy was validated using a case study: PGPR combined with humic acids to be applied on tomato plants. Among 200 bacterial strains isolated from tomato rhizosphere, 39 % were able to grow in presence of humic acids and shared the ability to solubilise phosphate. After the screening for traits related to product development, only 6 % of initial bacterial strains were sharing traits suitable for the further development as potential PBs. In fact, the selected bacterial strains were able to produce high cell mass and tolerated drought, aspects important for the mass production and formulation. These bacterial strains were not able to produce antibiotics, establish pathogenic interaction with plants and did not belong to bacterial species associated to human, animal and plant diseases. Most importantly, five of the selected bacterial strains were able to promote tomato seedling vigour in experiments carried out *in vitro*. These bacterial strains were furtherly characterised for their ability to colonize effectively tomato plant roots, produce phytohormones and solubilise soil minerals. This characterisation led to the selection of two candidates that showed the ability to promote tomato plant growth in experiments carried out in greenhouse conditions. Overall, this work provides a flow diagram for the selection of PGPR candidates to be successfully developed and commercialized as PBs. The validation of the flow diagram led to the selection of two bacterial strains belonging to *Pantoea* and *Pseudomonas* genera, potential active ingredients of new commercial PBs.

1. Introduction

Feeding the growing global population is one of the major challenges for agriculture (Rouphael and Colla, 2020). To sustain and guarantee an adequate yield, crop production is getting more and more dependent on chemical fertilisers (Berg, 2009), which has, unfortunately, a very negative impact on the environment (Vejan et al., 2016). Thus, the development of eco-friendly alternatives to chemical fertilisers greatly increased importance in recent time. One of them is the use of plant biostimulants (PBs), which is gaining interest globally (De Pascale et al., 2017). Indeed, the PB market is constantly increasing with an expected compound annual rate of 10.9 % until 2022 (Sessitsch et al., 2018). The

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main reasons of this growth are: (i) increasing importance of the organic farming, (ii) more PB use in developed countries and (iii) good acceptance of PBs among consumers (Biostimulant Market, 2014). Plant biostimulants include substances or microorganisms that enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits when applied to plants (du Jardin, 2015). Among PBs active ingredients, microorganisms residing in the plant rhizosphere received relevant attention, in particular plant growth promoting rhizobacteria (PGPR). In fact, PGPR promote plant growth through several mechanisms as modulation of the hormone balance in plants through the release of indole-3-acetic acid (IAA) and synthesis of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase (Glick, 2014; Spaepen and Vanderleyden, 2011). Moreover, PGPR make soil elements, such as iron, phosphorus and potassium, more available to plants thanks to the release of siderophores, organic acids and enzymes (Ahmed and Holmström, 2014; Parmar and Sindhu, 2013; Rodriguez and Fraga, 1999).

To develop commercial PBs based on PGPR, potential candidates are selected by following a step by step screening strategy based on testing different criteria, from laboratory to field-like conditions (du Jardin, 2015). However, without collaboration between stakeholders, farmers, researchers, and regulatory bodies to bring affordable and effective new bioproducts to the market is impossible (du Jardin, 2015). For instance, considering both the mode of action of the candidates and the market demand, can be an effective combined criterion to decide which strain would be the most competitive and successful product (Kamilova and De Bruyne, 2013). Many publications already described the general steps for the development of microbial bioproducts for agriculture (Backer et al., 2018; Nakkeeran et al., 2006; Pliego et al., 2011). Briefly, these include isolation of microorganisms, screening in laboratory and under greenhouse conditions, assessment of ecological safety, development of suitable formulations, marketing and registration. The characterization of the mode of action of selected PGPR is an important aspect taken under consideration when new bacterial isolates are selected as potential PBs (Rouphael and Colla, 2020). Particular attention is given to the ability of bacterial isolates to protect plants against abiotic stresses as drought, salinity and chilling (Albdaiwi et al., 2019; Subramanian et al., 2015; Tiwari et al., 2016). Similarly, PGPR are commonly evaluated for their impact on plant uptake of soil nutrients (De Pascale et al., 2017). It is undeniable that the enhancement of nitrogen (N) assimilation received most of the attention so far and N-fixing bacteria as *Rhizobium* spp. are already developed as commercial PBs (Remigi et al., 2016). At the same time, scarce attention has been given to the ability of PGPR to solubilise other soil nutrients as phosphorous (P) that will less available in the next future (Granada et al., 2018).

Many other factors need to be taken into account during the development of a commercial PB (Backer et al., 2018). Some of these parameters are high competitive saprophytic activity, high rhizosphere competence, affordable mass multiplication, broad spectrum of action and enhanced plant growth. In addition, tolerance to heat, desiccation, UV radiation and oxidizing agents are also criteria to be considered for a successful practical application (Nakkeeran et al., 2006). Moreover, it is also useful to take into consideration that the performance of commercial PBs may be region specifics due to the origin of the developed PGPR (Kristin and Miranda, 2013).

To support researchers and companies in the selection of PGPR to be developed as commercial PBs, we designed a stepwise screening strategy. It consists of several steps where various criteria are used, starting from the isolation of candidates to the effect of these candidates under greenhouse conditions. We validated this screening method by using a case study and efficiently selected PGPR able to fulfil all the criteria included in the program, ready to be scaled-up by companies.

2. Material and methods

2.1. Screening strategy

The screening strategy is made of six steps and the first consists of the selection of the target crop and the PB impact on the market (Fig. 1). The decision depend mainly on the market size, presence of competing products/solutions and advantages for the growers, which are all conditions that can create a sufficient market size to justify the investment for the development of the PB. The decision requires knowledge of the specific market and sufficient commercial experience to estimate possible economic constraints of the future PB and must also consider national/international regulations for PBs.

The second step is a crucial aspect and it is related to the decision of the most appropriate growth media to be used for the isolation of microbial candidates. In fact, the use of different growth media, *i.e.* synthetic, selective, poor or nutrient-rich media, will end up in the isolation of different microbial groups.

The third step consists of the preliminary screening based on the major agronomical advantages that the PB is expected to have. The availability/development of a high-throughput approach is crucial at this step, because screening of hundreds of candidates will increase the chances to select candidates with the desired features. The fourth step is the screening according to traits suitable for the development a successful PB product. At this stage, the industrial production and formulation approaches and the specific national/international standards and regulations laws must be carefully considered in the screening tests, because the economic and technical feasibility is highly depending on the specific microorganism's characteristics. For example, PBs candidates must reach a sufficient mass production on cheap growth medium in the industrial scale up, in order to achieve sustainable production costs. Another example is the tolerance to desiccation, if the final formulation is expected to be dry. This step must also include the safety issues, therefore taxonomic identification and detailed toxicological and ecotoxicological studies are needed to exclude human, plant or animal pathogens as well as microorganisms that can produce toxic metabolites. In this stage, a rapid throughput analysis consisting of *in vitro* bioassays on plants in controlled conditions is recommended allowing us to prescreen candidates able to guarantee plant growth promotion efficacy without time-consuming approaches or expensive resources.

