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REGULAR ARTICLE

Plant-assisted selection: a promising alternative for in vivo identification of wheat (*Triticum turgidum L. subsp. Durum*) growth promoting bacteria

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Abstract
Background and aims In this work we present the development of an easy and feasible in vivo alternative to identify promising Plant Growth Promoting Bacteria (PGPB), using wheat -as a model plant- growing under variable soil and climate conditions.
Methods The identification of promising strains was carried out by Plant-Assistant Selection (PAS) (compared with the conventional PGPB selection, named in this work as Metabolic Traits Selection or MTS). We validated the ability of the obtained strains by PAS to promote wheat growth, by analyzing biometric and nutrimental parameters, as well as the relative expressions of *NRT1.4*, *GluTR,* and *6-SFT1* genes.
Results Twenty strains were obtained by PAS (170 bacterial strains were originally co-inoculated to plants), of which, twelve strains showed the ability to promote wheat growth mainly by the stem development and the number of leaves. Moreover, thirteen strains up-regulated the *6-SFT1* gene, and three strains up-regulated the *GluTR* gen. Thus, the strains *Enterobacter cloacae* TS3, *Microbacterium foliorum* TS9, *Bacillus cereus* TS10, *Paenibacillus lautus* TE8, and *Paenibacillus lautus* TE10 were identified as promising PGPB, showing strong wheat growth promotion events compared with those strains obtained by MTS.
Conclusions PAS is an easy and feasible alternative for identification of PGPB. However, ecological and economic factors need to be investigated to use the obtained strains by PAS for commercial microbial inoculants formulations.

Keywords Microbial inoculants · Climate conditions · PGPB · Gene expression, endophytes

Abbreviations

16S rRNA	16S ribosomal RNA
18S rRNA	18S ribosomal RNA
6-SFT1	Suc:fructan 6-fructosyltransferase

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CFU	Colony forming units
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GluTR	Glutamyl-tRNA reductase 1
MTS	Metabolic Traits Selection
NRT1.4	Nitrate transporter 1.4
PAS	Plant-Assistant Selection
PGPB	Plant Growth Promoting Bacteria

Introduction

Projections of rapid growth in global human population states that by 2050 food demand will double compared to its current status, where 98% is supplied by the agricultural sector (Rao 2013; Tilman et al. 2002). This global food demand has led to an evolution of crop production systems towards the use of intensive agricultural practices (increased applications of synthetic fertilizers and pesticides), in order to find a solution to combat abiotic and biotic stresses, as well as to improve the nutrients uptake by plants for maintaining or increasing their yield and quality (Camelo et al. 2011; Kibblewhite et al. 2008). However, in the past 40 years, the use of nitrogen (N) in the agriculture has increased by 7.4 times, however, yield has increased only 2.4 times in the same period of time, indicating that crops have reduced their ability to use N efficiently (Hirel et al. 2011), which increase the economic and environmental (eutrophication, greenhouse gas emissions, desertification, and loss of microbial diversity) cost by food production (Moss et al. 2010; Sharip et al. 2012; Velten et al. 2015).

To mitigate these negative impacts of intensive agricultural practices in agro-systems, one of the used technologies is the application of microbial inoculants, which are eco-friendly and sustainable bio-products containing microorganisms, mainly bacteria [named Plant Growth Promoting Bacteria (PGPB)], with the ability to promote plant growth and health, and restore soil fertility (Canfora et al. 2016; Hassan and Bano 2015). These bio-products have been developed based on the ability of plants to interact with complex microbiomes, i.e. plants interact with ~ 1×10^9 microbial cells g^{-1} dry soil and 1×10^5 microbial species g^{-1} dry soil, which have the ability to regulate the growth, abiotic and biotic stress tolerance, nutrition, and to antagonize phyto-pathogens in the host (Dohrmann et al. 2013; Grover et al. 2011). The integration of PGPB to agricultural practices represents a promising sustainable alternative to address the food security issue, due to the fact that microorganisms sustain a very important ecosystemic services, such as: i) social and ecological sustainability, ii) adaptation and mitigation of climate change, iii) biotechnological resource for humanity, iv) water and nutrients cycling, and v) increase the food production (Compant et al. 2005; Hayat et al. 2010; Van Der Heijden et al. 2008). For these reasons, the design and commercialization of microbial inoculants has increased more importantly; at present, the international market for these bio-products has been valued at over US $1.72 billion in 2014. It is also expected to reach US $4.17 billion by 2023, with an annual growth rate of 9.9%, between 2015 and 2023 (Timmusk et al. 2017). However, the widespread commercial use and success of microbial inoculants requires a number of issues addressed in the field, such as: the microbial establishment, plant and soil colonization by inoculated strains, and microbial biosynthesis of active metabolites involved in the plant growth regulation, under specific conditions of soil, climate, agricultural practices, and plant genotypes (Bhattacharyya and Jha 2012; de Souza et al. 2015; Timmusk et al. 2017).

Wheat (*Triticum turgidum* L.) is one of the most important staple crops around the world, due to its high content of gluten (80%), essential amino acids, vitamins, minerals, fiber, and other phytochemical, therefore, it is necessary to enhance and assure its production (Arzani and Ashraf 2017; Brouns et al. 2013; Shewry and Hey 2015). Globally, in 2017, 754 million tons of wheat were produced in 220 million hectares, which fed over 1 billion people in developing countries (FAO 2018). Mexico contributes, to the global wheat demand, with 3.3 million tons, where the Yaqui Valley -located at the Southern end of the Sonora State-, is the most important agricultural region for wheat production in this country, contributing with ~50% (1.7×10^6 tons $year^{-1}$) of the national production (CIMMYT 2018; SIAP 2018). The Yaqui Valley, the nucleus of the Green Revolution, has conducted successful researches to the development of more efficient genotypes and agricultural practices for global wheat production (Reynolds and Borlaug 2006). However, these intensive agricultural practices in the region [over ploughing and high fertilization doses (250 kg N ha^{-1}, and 100 kg monoammonium phosphate ha^{-1})], combined with climatic (semi-arid) conditions probably have negatively impacted soil fertility, causing

low organic matter content (<1%), saline (> 4 dS m^{-1}), and alkaline (pH 8.1) soils (Alvarado et al. 2014; Lares-orozco et al. 2016). This soil disturbance modifies the structure, and diversity of native soil microorganisms in agro-systems, and consequently their potential for food production (Ambrosini et al. 2016; Brahmaprakash and Sahu 2012; Zhen et al. 2014). In order to mitigate these agro-system disturbances, microbial inoculants have been sporadically applied to wheat in the Yaqui Valley due to those have showed low efficiency in the field, which could be explained by the origin and source of the used strains, because all these bio-products are imported from region or countries having completely different soil and climate conditions, as well as agricultural practices compared with those observed in the Yaqui Valley. In addition, the selection of PGPB (for those microbial inoculants formulations) is carried out by traditional in vitro microbial metabolic traits [named in this work as Metabolic Traits Selection (MTS)], i.e. firstly, PGPB are selected by their ability to produce widely studied metabolites and/or pathways involved in plant growth promotion, such as: phyto-hormones, solubilization of phosphates, production of ACC-deaminase, siderophore production, lytic enzymes activities, biological nitrogen fixation, lipopeptides biosynthesis, and antibiotic production (Barra et al. 2016; Berendsen et al. 2012; Mahmood et al. 2014; Trabelsi and Mhamdi 2013; Vejan et al. 2016). Then, the selected PGPB are evaluated in vitro and in greenhouse assays, and finally, the promissory ones are evaluated and validated in the field, generating microbial inoculants used successfully in crops production in fertile soil, under un-variable climatic conditions (Timmusk et al. 2017). However, a large number of these strains do not show the positive expected effect in degraded agro-systems (such as the Yaqui Valley), due to their inability to i) colonize plant tissues, and/or ii) bio-synthesize metabolites involved in plant growth regulation; both due to the plant genotype (exudates production, such as polysaccharides, mucilage, proteins, vitamins, and organic acids), type of soil, climate conditions, and agricultural practices (Berendsen et al. 2012; Lugtenberg and Kamilova 2009; Philippot et al. 2013). Another limitation of selecting strains by MTS is that there is a large number of novel and still poorly understood microbial mechanisms involved in plant growth promotion, such as: nitric oxide production, jasmonic acid signaling, production of bacteriocins and polyamides, and quorum sensing molecules (Ilangumaran and Smith 2017; Saraf et al. 2014; Vejan et al. 2016; Zhou et al. 2017), or even undiscovered, limiting the use of the large diversity and function of bacteria.

The aim of this work was to develop and validate an easy and feasible in vivo alternative to identify promising PGPB, named Plant-Assisted Selection (PAS). It was based on the microbial community modulation by plant species and specific soil/climatic conditions in agro-systems. Wheat growing under Yaqui Valley edapho-climatic conditions was used as a model system. The identification of promising PGPB was validated for their ability to promote wheat growth, analyzing biometric parameters, plant nitrogen content, as well as the relative expressions of genes involved in nitrate transport (*NRT1.4*), chlorophyll synthesis (*GluTR*), and water-soluble carbohydrate content (*6-SFT1*).

Materials and methods

Studied bacterial strains

A total of 170 uncharacterized bacterial strains for Plant-Assisted Selection (PAS), and five *Bacillus* strains identified as PGPB by Metabolic Traits Selection (MTS) were studied to compared both strategies for the identification of promising PGPB (PAS vs. MTS) (Fig. 1). All these strains were isolated from wheat crop rhizosphere in commercial fields located in the Yaqui Valley, and preserved in Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA, www.itson.edu.mx/COLMENA) (de los Santos-Villalobos et al. 2018).

Soil sample

Soil used in this work was a composite sample obtained from 6 individual samples collected from wheat commercial fields, located in the Yaqui Valley (27°35′53.14″ N and 110°2′53.26″W), under conventional agricultural practices, for at least 40 years. Then, soil samples were transferred to paper bags to dry (60° C), and then physicochemical and nutrient analyses were carried out according to NOM-021-RECNAT-2000 (DOF 2000).

Phase I: Pre-identification of promising bacterial strains by plant-assisted selection (PAS)

Wheat (*Triticum turgidum L. subsp. Durum*) seeds Var. CIRNO C2008 -the most widely wheat variety used in