The fifth step is dedicated to the characterisation of the mode of actions of the candidates to obtain knowledge to optimize the application and maximize their efficacy. In fact, candidates will be evaluated for their ability to make soil elements more available to the plants and to promote plant growth through the production of phytohormones and/or the modulation of plant hormone balance. Finally, in the sixth step, plant growth promoting efficacy of candidates is evaluated under small-scale experiments mimicking the real environmental conditions, in order to select the most efficient one(s). In general, greenhouse experiments are proposed at this stage in order to control environmental conditions, while maintaining them as close as possible as the expected ones under future practical application.

2.2. Assessment of target crops and design of a commercial strategy

Target crop was chosen by consulting statistics reported by Food and Agriculture Organization (http://www.fao.org/faostat/) and Eurostat (https://ec.europa.eu/eurostat/data/database). The choice was oriented on a crop whose cultivation, production and organic production was grown constantly during the recent years. The commercial strategy was designed starting from a review process of the scientific works and patents related to PBs and PGPR published in the last five years (2015–2019) using the databases Espacenet (https://worldwide.espacenet.com) and Web of Science (https://apps.webofknowledge.com/). In Web of Science, an Advanced Search was carried out using the formulae: TI = plant biostimulants AND SU = agriculture and TI = plant

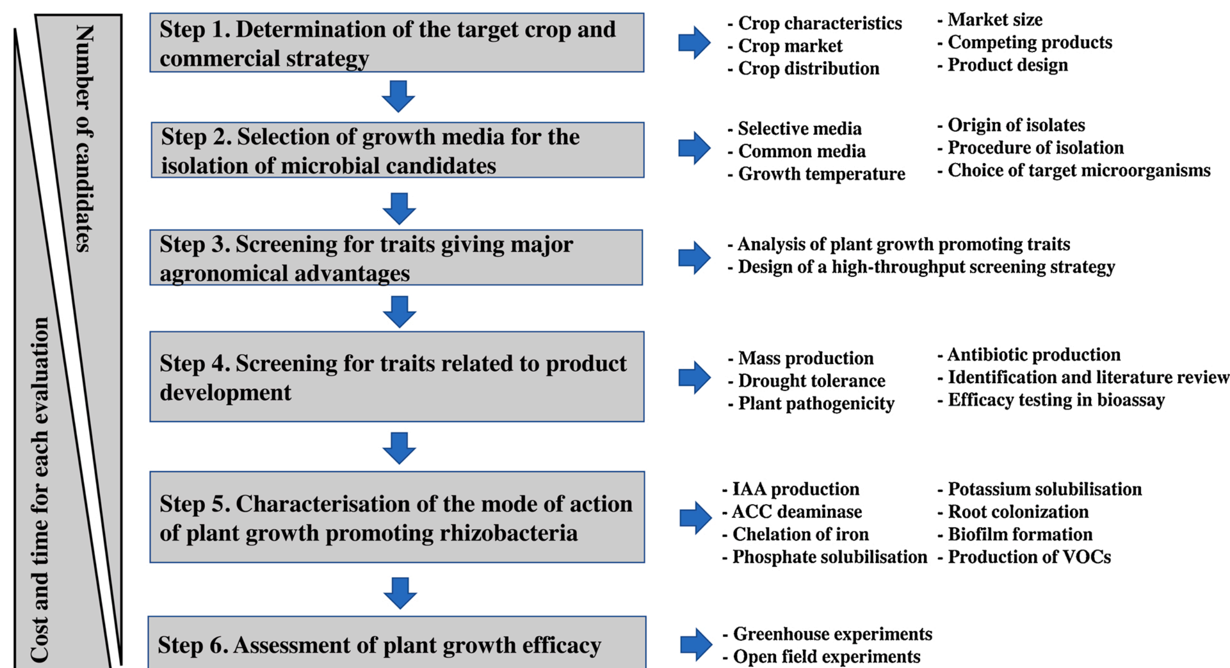

Fig. 1. Stepwise screening strategy of microorganisms for commercial use as biostimulants.

growth promoting rhizobacteria AND SU = agriculture. Similarly, the formulae ti = "Plant" OR ti = "Biostimulants" and ti = "Plant growth promoting" OR ti = "rhizobacteria" were used to carry out an Advanced Search in Espacenet. The products were analysed to have a picture of: i) the progresses reached in the analysis of PGPR to be developed as PBs; ii) the procedures adopted to screen PGPR in the majority of the published literature; iii) new strategies that might be implemented to design next generation PBs. After the analysis, brainstorming meetings were programmed to formulate new commercial strategies. The ideas were subsequently discussed for their feasibility and impact on the market.

2.3. Isolation of bacteria from tomato rhizosphere soil

Soil adhering to tomato (*Solanum lycopersicum* cv. Tondo rosso) plant roots was collected, sieved at 2 mm mesh, placed in sterile 50 mL tubes and stored at 4 °C until time of processing (approximately 24 h). Isolation of culturable bacteria was carried out by dilution plating method using the Rhizosphere Mimicking Agar (RMA, Brescia et al., 2020). Soil suspensions were prepared mixing 5 g of root-adhering soil in 45 mL of sterile saline solution (NaCl 0.85 % w/v) contained in sterile 50 mL tubes. Subsequently, tubes were shaken at 200 rpm for 1 h at room temperature. At the end of the shaking, soil mixtures were serially diluted (from 10^{-1} to 10^{-7}) in triplicate. A volume of 100 μL of diluted suspension (from 10^{-3} to 10^{-7}) was spread onto RMA medium amended with cycloheximide (100 mg/l). Petri dishes were incubated at 27 °C and bacterial colonies selected after 72 h based on their morphology. Selected bacterial isolates were stored at length in glycerol 40 % at -80 °C and routinely grown on Nutrient Agar (NA, Oxoid, United Kingdom) in Petri dishes (90 mm diameter). In all the experiments enlisted in the flow diagram (Fig. 1), bacteria were discarded if they did not share traits selected in each step except when otherwise indicated.