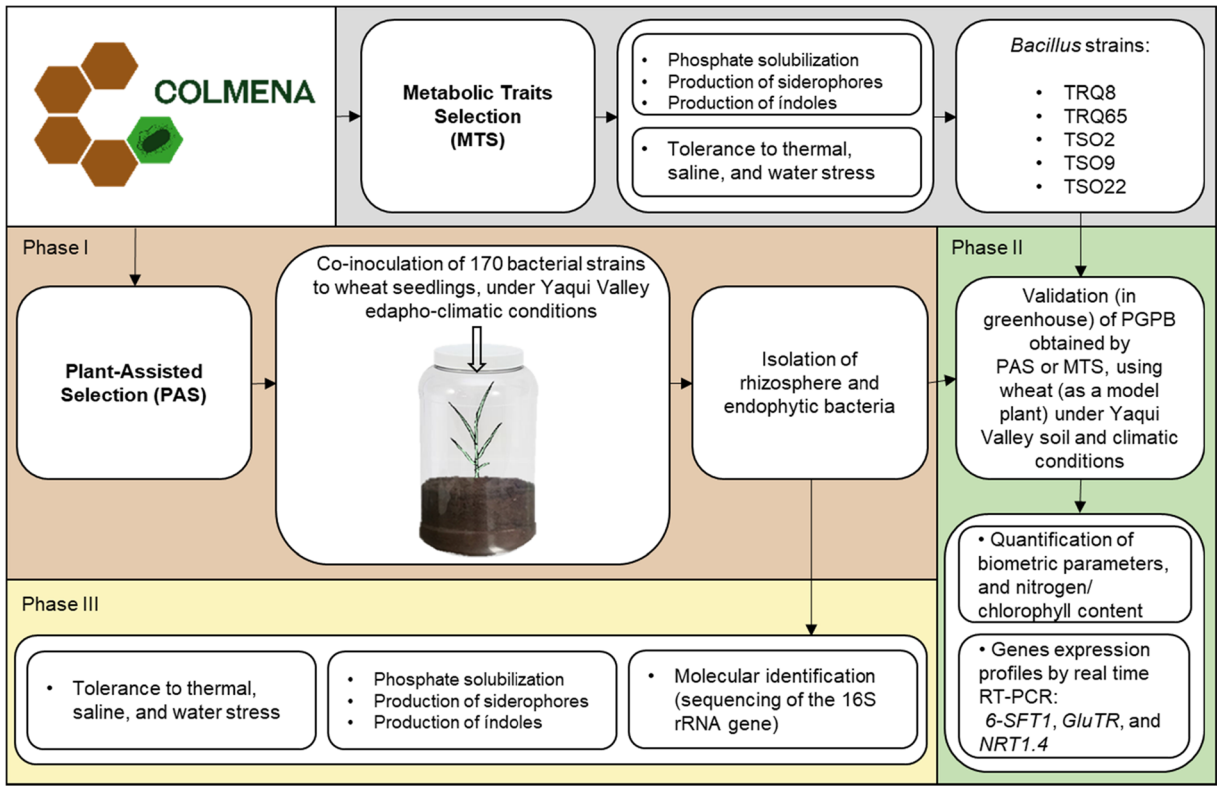

Fig. 1 Experimental design used in this work in order to compared the efficiency of PGPB identification by Plant-Assisted Selection (PAS) vs. Metabolic Traits Selection (MTS)

the Yaqui Valley-, were disinfected by the following protocol: 30 wheat seeds were washed thrice with sterile (121° C and 15 psi for 15 min) distilled water, then soaked in 70% (vol/vol) ethanol for one minute, followed by a wash with 3% (vol/vol) sodium hypochlorite for 10 min, and finally five washes with sterile distilled water (Groppa et al. 2007; Wang et al. 2014). Disinfected seeds were germinated on Petri Dishes containing agar-agar (8 g L^{-1}), for 5 days at 28 °C. Then, each germinated seedlings were placed into sterilized hermetic containers (washed with 70% vol/vol ethanol, and UV light treatment for 1.5 h), which contained 1.5 kg sterile soil (autoclaved for five consecutive days at 121° C and 15 psi pressure, for 1 h). Those disinfected seedlings growing in sterilized hermetic containers were co-inoculated with 4×10^7 Colony Forming Units (CFU) $plant^{-1}$ of each 170 bacterial strains (un-inoculated disinfected seedlings were used as the control treatment), for a total bacterial population of 8×10^9 CFU $plant^{-1}$ or 5×10^6 CFU g^{-1} dry soil [1.0×10^3 CFU of each 170 bacterial strains was inoculated separately into 30 mL of sterile nutrient broth contained in a falcon tube (50 mL), and incubated at 28 °C for 2 days at 5 *g* (using a rotary shaker). After the incubation period, each bacterial suspension was centrifuged at 3600 *g* for 10 min, and the pellet was washed twice and re-suspended in sterile distilled water, and the optical density (630 nm) of each bacteria was adjusted to 0.5 (~10^8 CFU mL^{-1})]. In addition, PAS was conducted under climatic conditions registered in the Yaqui Valley; thus, a growth chamber (BJPX-A450, BIOBASE) with the following parameters was used, 13 h of darkness at 14 °C, 2 h of light at 18 °C, 7 h of light at 25 °C, and 2 h of light at 18 °C, based on historical records (for the last 3 yrs) of the REMAS weather station (27°22′12.28“N and 109°55’51.71”O) located at the Yaqui Valley.

Isolation of rhizosphere and endophytic bacteria from PAS, phase I

The isolation of wheat rhizosphere and endophytic bacteria was performed 30 days post-inoculation [GS 13 growth stage (Zadoks et al. 1974)], by serial dilutions (1:10) method (for the isolation of endophytic strains

wheat plants were disinfected as described previously). Thus, 10 g of rhizosphere soil or 1 g of disinfected plant tissue were macerated and homogenized, during 1 h at 5 *g* (using a rotary shaker), with 90 mL or 9 mL of sterile distilled water, respectively. Later, serial dilutions were prepared up to 10^{-3}, and 1 mL of these dilutions was spread, by triplicate, onto the surface of Petri-dishes containing nutrient agar as culture medium, and incubated for 2 days, at 28 °C. Then, CFU and macroscopic and microscopic analysis of bacterial isolates were carried out, in order to estimate the total bacterial population and diversity (Rojas-Solís et al. 2018).