2.4. Screening of bacterial isolates for compatibility with humic acids and phosphate solubilisation

The National Botanical Research Institute's phosphate growth medium (NBRIP) and R2A medium amended with 0.003 % of HA (Sigma Aldrich, United Kingdom) were respectively used to select bacterial isolates having the ability to solubilize phosphate and to be combined with humic acids. Briefly, bacterial isolates were grown in sterile 15 mL tubes containing 5 mL of Nutrient Broth (NB, Oxoid) at 27 °C on an orbital shaker (200 rpm). After 24 h, a volume of one mL of bacterial cell suspensions was centrifuged (13,000 rpm, 2 min) and pellets were suspended in NaCl (0.85 % w/v) to a final optical density at 600 nm ($A_{OD600nm}$) of 0.1 corresponding to $\simeq 1 \times 10^7$ colony forming units (CFU)/mL . A volume of five μL of bacterial cell suspension was spot inoculated into the above-mentioned growth media and incubated for 48 h at 28 °C. Bacterial isolates having both the ability to grow onto R2A amended with HA and develop a halo around the macrocolony on the NBRIP medium (Campisano et al., 2015) were selected for further characterisation.

2.5. Selection of bacterial isolates sharing traits useful for their development as a bioproduct

2.5.1. Evaluation of cell mass production and tolerance to desiccation

To assess cell mass production, bacterial isolates were grown in steile 15 mL tubes containing NB (5 ml) at 27 °C on an orbital shaker (200 rpm). After 16 h, bacterial cell suspensions were serially diluted (from 10^{-1} to 10^{-8}) and dilutions (from 10^{-5} to 10^{-8}) were plated onto NA. Once inoculated, Petri dishes were incubated at 27 °C and the developed CFU were counted after 48 h incubation. Results were expressed as $\log_{10}$ CFU/mL.

Tolerance to desiccation was assessed by growing bacterial isolates in sterile 15 mL tubes containing NB (5 mL) at 27 °C on an orbital shaker (200 rpm) for 16 h. Subsequently, a volume of five μL of bacterial cell suspensions was spot inoculated (in triplicate) onto NA amended with sorbitol (0.53 M) to mimic drought condition [-2.5 MPa water potential (w.p.)]. Development of bacterial macrocolonies was assessed after 24 h incubation at 28 °C.

Bacterial isolates able to reach a concentration $\geq 10^9$ CFU/mL and to develop macrocolonies on NA amended with sorbitol were selected and further characterised.

2.5.2. Assessment of plant pathogenicity and production of toxic secondary metabolites

The ability to establish a pathogenic interaction with plants was assessed according to Klement et al. (1964). Briefly, bacterial cell

suspensions (1×10^8 CFU/mL) in sterile distilled water (SDW) were injected (in triplicate) into the intercellular spaces of healthy tobacco (*Nicotiana tabacum*) leaves. Tobacco plant leaves injected with SDW only were used as untreated control. Tobacco plants were kept in the greenhouse (25 ± 1 °C; 70 ± 10 % RH; 16 h photoperiod) and occurrence of a hypersensitivity reaction was visually assessed after 24 h.

Production of antibiotics was assessed by dual plate assay according to the procedure described by Puopolo et al. (2014a) with some modifications. Briefly, five μL of bacterial cell suspension (1×10^8 CFU/mL) was spot inoculated at 30 mm of the border of Petri dishes (90 mm diameter) containing Potato Dextrose Agar (PDA, Oxoid). After 24 h at 27 °C, plugs of mycelium of the phytopathogenic oomycete *Pythium* (*Py.*) *ultimum* (5 mm of diameter) were cut from the youngest region of the mycelium and placed at 25 mm far from the bacterial macrocolonies. *Pythium ultimum* was used as test microorganism based on its high sensitivity to microbial toxic secondary metabolites (Santos and Melo, 2016). PDA dishes seeded with mycelium plugs only were used as untreated controls. Inhibition of the mycelial growth was evaluated by measuring the *Py. ultimum* colony diameter after 72 h incubation at 25 °C.

Bacterial isolates not able to cause hypersensitivity reaction in tobacco plants and/or release secondary metabolites toxic against *Py. ultimum* were selected and further characterised.

2.5.3. Identification of bacterial isolates by 16S rDNA and literature review

Bacterial genomic DNA was extracted using the kit Power Soil™DNA Isolation Kit (Mo Bio Laboratories, Inc., Carslband CA, USA) according to the manufacturer's instructions. Five μL of genomic DNA were used as template in PCR for the amplification of 16S rDNA region. Reaction mixtures (25 μL) containing 12.5 μL Go Taq Green Master Mix (Promega GmbH, Mannheim, Germany), 11.5 μL sterile deionized water and 0.5 μL of universal primers 16S-27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1492R (5′GGTTACCTTGTTACGACTT-3′). Reaction was carried out in an automated thermal cycler (Biometra GmbH, Göttingen, Germany) with the following protocol: initial denaturation at 95 °C for 2 min, 36 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min), extension (72 °C for 1 min) and final extension at 72 °C for 4 min.

PCR products were purified using Illustra ExoProstar Kit (Euroclone S.p.A., Italy) according to the manufacturer's instructions and subsequently sequenced at the Sequencing and Genotyping Technological Platform of Research and Innovation Centre, Fondazione Edmund Mach (Italy). To identify at the species level preferably, resulting nucleotide sequences were compared to known sequences deposited in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/) using BLASTN (Basic Local Alignment Tool).

Bacterial isolates belonging to human and/or animal harmful bacterial species were discarded.

2.5.4. Effect of bacterial seed treatment on the in vitro tomato seedling vigour

The ability of bacterial isolates to promote plant growth was carried out by a seed germination assay according to the procedure described by Smyth et al. (2011) with some modifications. Tomato seeds (*S. lycopersicum* var. Moneymaker, Justseed, Wrexham, UK) were surface sterilized by submerging them in the following solutions: 70 % ethanol for 2 min, 1 % sodium hypochlorite (NaOCl) for 5 min. After that, surface-sterilized seeds were washed five times with SDW. Inoculation of bacterial strains was carried out by soaking seeds in either 1 mL of bacterial cell suspension (1×10^8 CFU/mL; treated) or SDW (untreated) for 1 h and drying for 30 min under the laminar flow cabinet. Five seeds per bacterial isolates were placed on the top of four sterile filter papers soaked with 5 mL of SDW contained in sterile Petri dishes. Subsequently, Petri dishes were incubated in darkness for five days at 20 °C. Germination was considered to occur if at least 1 cm of radicles appeared. To determine the vigour index, the following formula was applied:

Vigour index = (Average root length + Average shoot length) / Germination rate (%)

After this calculation, the change in the vigour index was evaluated as follows:

Change Vigour Index (%) = (Average vigour index treated- Average vigour index untreated) / Average vigour index untreated

Three replicates (Petri dishes) were used for each treatment and the experiment was carried out five times. Bacterial strains able to increase the vigour index of tomato seedlings at least in three out of five experiments were selected and further characterised.

2.6. Characterisation of bacterial strains for plant growth promoting activities

2.6.1. Chelation of iron and solubilisation of phosphate and potassium

To determine the ability of bacterial strains to chelate iron through the release of siderophores, King's B Agar dishes were overlaid with Chrome Azurol S (CAS) Agar medium (Schwyn and Neilands, 1987). Solubilisation of phosphate and potassium were evaluated using the NBRIP medium and Aleksandrow Agar (HiMedia GmbH, Germany) respectively. In all the tests, a volume of five μL of bacterial cell suspension (1×10^8 CFU/mL) was spot inoculated onto these media and incubated for 72 h at 28 °C. At the end of incubation, the areas of orange haloes (release of siderophores) and the clarification haloes (solubilisation of phosphate and potassium) formed around bacterial macrocolonies were determined by capturing digital images with Bio-Rad Quantity One software implemented in a Bio-Rad Geldoc system (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.). The halo areas were subsequently measured using Fiji software (ImageJ1.50i; Schneider et al., 2012). For all the tests, three replicates (Petri dishes) were used and the experiment was repeated.

2.6.2. Production of indole-3-acetic acid and 1-Aminocyclopropane-1-carboxylate deaminase

Indole-3-acetic acid (IAA) production was evaluated by a colorimetric detection test in liquid culture according to the procedure described by Campisano et al. (2015) with some modifications. Briefly, a volume of 500 μL of bacterial cell suspension (1×10^8 CFU/mL) was grown in five mL of DF salt minimal broth amended with 500 μg/mL of L-Tryptophan (Sigma-Aldrich) contained in sterile 15 mL tubes at 28 °C on an orbital shaker (200 rpm). After 120 h, final cell densities were determined by measuring the absorbance at 600 nm ($A_{OD600nm}$). A volume of one mL of bacterial cell suspensions was centrifuged (13,000 rpm, 10 min) and 250 μL of supernatant were mixed with one mL of Salkowski's reagent. After 30 min incubation at room temperature, 150 μL of the mixture were transferred to 96-well polystyrene dishes (Thermo Fisher Scientific, Waltham, MA, USA). The intensity of pink red colour was quantified by measuring the absorbance at 530 nm ($A_{OD530nm}$) by Synergy 2 Multiplate Reader (Biotek, Winooski, VT, USA) and IAA concentration was determined by a standard curve prepared from pure IAA solutions (Sigma-Aldrich) in a range from 0.5–100 μg/mL (Fig. S1). Quantity of IAA produced by bacterial cells was expressed as the ratio between $A_{OD530nm}$ and $A_{OD600nm}$.

1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity was assessed by a colorimetric ninhydrin assay according to the procedure described by Li et al. (2011). Briefly, bacterial cell suspensions (1×10^8 CFU/mL) were grown in sterile 15 mL tubes containing 5 mL of DF salt minimal medium supplemented with 3 mmol/L of ACC as the only nitrogen source. The resulted Ruhemann's Purple depth was measured as absorbance at 570 nm ($A_{OD570\ nm}$) and associated to presence of ACC deaminase. ACC deaminase activity was calculated by dividing $A_{OD570\ nm}$ by the bacterial cell density ($A_{OD600nm}$).

In both experiments, three replicates (15 mL tubes) were used for each bacterial strain and experiments were repeated.

2.6.3. Biofilm formation

Bacterial strains were evaluated for their ability to form biofilm using the procedure described by Puopolo et al. (2014b). Briefly, 1.5 μL of bacterial cell suspension (1×10^8 CFU/mL) was inoculated in 150 μL of NB distributed in sterile 96-well polystyrene dishes and incubated at 27 °C without shaking. NB not inoculated with bacterial strains was used as the untreated control. After 72 h, final cell density ($A_{OD600nm}$) was determined. Unattached cells were removed by inverting the plate and the remaining bacterial cells were attached to the well surfaces by an incubation period for 20 min at 50 °C. Bacterial cells were then stained and washed with 200 μL of crystal violet solution and SDW respectively. Adherent cells were decolorized with 200 μL of acetone/ethanol (20 %/80 %). A volume of 100 μL of each suspension was transferred to a new 96-well polystyrene plate. The density of adherent cells (biofilm formation) was determined by measuring the absorbance at 540 nm ($A_{OD540nm}$). To determine the Specific Biofilm Formation (SBF) the following formula was applied:

$$SBF = (A_{OD540nm}X - A_{OD540nm}C) / (A_{OD600nm}X - A_{OD600nm}C)$$

where X indicated the treated samples whereas C indicated the untreated control.

For each bacterial strain, three replicates (wells) were used and the experiment was repeated.

2.6.4. Root colonisation

Seeds of tomato plants (*S. lycopersicum* var. Moneymaker, Justseed, Wrexham, UK) were surface-sterilized and bacterial strains were inoculated as mentioned above. Treated and untreated seeds (in triplicate) were dipped into perlite (3 g) contained in 95 mL glass tubes (Artiglass, Padova, Italy) and moistened with 10 mL of Hoagland nutrient solution. Subsequently, tubes were kept in a growth chamber (25 ± 1 °C; 70 ± 10 % RH; 16 h photoperiod). After 144 h, roots were weighted, cut and mixed in 5 mL of $MgSO_4$ 10 mM contained in sterile 15 mL tubes. Subsequently, tubes were shaken at 200 rpm for 30 min at room temperature. After shaking, suspensions were serially diluted (from 10^{-1} to 10^{-7}). A volume of 10 μL of diluted suspension (from 10^{-4} to 10^{-7}) was spot inoculated onto NA (Oxoid). Once inoculated, Petri dishes were incubated at 27 °C and the developed CFU were counted after 48 h. Results were expressed as $\log_{10}$ CFU/mg of tomato roots. Three replicates (tomato seedlings) were processed for each bacterial strain and the experiment was repeated.

2.7. Greenhouse experiment

The effect of the application of two selected bacterial strains on tomato plant growth was assessed under greenhouse conditions. Tomato seeds (*S. lycopersicum* var. Moneymaker, Justseed, Wrexham, UK) were seeded in 100 mL pots containing DCM Ecoterra ® Zaaien & Stekken potting mix (DCM; Grobbendonk; Belgium) and grown in the greenhouse with an average temperature 22 °C ± 2 at night and 24 °C ± 2 during the day until the plants had produced one shoot with at least two true leaves. At this stage, a volume of 10 mL of three days old bacterial cell suspension (1×10^8 CFU/mL) was applied into the pots to reach a final cell density of 1×10^7 CFU/pot and plants were then kept in the greenhouse. After two days, tomato plants were transplanted to 4 L pots containing potting mix without fertilizer (DCM; Grobbendonk; Belgium) mixed with an organic fertilizer: DCM ECO-PLANT 2 (DCM; Grobbendonk; Belgium), and an organic amendment enriched in trace element: MICRO MIX DCM (DCM; Grobbendonk; Belgium) at 6 g/L and 0.3 g/L respectively. One week after soil transplantation, a volume of 100 mL of three days old bacterial cell suspension (1×10^9 CFU/mL) was applied into the pots to reach a final cell density of 2.5×10^7 CFU/pot. For both inoculations, the untreated control was treated with only water. Six weeks after the second application of bacterial strains, shoot length and shoot dry biomass (48 h incubation at 65 °C) was evaluated. Twenty replicates (tomato plants) were used for each treatment.