Phase II: Validation of PGPB obtained by PAS or MTS, in a greenhouse

The wheat growth promotion by bacterial strains obtained by PAS, rhizosphere (11) and endophytic (9) strains, as well as by MTS (5) was evaluated in a greenhouse assay. Thus, 400 wheat seeds Var. CIRNO C2008 were germinated on Petri Dishes containing agar-agar (8 g L^{-1}); 7 d post-germination, individual wheat seedlings were transplanted into pots containing 1.5 kg of solarized soil (15 days at ~30 °C) previously collected and characterized (Phase I). Then, 3 d post-transplantation, treatments having 15 wheat plants were individually inoculated with 5×10^8 CFU of each 25 bacterial strains (the negative control was sprayed with sterile distilled water). This assay was carried out under climatic condition observed in the Yaqui Valley, from December to February, 2017 (recommended period of time for wheat production in commercial fields; average temperature 17 °C, minimum temperature 7 °C, maximum temperature 29 °C, and relative humidity 60%) (Meisner et al. 1992; REMAS 2018). Sixty days post inoculation (35 days pre-anthesis), the chlorophyll (SPAD units), and plant biometric parameters, such as: shoot and root length, stem diameter, number of tillers, and dry weight of wheat plants, were measured. Leaves and stems were collected and dried (60° C) in order to quantify the total N content, using the HACH method by a DR 3900 spectrophotometer according to Alcántar and Sandoval (1999). In addition, wheat tissue (leaves and stems) of 9 plants per treatment were collected and frozen, using liquid nitrogen, and stored at -70 °C, during 8 weeks, until the quantification of gene expression profiles by Real Time RT-PCR.

RNA extraction of wheat plants and real time RT-PCR

Total wheat RNA was extracted by using Trizol™ reagent (Invitrogen) according to the manufacturer's instructions. The total RNA obtained was used as template for cDNA synthesis by PCR amplification using SensiFAST™ cDNA Kit (4000 ng total RNA, 4 μL 5x TransAmp Buffer, and DNase/RNase free-water up to 20 μL, at 10 min at 25 °C, 15 min at 42 °C, and 5 min at 85 °C). The expression profiles, by Real Time RT-PCR, of genes associated to plant growth promotion were evaluated, i.e. the *NRT1.4* (Nitrate transporter 1.4), the *GluTR* (Glutamyl-tRNA reductase 1), and the *6-SFT1* (Suc:fructan 6-fructosyltransferase) genes, involved in nitrate transport, chlorophyll synthesis, and water soluble carbohydrate (fructose and sucrose) synthesis, respectively (Guo et al. 2014; Stephenson et al. 2011; Xue et al. 2008).

The Real Time RT-PCR reactions contained, in a total volume of 20 μL, 2 μL cDNA (~1600 ng), 0.8 μL each forward and reverse oligonucleotide (10 μM), 10 μL SensiFAST SYBR 2X, and 7.4 μL molecular grade water. The gene expression profile was carried out using a Tprofessional Thermocycler (Analitikjena), under the following thermal cycling conditions: 3 min at 95 °C to activate the Taq Polymerase, 40 cycles of denaturation at 95 °C for 15 s, and 1 min for the oligonucleotides at the obtained temperature. Specificity of Real Time RT-PCR amplicons was conducted by a melting curve analysis with continual fluorescence data acquisition during the 60–90 °C melting event. The baseline and threshold cycle (Ct) were automatically determined using the qPCRsoft 3.1 program. The relative transcript abundance was calculated according to $2^{-\Delta\Delta Ct}$ method (Livak and Schimttgen 2001), and normalized to *18S rRNA* (18S ribosomal RNA) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) genes, as well as to the control (un-inoculated plants) treatment. The primer amplification efficiency and specificity were determined by amplification of cDNA dilution series (1:5) (obtained efficiency interval: 90–110%), and sequencing of amplicons by Sanger platform. The oligonucleotides design for real time RT-PCR was carried out using the program AlleleID 7.

Phase III: Molecular identification of PGPB

Genomic DNA from all PGPB obtained by PAS was extracted according to Raeder and Broda (1985). The

bacterial molecular identification was carried out amplifying the *16S rRNA* gene (16S ribosomal gene), using FD1 (5′- CCGAATTCGTCGACAACAGA GTTTGATCCTGGCTCAG -3′) and RD1 (5′- CCCGGGATCCAAGCTTAAGGAGGTGA TCCAGCC -3′) oligonucleotides (Weisburg et al. 1991). The used PCR protocol was: an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 40 s at 57 °C, and 2 min at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were verified by 2% agarose gel electrophoresis, and purified using ISOLATE II PCR and Gel Kit of Bioline, and then sequenced by Sanger platform. The obtained DNA sequences were edited and analyzed using the software FinchTV 1.4.0 by Geospiza, Seattle, WA; and BLAST (NCBI, www.ncbi.nlm.nih.gov), respectively. Then, those were aligned using CLC Sequence viewer 7, and MEGA 7.0 was used for the construction of a phylogenetic tree by the Neighbor-Joining method, the nucleotide sequence of the *16S rRNA* gene belongs to *Acidicapsa acidisoli* (NR_148580.1) was used as the out-group, and the stability of clades was assessed with 1000 bootstrap replications.

Metabolic characterization and abiotic stress tolerance of PGPB

The obtained strains by PAS or MTS were characterized focused on the most studied mechanisms of plant growth promotion, i.e. production of indoles (de los Santos Villalobos et al. 2013), phosphate solubilization (Onyia and Anyanwu 2013; Smith et al. 2016), and production of siderophores (Alexander 1991). In addition, the PGPB tolerance to abiotic stress was carried out as follow: 1×10^5 CFU of each strain was inoculated on Petri dishes containing nutrient agar as a culture medium, supplemented with i) Sodium Chloride (5%, 6.8 dS m^{-1}) for simulating saline stress, and ii) Polyethylene Glycol 6000 (10%, −0.84 mPa) for simulating hydric stress (both assays were incubated for 3 days at 28 °C), as well as using nutrient agar, and incubated at 43.5 °C for 3 days for simulating thermal stress. The control treatment was conducted inoculating 1×10^5 CFU of each strain on Petri dishes containing only nutritive agar and incubated at 28° C (Meléndez et al. 2017).