2.8. Statistical analysis

All experiments were carried out twice except seed germination test which was repeated five times. Normality (Shapiro-Wilk test, $p > 0.05$) and variance homogeneity (Levene's test, $p > 0.05$) were checked and parametric tests were used. Data from experiments were pooled when two-way ANOVA demonstrated non-significant differences between two experiments ($p > 0.05$). When significant differences between experiments were found, data presented were analysed from one representative experiment with similar results. Data were subsequently analysed using one-way ANOVA and mean comparisons between treatments were assessed by Tukey's test ($\alpha = 0.05$). Data were analysed with IBM SPSS software (Version 21).

3. Results

3.1. Determination of target crop and product design

Consultation of statistics from Eurostat and FAO (FAO, 2018) led us to choose tomato as the target crop for a future PB. In particular, the constant increase of world production and the increase of hectares deputed to organic production of fresh vegetables in EU played a major role in this choice (Fig. S2). The advanced research on Web of Science revealed an increase of published articles having the terms PGPR and PBs in the title in the period of 2015−2019. In details, 352 published articles contained the term PGPR in the title, whereas the term PBs appeared in 48 published articles only (Fig. S3). Regarding patents, the advanced search in Espacenet showed the presence of 15 patents having the term "plant biostimulants" in the title, but only few of them were related to PGPR. A higher number of patents (27) contained the term "plant growth promoting rhizobacteria" in the title and most of them referred to the application of rhizobacteria to stimulate plant growth.

Overall, the advanced searches carried out gave us a better idea in the steps that might be included in the screening procedure to make it more suitable for the selection of PGPR having traits useful for their future development as commercial PBs. Moreover, results from these activities were discussed and the idea of a new commercial PB was conceived. Firstly, ability to solubilise phosphate was chosen as a plant-growth promoting activity that might characterise the future PB. Although the input of phosphorous in agriculture is lower than nitrogen (Fig. S4), a relevant number of commercial PBs including PGPR able to make nitrogen more available to crop plants are already available on the market and this might represent a strong competition for a new commercial PB. Based on an internal research, the margin of a success of a future PB including PGPR able to solubilise phosphate is higher due to the small number of PBs with this plant-growth promoting activity. Based on the most recent published articles focused on PBs, we discussed and agreed on the fact that the combination of PGPR and humic acids might represent a competitive advantage of the future commercial PB.

3.2. Isolation of bacteria from rhizosphere soil of tomato plants and first screening

Once established the target crop and the potential of a new PB competitive products, 200 culturable bacteria were isolated from the rhizosphere soil of tomato plants and rapidly screened for their ability to solubilize phosphate and grow in presence of humic acids. From this screening, 78 bacterial isolates having both the traits were selected and furtherly evaluated.

3.3. Selection of bacterial isolates sharing traits useful for their development as a bioproduct

Out of 78 bacterial isolates selected from prior steps, 25 were able to reach 10^9 CFU/mL on NB after 24 h and tolerated desiccation condition. Seven of this group were discarded since they showed antifungal activity against *Py. ultimum* while the remaining 18 bacterial isolates were tested for their phytopathogenicity. After injection of bacterial isolates on tobacco leaves, only one showed hypersensitive reaction and discarded from the selection since it might represent a plant pathogenic bacterium.

Taxonomic identification of selected bacterial isolates showed different percentage of identity based on the similarity between nucleotides (Table 1). After a literature review, the bacterial isolates showing high sequence identity level with *Bacillus cereus, Kluyveria intermedia*, *Pseudomonas* (*Ps.*) *plecoglossicida* and *Pseudomonas lini* were discarded for being human, animal and/or plant pathogen (Bottone, 2010; Nishimori et al., 2000; Sarria et al., 2001). The effect on the vigour index of tomato seedlings compared to the untreated seeds varied among the different bacterial strains (Fig. 2). Among 12 bacterial strains previously selected, seven of them showed either slightly positive results, negative results or no differences in the vigour index compared to the untreated plants in most of the experiments carried out (Fig. 2). In contrast, tomato seeds treated with *Paenarthrobacter* (*Pae.*) *nitroguajacolicus* MVC 6 showed a positive modulation of vigour index in all the experiments carried out. Similarly, *Arthrobacter enclensis* MVC 3, *Pseudomonas putida* MVC 17, *Pantoea agglomerans* MVC 21, and *A. oxydans* MVC 13 determined an enhancement in vigour index in four or three experiments carried out (Fig. 2). Based on these results, five bacterial strains that showed positive results in at least three independent experiments were selected to be further characterised.

3.4. Screening for plant- growth promoting activities

Pantoea agglomerans MVC 21 was the best phosphate solubilising strain showing the largest clarification halo areas (Fig. 3A). Three bacterial strains were able to solubilise potassium and, in particular, *P. agglomerans* MVC 21, showed the largest clarification halo area (74.28 ± 6.30 mm^2, Fig. 3B). Similarly, three bacterial strains released siderophores and the most active bacterial strain was *Ps. putida* MVC 17 (1042 ± 24.055 mm^2; Fig. 3C). All the tested bacterial strains produced IAA and *P. agglomerans* MVC 21, with 281.84 ± 3.79 µg/CFU, showed the highest production (Fig. 3D). ACC deaminase activity was not detected in any tested bacterial strain.

Table 1
Taxonomic identification of bacterial isolates by 16S rDNA.

Strain code	Bacterial species	Identity level (%)	Accession number
MVC 1	*Kluyvera intermedia*	99.7	MT374833
MVC 3	*Arthrobacter enclensis*	99.2	MT374834
MVC 6	*Paenarthrobacter nitroguajacolicus*	99.8	MT374835
MVC 11	*Bacillus aryabhattai*	99.3	MT374836
MVC 13	*Pseudarthrobacter siccitolerans*	99.4	MT374837
MVC 15	*Pseudomonas plecoglossicida*	99.0	MT374838
MVC 16	*Arthrobacter pascens*	99.9	MT374839
MVC 17	*Pseudomonas putida*	99.8	MT374840
MVC 18	*Pseudomonas lini*	98.9	MT374841
MVC 21	*Pantoea agglomerans*	99.6	MT374842
MVC 22	*Paenarthrobacter nitroguajacolicus*	97.7	MT374843
MVC 23	*Paenarthrobacter nitroguajacolicus*	99.7	MT374844
MVC 31	*Bacillus pumilus*	99.1	MT374845
MVC 33	*Pseudarthrobacter oxydans*	99.6	MT374846
MVC 41	*Erwinia endophytica*	98.3	MT374847
MVC 107	*Bacillus cereus*	99.9	MT374848
MVC 109	*Bacillus cereus*	99.7	MT374849

All the tested bacterial strains were able to form biofilm and colonize tomato roots. *Artrhrobacterenclensis* MVC 3 was the best biofilm producer under the conditions tested (Fig. 3E) whereas *P. agglomerans* MVC 21 showed the highest values of root colonisation (6.47 ± 0.23 $\log_{10}$ CFU/mg of tomato roots; Fig. 3F).