Statistical analysis

All data were expressed as mean of randomized block design experiments. Significant differences were analyzed by one-way analysis of variance (ANOVA) test and Tukey–Kramer test ($P < 0.05$), using Statgraphics Centurion XVI.II.

Results

Phase I: Pre-identification of promising bacterial strains by PAS

Plant-Assisted Selection (PAS) was carried out under completely sterile conditions, simulating soil and climatic conditions in the Yaqui Valley. Soil used in this work was collected from wheat commercial fields in the region, which showed typical physicochemical and nutrimental properties, where the low organic matter content (1.1%) helps explain the soil degradation observed in this region. In addition, the low content of the major nutrient and a slightly alkaline pH (Table 1) could be involved in regulating the microbial diversity and population in this agro-system.

In order to identify native bacterial strains with the ability to promote wheat growth under edapho-climatic conditions of the Yaqui Valley, one hundred seventy native bacterial strains were co-inoculated to wheat seedlings for PAS. Based on molecular methods, those belonged to 22 bacterial genera, being the most abundant *Bacillus* (42%), followed by *Pseudomonas* (10%), *Stenotrophomonas* (8%), and *Paenibacillus* (7%) (Table 2).

Thirty days post-bacterial co-inoculation to wheat seedlings (PAS), the root and stem length, and dry weight of plants were measured to quantify the effect of co-inoculated strains on biometric parameters involved on wheat growth promotion, as well as the modulation of the co-inoculated microbial diversity by the host was determined. Biometric parameters data, even when there were no significant differences between co-inoculated treatments vs. control (probably due to a short-term assay), showed a positive tendency of bacterial strains to promote wheat growth compared with un-inoculated treatment, i.e. the stem height, root length, and dry weight per plant in the co-inoculated treatment was higher than the control treatment, with an increment of 5%, 35% and 20%, respectively (Table 3).

Table 1 Physicochemical properties of soil collected from the Yaqui Valley, used for the identification of promising PGPB

Texture	pH	OM (%)	N (kg Ha^{-1})	P (kg Ha^{-1})	K (kg Ha^{-1})
Clay	7.9 ± 0.1	1.1 ± 0.2	47 ± 4.3	58 ± 3.7	2.5 ± 0.8

OM: Organic Matter, N: Total Nitrogen, P: Total Phosphorus, K: Exchangeable Potassium

In addition, the final bacterial population increased from 5×10^6 CFU g^{-1} dry soil (initial co-inoculated bacterial population) to 6.8×10^8 CFU g^{-1} dry soil and 4×10^2 CFU g^{-1} fresh plant. The morphological bacterial diversity was reduced from 170 strains (initially co-inoculated) to 11 rhizosphere and 9 endophytic strains. All these 20 bacterial strains obtained by PAS were studied in order to explore their ability to promote wheat growth in a greenhouse assay, measuring biometric parameters, plant nitrogen and chlorophyll content, and expression profiles of genes involved in wheat yield.

Table 2 Bacterial genera (based on the *16S rRNA* gene sequencing) used for the identification of PGPB by Plant-Assisted Selection (PAS)

Genera	Number of strains
Achromobacter	11
Acinetobacter	3
Arthrobacter	4
Bacillus	72
Brevundimonas	2
Cellulosimicrobium	1
Cupriavidus	3
Delftia	4
Enterobacter	7
Kosakonia	1
Lysinibacillus	1
Microbacterium	7
Ochrobactrum	1
Paenibacillus	12
Pseudomonas	18
Rhizobium	2
Rhodococcus	1
Serratia	1
Shinella	2
Staphylococcus	1
Stenotrophomonas	14
Streptomyces	2

Phase II: Validation of PGPB obtained by PAS or MTS, in a greenhouse

Twenty-five bacterial strains [11 rhizosphere and 9 endophyte strains (obtained by PAS), and 5 pre-selected strains according to their metabolic traits (MTS)] were evaluated as wheat growth promoting bacteria in a greenhouse assay, under soil and climatic conditions observed in the Yaqui Valley. Sixty days post-inoculation [GS 39 growth stage] biometric parameters were measured, observing that 14 of 25 bacterial strains showed a significant positive effects, at least, in one of plant biometric parameters evaluated, such as: number of tillers, stem diameter, and number of leaves (Table 4). The last two biometric parameters were the most representative effects on wheat plants caused by the inoculated strains, observing a positive correlation between stem diameter and number of leaves for all inoculated bacterial strains (Fig. 2). Thus, 11 strains (TS1, TS3, TS6, TS7, TS8, TS9, TS10, TE6, TE8, and TE10 identified by PAS; and TSO22 identified by MTS) were identified as wheat growth promoting bacteria, observing a higher number of leaves and stem diameter compared with the control treatment, suggesting that those strains could regulate mechanisms involved in carbohydrates biosynthesis and/or accumulation in wheat. In addition, even when there were no significant differences between inoculated treatments vs. control, several bacterial strains showed a tendency to increase the other biometric parameters evaluated, probably a long-term assay will show a stronger difference (Table 4).

Table 3 Wheat growth promotion by the co-inoculation of 170 native bacterial strains, under Plant-Assisted Selection (PAS)

Treatment	Stem Height (cm)	Root length (cm)	Plant dry weight (mg)
Un-inoculated (Control)	14.5 ± 3.4 a	2.8 ± 1.3 a	49.5 ± 12.0 a
Co-inoculated	15.3 ± 4.8 a	3.8 ± 1.3 a	59.4 ± 15.2 a

Means ($n = 30$) with the same letter are not significantly different, according to Turkey-Kramer test ($P = 0.05$)

The total N content per dry plant and chlorophyll content in inoculated plants did not show statistically significant differences compared with the control treatment; however, wheat plants inoculated with the strain TS10 showed a tendency of a higher value compared with un-inoculated plants, 64.1 μg N plant^{-1} vs. 53.7 μg N plant^{-1}, respectively. In addition, the chlorophyll content in plants inoculated with the strain TE5 or TE3 showed the same positive tendency, 58.7 SPAD Unit and 57.2 SPAD Unit vs. 43.1 SPAD Unit (un-inoculate treatment), respectively (Table 5).

Wheat gene expression profiles by bacterial strains obtained by PAS or MTS

The impact of the bacterial strains inoculation on the expression profile of genes involved in wheat growth promotion was studied. Thus, plant genes associated to *Nitrate transporter 1.4* (*NRT1.4*), *Glutamyl-tRNA reductase 1* (*GluTR*), and *Suc:fructan 6-fructosyltransferase* (*6-SFT1*) were quantified by Real Time RT-PCR, using *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH),* and *18S ribosomal RNA (18S)* genes as endogenous (Table 6). The sequencing of obtained amplicons by each oligonucleotide pairs showed a high identity and coverage percentages to target genes, > 95%.

As shown in Fig. 3, the relative quantification (RQ) of studied wheat genes normalized to *GAPDH* and *18S rRNA* genes and un-inoculated (control) treatment, showed that *6-SFT1* was the most strongly up-regulated gene (RQ from 2.2 to 6.3) by the inoculation of studied bacterial strains (72%), followed by *GluTR* (12% of bacterial strains showed RQ from 2.6 to 4.1), and *NRT1.4* (32% of bacterial strains showed a slightly down-regulation, RQ from 0.45 to 0.24). These findings showed that bacterial strains TS1, TS2, TS3, TS4, TS8, TS9, TS10, TS11, TE2, TE5, TE8, TE9, TE10, TRQ8, TRQ65, TSO2, TSO9, and TSO22 positively regulated the water-soluble carbohydrates synthesis (fructose and sucrose), and fructan concentration in the wheat plant stems (*6-SFT1* gene up-regulating), where all bacterial strains selected by MTS showed this effect. In addition, the chlorophyll biosynthesis gene (*GluTR*) was up-regulated only by the strains TS1, TS2, and TS3; however, the strains TS1 and TS3 showed significant differences in, at least, wheat stem diameter and number of leaves. The nitrate transporter (*NRT1.4*) in wheat leaves was slightly down-regulated by bacterial strains TS1, TS4, TS10, TE5, TE6, TE7, TE9, and TE10.

Phase III: Molecular identification, metabolic characterization, and abiotic stress tolerance of evaluated PGPB

Phylogenetic analysis based on the *16S rRNA* gene of 20 bacterial strains selected by PAS (Fig. 4), showed that nine bacterial genera were isolated by this strategy. Most bacterial genera were obtained from wheat rhizosphere (45%), while 22% were isolated as endophytes; in addition, 33% of the genera were found in both sources, i.e. *Microbacterium* (TS9), *Stenotrophomonas* (TS1, TS6, and TS7), *Achromobacter* (TS5), and *Pseudomonas* (TS2 and TS4) were isolated from the wheat rhizosphere, while *Staphylococcus* (TE2) and *Cellulosimicrobium* (TE6) were isolated as endophytic strains. In addition, *Bacillus* (TS8, TS10, TE3, TE4, and TE5), *Paenibacillus* (TS11, TE7, TE8 and TE10), and *Enterobacter* (TS3 and TE9) were isolated from both plant tissue and the rhizosphere.

The metabolic characterization of bacterial strains studied (Table 7), showed that 90% of those strains obtained by PAS showed the ability to solubilize inorganic phosphate, ranged from 6% to 80%, where 25% of strains showed a low level (< 20%), 40% a medium level (≥ 20% and < 40%), and 25% a high level of phosphate solubilization (≥ 40%). The bacterial strains identified by MTS were grouped at medium level (3 strains, TRQ8, TSO2, and TSO22) and high level of phosphate solubilization (1 strain, TSO9); in addition, the strain TRQ65 (identified by MTS) did not show the ability to solubilize this nutrient. The more efficient phosphate solubilizing strains obtained by PAS or MTS were TS4 (80.1%), and TSO9 (54%), respectively. Regarding siderophore production (based on the previous classification), 25% of strains obtained by PAS were siderophores producers, where 20% of strains showed a low production, and 5% of strains showed high production of siderophores. One strain (identified by MTS) showed the ability to produce siderophores, TRQ8 with a low production. The indole production by evaluated bacteria showed that 70% of strains produced this compound (>3 ppm) [60% showed low indole production (>3 ppm and < 10 ppm), 5% of strains showed a medium production (≥10 ppm and < 30 ppm), and 5% showed high production (≥30 ppm)]. In addition, 80% of strains obtained by MTS showed indole production [20% with a low level, 40% with a medium level, and 20% with a high

Table 4 Wheat growth promotion by bacterial strains obtained by Plant-Assisted Selection (PAS) or Metabolic Traits Selection (MTS), in a greenhouse assay