3.5. Effect of selected bacterial strains under greenhouse conditions

Under greenhouse conditions, tomato plants inoculated with either *P. agglomerans* MVC 21 or *Ps. putida* MVC 17 showed a significant increase in the shoot length and biomass compared to the untreated plants. Application of *P. agglomerans* MVC 21 determined the most significant increase in terms of shoot length (444.50 ± 16.06 mm; Fig. 4A). Regarding the shoot dry biomass, the application of *P. agglomerans* MVC 21 (12.45 ± 0.93 mg) and *Ps. putida* MVC 17 (11.25 ± 0.75 mg) determined a significant increase compared to the untreated tomato plants (8.76 ± 0.66 mg; Fig. 4B).

4. Discussion

The design of flow diagrams including all the steps needed for the selection of microorganisms and their development as novel commercial bioproducts was addressed in the case of biocontrol agents (Köhl et al., 2011; Segarra et al., 2015). In contrast, this topic received scarce consideration in the case of the selection and characterization of PBs (Povero et al., 2016). In this work, we designed a straightforward stepwise screening program and we validated it through the selection of two PGPR candidates able to fulfil all the requirements needed to be successfully developed as commercial PBs.

The first step of our stepwise screening program was dedicated to the choice of the target crop and the strategy to realize a novel PB that might compete with PBs already available on the market. To decide the crop, the statistics published by Eurostat and FAO were examined (De Cicco, 2019; FAO, 2018). It is widely accepted that tomato is one of the most consumed vegetable worldwide (Nicola et al., 2009) and this is corroborated by the constant increase of world production in the last 20 years (FAO, 2018). The EU tomato production accounted for the 21.1 % of the total value of total fresh vegetable production in 2017 corresponding to 7.3 billion € with the greatest planted area, corresponding to the 10.8 % of the total EU area planted with fresh vegetables (De Cicco, 2019). Moreover, the increase of the organic farming in EU in the last years might contribute to a stimulus for the demand of PBs to be applied in organic production. Based on these evidences, we decided to focus our attention on the selection of PGPR having a positive effect on the growth of tomato plants as this choice will guarantee a broad market to a future commercial PB.

Based on the analysis of patents and recently published articles, we considered that the combination of a PGPR and humic acids might have a competitive advantage on the market. Indeed, the combination of non-microbial and microbial biostimulants was recently suggested for the design and development of the second generation of PB products (Rouphael and Colla, 2018). Among non-microbial biostimulants, humic acids play an important role in the soil fertility and the PBs containing humic acids have been already evaluated for their efficacy in improving crop production (Canellas et al., 2015; Nardi et al., 2002; Olivares et al., 2017). A synergistic effect was already observed in the case of the combined application of humic acids with endophytic bacteria on tomato plants and the mycorrhizal fungus *Rhizophagus irregulare* on onion seedlings and perennial ryegrass (Bettoni et al., 2014; Galambos et al., 2020; Nikbakht et al., 2014). Furthermore, humic acids can be used as a vehicle to introduce beneficial microorganisms to the soil, as they are considered recalcitrant to microbiological attack (Canellas and Olivares, 2014).

Based on these ideas, the work started from the isolation of bacteria from the rhizosphere soil of tomato plants. In this context, different growth media may be used to collect a sound number of bacterial

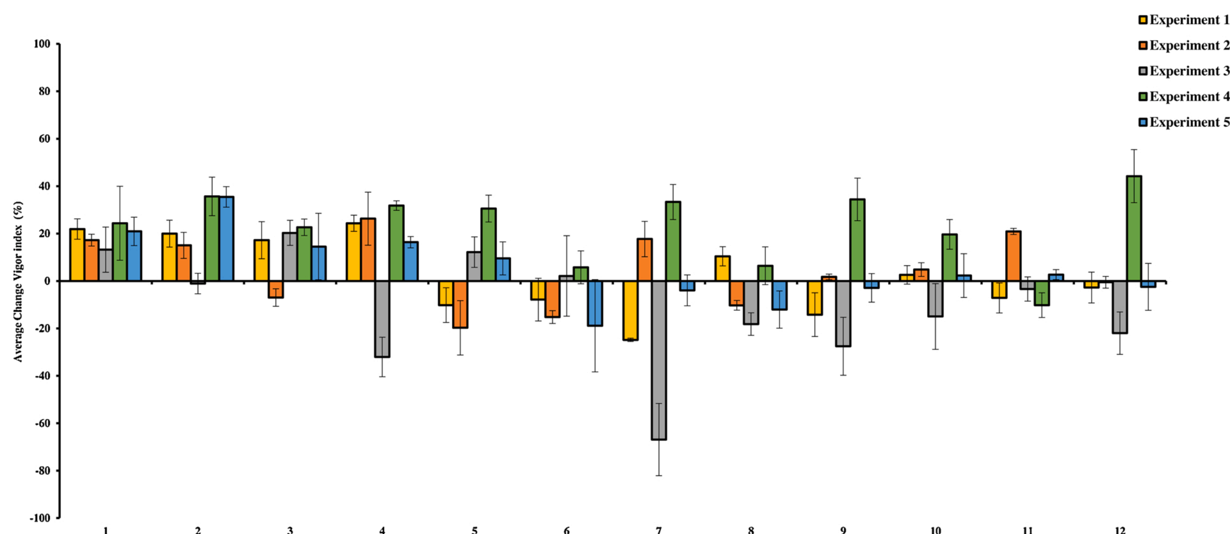

Fig. 2. Efficacy of bacterial isolates on the *in vitro* seedling germination of tomato seeds. The vigour index was assessed from each treatment by the germination rate and the root and shoot elongation. 1. *Paenarthrobacter nitroguajacolicus* MVC 6; 2. *Arthrobacter enclensis* MVC 3; 3. *Pseudomonas putida* MVC 17; 4. *Pantoea agglomerans* MVC 21; 5. *Arthrobacter oxydans* MVC 13; 6. *Bacillus aryabhattai* MVC 11; 7. *Arthrobacter globiformis* MVC 16; 8. *Paenarthrobacter nitroguajacolicus* MVC 22; 9. *Paenarthrobacter nitroguajacolicus* MVC 23; 10. *Bacillus pumillus* MVC 31; 11. *Arthrobacter oxydans* MVC 33; 12. *Pantoea cedenensis* MVC 41. Values were compared with the untreated (change vigour index) for five experiments (columns). Mean and standard error values (columns) from fifteen replicates are represented for each treatment.