Strategy of PGPB identification	Bacterial strain	Stem height (cm)	Root length (cm)	Tiller number	Stem diameter (cm)	Leaves number	Stem dry weight (mg)	Root dry weight (mg)
	Un-inoculated	31.7 ± 7.1 ab	25.9 ± 1.7 ab	2.2 ± 1.0 a	4.3 ± 2.2 a	7.2 ± 2.5 a	2.0 ± 0.6 a	2.5 ± 0.8 a
PAS	TS1	33.8 ± 6.1 ab	25.8 ± 1.3 ab	3.5 ± 0.5 ab	9.2 ± 2.9 bc	12.0 ± 2.7 b	1.7 ± 0.5 a	2.2 ± 0.6 a
	TS2	36.7 ± 2.3 ab	28.1 ± 3.7 ab	3.2 ± 1.4 ab	8.1 ± 4.0 abc	11.5 ± 4.5 ab	2.0 ± 0.4 a	4.1 ± 0.6 a
	TS3	35.3 ± 2.5 ab	27.4 ± 7.3 ab	3.7 ± 0.7 b	9.7 ± 1.0 bc	13.9 ± 2.1 b	1.9 ± 0.3 a	2.4 ± 0.5 a
	TS4	33.5 ± 3.6 ab	29.3 ± 4.1 ab	2.9 ± 1.2 ab	7.5 ± 3.9 abc	11.1 ± 4.1 ab	1.7 ± 0.9 a	1.4 ± 0.6 a
	TS5	32.6 ± 4.9 ab	28.3 ± 5.7 ab	3.8 ± 0.7 b	7.3 ± 4.4 abc	10.5 ± 4.7 ab	2.6 ± 0.7 a	1.8 ± 0.6 a
	TS6	36.3 ± 1.5 ab	26.4 ± 2.5 ab	3.4 ± 0.7 ab	11.2 ± 4.2 c	13.1 ± 3.6 b	2.4 ± 0.4 a	2.5 ± 0.8 a
	TS7	34.5 ± 2.7 ab	28.5 ± 2.4 ab	2.9 ± 1.1 ab	9.8 ± 4.2 bc	12.1 ± 3.8 b	1.7 ± 0.4 a	1.5 ± 0.7 a
	TS8	28.8 ± 7.5 a	37.3 ± 7.9 b	2.8 ± 1.8 ab	9.3 ± 6.3 bc	11.6 ± 5.6 b	1.0 ± 0.3 a	1.0 ± 0.5 a
	TS9	28.9 ± 5.5 a	32.5 ± 1.8 ab	2.9 ± 1.3 ab	9.5 ± 5.1 bc	12.8 ± 5.0 b	2.6 ± 0.6 a	3.1 ± 0.3 a
	TS10	34.5 ± 1.7 ab	33.9 ± 3.6 ab	3.0 ± 1.5 ab	9.5 ± 4.8 bc	12.6 ± 5.5 b	2.7 ± 0.6 a	3.8 ± 0.6 a
	TS11	30.6 ± 5.1 ab	25.5 ± 3.0 ab	2.7 ± 1.2 ab	6.5 ± 3.1 abc	10.2 ± 4.2 ab	2.0 ± 0.3 a	1.7 ± 0.5 a
	TE2	29.9 ± 7.4 a	31.0 ± 5.5 ab	3.2 ± 1.5 ab	6.6 ± 3.5 abc	10.5 ± 4.7 ab	2.4 ± 0.7 a	2.5 ± 0.7 a
	TE3	33.5 ± 5.4 ab	25.7 ± 2.9 ab	3.3 ± 1.9 ab	7.4 ± 4.5 abc	10.8 ± 6.0 ab	2.2 ± 0.2 a	4.0 ± 0.8 a
	TE4	36.0 ± 5.8 ab	20.4 ± 8.3 ab	2.4 ± 0.7 ab	5.5 ± 2.7 abc	9.0 ± 2.3 ab	1.4 ± 0.7 a	1.2 ± 0.9 a
	TE5	30.5 ± 8.7 ab	20.5 ± 0.4 ab	2.3 ± 1.2 ab	6.9 ± 3.8 abc	12.0 ± 4.9 ab	0.5 ± 0.1 a	0.3 ± 0.1 a
	TE6	34.3 ± 5.0 ab	33.3 ± 11.5 ab	2.5 ± 0.8 ab	9.4 ± 1.6 bc	13.8 ± 1.9 b	1.8 ± 0.5 a	3.4 ± 1.0 a
	TE7	30.8 ± 8.0 ab	29.4 ± 11.1 ab	2.2 ± 1.0 a	7.3 ± 4.0 abc	12.3 ± 4.2 b	1.6 ± 0.5 a	2.6 ± 0.9 a
	TE8	31.8 ± 5.8 ab	19.3 ± 4.9 a	2.8 ± 0.7 ab	9.2 ± 2.3 bc	13.3 ± 1.6 b	1.7 ± 0.3 a	3.9 ± 1.1 a
	TE9	31.8 ± 6.7 ab	27.3 ± 4.3 ab	2.5 ± 1.0 ab	7.5 ± 2.6 abc	11.9 ± 3.5 ab	2.1 ± 0.5 a	3.6 ± 0.9 a
	TE10	36.7 ± 2.1 ab	24.4 ± 8.3 ab	2.6 ± 0.9 ab	8.8 ± 3.1 bc	13.3 ± 3.8 b	2.1 ± 0.3 a	4.2 ± 1.1 a
MTS	TRQ8	36.0 ± 1.6 ab	22.8 ± 3.2 ab	2.5 ± 0.8 ab	7.5 ± 3.5 abc	11.5 ± 4.3 ab	1.8 ± 0.7 a	2.4 ± 0.8 a
	TRQ65	38.3 ± 3.3 b	30.3 ± 8.0 ab	2.9 ± 0.3 ab	4.9 ± 1.6 ab	11.0 ± 2.9 ab	1.4 ± 0.8 a	2.8 ± 0.9 a
	TSO2	37.0 ± 2.8 ab	27.2 ± 0.7 ab	2.2 ± 0.8 ab	7.2 ± 2.5 abc	10.9 ± 3.1 ab	1.5 ± 0.4 a	3.9 ± 1.1 a
	TSO9	35.5 ± 1.2 ab	29.4 ± 2.8 ab	2.5 ± 1.0 ab	8.1 ± 3.4 abc	12.1 ± 4.7 b	1.7 ± 0.3 a	3.1 ± 0.8 a
	TSO22	37.6 ± 2.6 bcd	25.8 ± 7.4 ab	3.1 ± 1.0 ab	10.3 ± 3.5 bc	14.7 ± 3.3 b	1.8 ± 0.3 a	3.2 ± 0.6 a