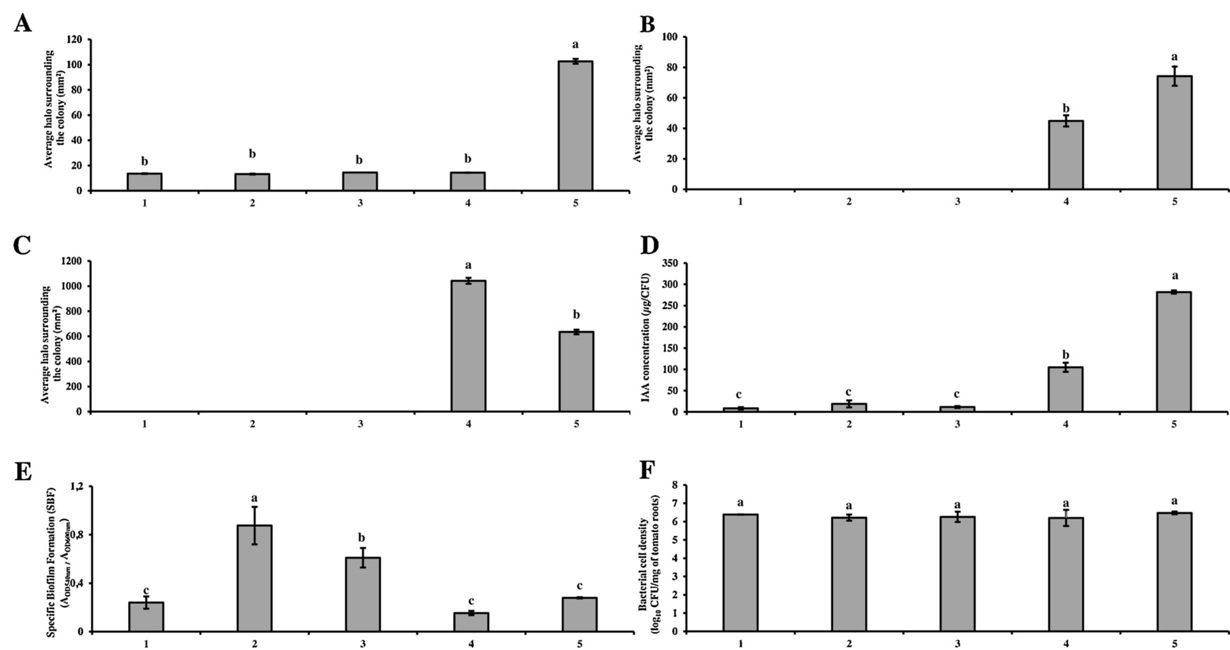

Fig. 3. Quantitative assessment of plant growth promoting activities. Phosphate (A) and potassium (B) solubilization, chelation of iron (C), IAA production (D), biofilm formation (E) and root colonisation (F) were assessed for *Paenarthrobacter nitroguajacolicus* MVC 6 (1), *Arthrobacter oxydans* MVC 13 (2), *Arthrobacter enclensis* MVC 3 (3), *Pseudomonas putida* MVC 17 (4), and *Pantoea agglomerans* MVC 21 (5),. Mean and standard error values (columns) of six replicates from the two experiments are presented for each treatment (bacteria). Different letters indicate significant differences among treatments according to Tukey's test ($\alpha = 0.05$).

isolates. Frequently, common growth media are used to isolate bacteria from environmental samples. In our case, we opted for a semi-selective growth medium mimicking the nutrient conditions that the bacterial cells may find in the tomato rhizosphere (Brescia et al., 2020). Thus, all the bacterial isolates collected from rhizosphere soil shared the ability to grow in the presence of these nutrients, representing a first indication of their rhizosphere competence. In future, growth media mimicking the nutrient conditions of rhizosphere might be used in the isolation of microorganisms inhabiting the rhizosphere soils of various crop plants.

To proceed with a first screening step, we decided to take into consideration the compatibility with humic acids and a plant-growth promoting activity. As plant-growth promoting activity, we focused our attention on the phosphate solubilisation. Indeed, phosphorous is the second of the most important inorganic nutrients necessary for plant growth (Alori et al., 2017). However, plants absorb and assimilate phosphorus as inorganic orthophosphate, a form with low availability in most soils (Herrera-Estrella and López-Arredondo, 2016). Microorganisms able to convert the inorganic orthophosphate to soluble forms will increase the uptake of this nutrient by crop plants (Khan et al., 2008). Moreover, we thought that the choice of phosphate solubilisation might

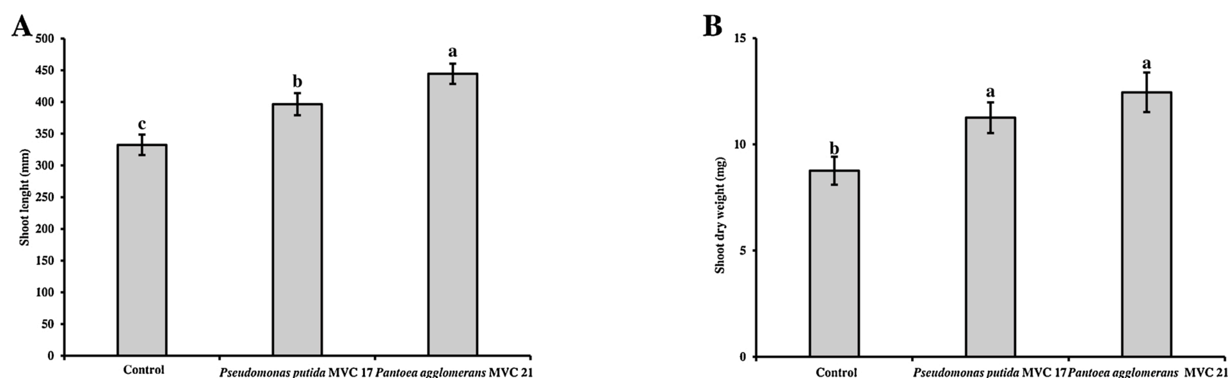

Fig. 4. Plant growth promoting effects of *Pantoea agglomerans* MVC 21 and *Pseudomonas putida* MVC 17 under greenhouse conditions. Shoot length (A) and shoot biomass (B) were evaluated after 6 weeks from the second inoculation. Mean and standard error values from 20 replicates are represented for each treatment. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

present an advantage from a commercial point of view. Indeed, a relevant number of microbial PBs includes PGPR able to fix nitrogen whereas the number of PBs with the ability to solubilise phosphate is increasing slowly (Parnell et al., 2016). Consequently, the designed PBs having PGPR able to solubilize phosphate might face a less strong competition when it will be commercialized.

Based on these aspects, the collection of bacterial isolates was screened for the ability to grow in presence of humic acids and solubilize phosphate. A sensitive reduction of the bacterial isolate collection was registered with only the 39 % of bacterial isolates able to grow in presence of humic acids and solubilise phosphate.

To narrow the list of bacterial isolates that might be developed as a commercial PB, we decided to introduce steps that are not frequently used in the published articles dedicated to the characterization of potential PGPR. For instance, we took into consideration parameters related to the mass production, as they might impact the costs related to the production of bacterial cells. Firstly, we selected the bacterial isolates able to reach a cell concentration $\geq 10^9$ CFU/mL in 16 h. This threshold was chosen since minimum incubation time to reach highest cell density may allow a reduction in production cost (Posada-Uribe et al., 2015). Moreover, this value represents an optimal concentration to develop a successful commercial strain since a threshold of approximately from 10^8 to 10^9 CFU/g or CFU/mL of formulation is required for inoculant quality standards (Malusá and Vassilev, 2014). We included also another selecting step concerning the resistance to desiccation since drying procedures may be included in the formulation of microbial inoculants (Validov et al., 2007). To simulate desiccation, we used sorbitol since it is the most commonly used stress-inducing agent in solid medium which acts by lowering the w.p. of the medium (Claeys et al., 2014). We considered -2.5 MPa w.p. an acceptable value to select bacterial isolates able to survive desiccation conditions since it is reported that rhizobacteria are able to survive to up to -3.5 MPa w.p. (Abolhasani et al., 2010).

As PBs may not act as biocontrol agents (Validov et al., 2007), we introduced an additional step that is not frequently reported in the published articles dedicated to the screening of bacterial isolate collection for the identification of new PGPR. We decided to discard bacterial isolates able to produce antibiotics active against *Py. ultimum*, a soil-borne plant pathogen commonly found in soils (Rai et al., 2020) that shows high sensitivity to secondary metabolites with antibiotic activity (Santos and Melo, 2016). Noteworthy, bacterial isolates able to produce antibiotics might represent potential active ingredients for new commercial biopesticides. Thus, research groups and companies might discard these bacterial isolates or considering their transfer in the pipeline for the characterisation of microorganisms for the development of microbial biopesticides.

Looking at the final commercial PB product, bacterial isolates that may be a threat to animals, crop plants and humans were also discarded (Tabassum et al., 2017; Köhl et al., 2011). We decided to use the classical hypersensitive reaction on tobacco leaves to assess the potential to establish a pathogenic interaction with plants. This additional step is not found frequently in published screening strategies even if it is a cheap and fast method and might guarantee the discard of unmarketable bacterial isolates before proceeding with more expensive steps.

Hypersensitive reaction on tobacco leaves was followed by the identification at species level based on 16S rDNA sequencing to determine if the bacterial isolates belonged to bacterial species potentially pathogenic to animal, human and plants. Notably, the 16S rDNA sequencing allowed to discard *Ps. lini* MVC 18 even if this bacterial isolate did not trigger the hypersensitive reaction in tobacco leaves. This result proves the importance of the identification of the microorganisms based on molecular tools to correctly discard microorganisms that may cause problems during their registration. Moreover, the sequencing results also highlighted how human opportunistic pathogens find in the rhizosphere their ideal niche (Berg et al., 2005) as the two bacterial strains belonging to *B. cereus* and one bacterial strain belonging to *K. intermedia* that were discarded. At the end of this screening procedure, the 6 % of the initial 200 bacterial isolates showed all the traits suitable for the development as a PB product.

As efficacy testing in bioassays and field conditions is time consuming and expensive, candidates might be screened first under *in vitro* or *in planta* conditions selecting the most promising candidates (Köhl et al., 2011). We evaluated the effect of bacterial inoculation on the seed germination, root and shoot elongation to quickly select plant growth promoter candidates since seed vigour and viability determine seedling establishment, crop growth and productivity (Nehra et al., 2016). Only five bacterial strains (*A. enclensis* MVC 3, *A. oxydans* MVC 13, *Pae. nitroguajacolicus* MVC 6, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17) guaranteed a reproducibility of the results registering a positive effect at least in three out of the five experiments carried out.

Using the growth medium reproducing the rhizosphere conditions contributed to select bacterial strains able to grow using nutrients released by plants in the rhizosphere (Brescia et al., 2020). Indeed, all the selected bacterial strains were able to colonize actively roots of tomato seedlings and this ability was corroborated by their ability to form biofilm, structures that help bacteria to survive in hostile environments leading to increase the chance of survival (Seneviratne et al., 2010).

Although plate assays are not indicative of the mechanisms of action implemented by PGPR during their interaction with crop plants (Cardinale et al., 2015), understanding plant-growth promoting activities endowed by PGPR may represent a benefit for a future PB product from a commercial point of view (Backer et al., 2018). Thus, the selected bacterial strains were evaluated for their ability to produce plant growth regulators and increase the nutrient availability in the rhizosphere favouring the nutrient uptake (Vejan et al., 2016). *Pantoea agglomerans* MVC 21 and *Ps. putida* MVC 17 were the most effective strains for the

release of siderophores and solubilisation of phosphate and potassium in agreement with previous results (Ahmad et al., 2008; Mukherjee et al., 2020). Similarly, these bacterial strains were the best producer of IAA, a phytohormone involved in plant cell enlargement, division, tissue differentiation and responses to light and gravity (Sureshbabu et al., 2016). *Pantoea agglomerans* MVC 21 and *Ps. putida* MVC 17 were endowed by all these plant-growth promoting activities and were able to positively stimulate tomato plant growth in greenhouse conditions. However, further investigation will be carried out to better elucidate the mechanisms of actions that are implemented by these two PGPR during their interaction with tomato plants.

Overall, most of the methods used in this screening procedure are cheap and fast, two important factors that need to be taken into consideration for the development of PGPR at industrial scale. Moreover, this screening procedure is provided with steps dedicated to the evaluation of traits related to the product development that are not frequently used in published articles dedicated to the identification and characterization of PGPR. This complete screening strategy might contribute to the selection of new candidates endowed with traits that might encounter the interest of private companies involved in the development of PBs. Indeed, this strategy led to the selection of *P. agglomerans* MVC 21 and *Ps. putida* MVC 17, which represent good candidates for future steps aimed at designing a novel formulation with these bacterial strains.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126672.

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