The gray squares indicate statistically significant differences between inoculated vs. un-inoculated treatment. Means ($n = 15$) with the same letter are not significantly different, according to Turkey-Kramer test ($P = 0.05$)

level]. On the other hand, 50% of strains obtained by PAS were stress tolerant (saline, hydric, and thermal), 30% saline and hydric stress tolerant, 10% thermal and hydric tolerant, and 10% hydric stress tolerant. All five strains obtained by MTS were thermal, saline, and hydric stress tolerant, due to these traits were used as criteria for selecting bacterial strains to be used in this work.

Discussion

The key factor for the success of microbial inoculants in the field is the correct selection of PGPB for their formulations. This selection has been carried out using as a main criterion the presence of bacterial metabolic traits involved in plant growth promotion events.

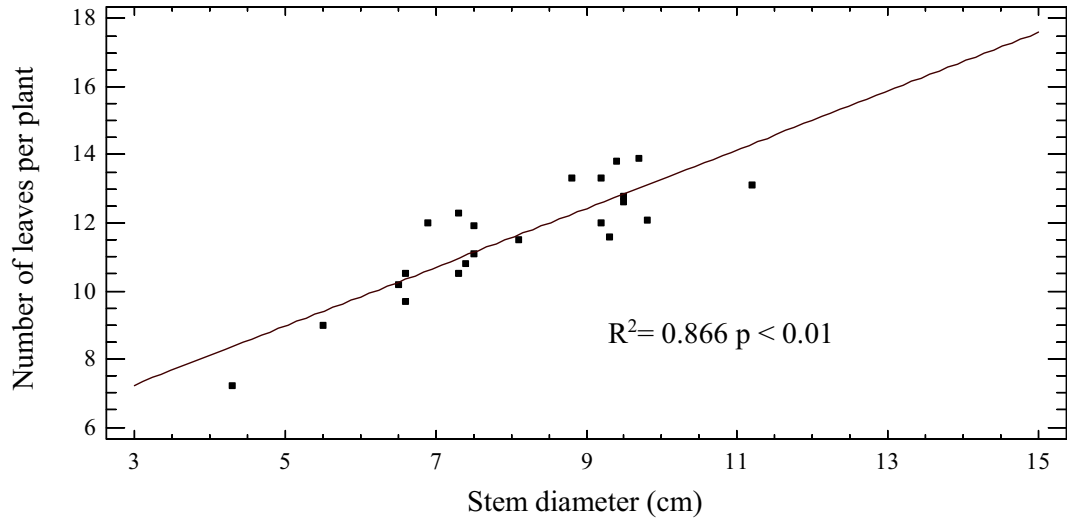

Fig. 2 Correlation between the stem diameter and the number of leaves in wheat plants, inoculated by each bacterial strain evaluated

Table 5 Total N per dry plant and chlorophyll units in inoculated wheat plants (sixty days post-inoculation under greenhouse conditions)

Strategy of PGPB identification	Bacterial strain	Total nitrogen per dry plant (μg plant^{-1})	Chlorophyll (SPAD unit)
	Un-inoculated	53.67 ± 15.1 a	43.1 ± 5.0 a
PAS	TS1	44.86 ± 4.2 a	43.1 ± 5.8 a
	TS2	52.55 ± 18.8 a	41.8 ± 1.6 a
	TS3	34.52 ± 5.0 a	42.0 ± 6.1 a
	TS4	36.85 ± 19.1 a	41.9 ± 4.0 a
	TS5	59.28 ± 15.4 a	42.6 ± 7.7 a
	TS6	57.83 ± 9.0 a	45.1 ± 3.9 a
	TS7	36.33 ± 9.2 a	45.8 ± 4.4 a
	TS8	29.43 ± 9.1 a	44.3 ± 7.2 a
	TS9	42.12 ± 9.9 a	41.8 ± 5.1 a
	TS10	64.12 ± 14.0 a	43.4 ± 3.4 a
	TS11	34.00 ± 5.3 a	42.7 ± 4.8 a
	TE2	32.37 ± 7.3 a	47.6 ± 9.7 a
	TE3	43.62 ± 11.7 a	57.2 ± 4.8 a
	TE4	37.54 ± 14.2 a	54.1 ± 2.2 a
	TE5	31.21 ± 4.0 a	58.7 ± 14.3 a
	TE6	39.96 ± 12.3 a	51.8 ± 16.7 a
	TE7	29.08 ± 9.9 a	39.8 ± 3.7 a
	TE8	52.12 ± 9.1 a	45.2 ± 10.7 a
	TE9	42.85 ± 17.0 a	47.8 ± 15.2 a
	TE10	36.02 ± 4.3 a	41.6 ± 5.1 a
MTS	TRQ8	31.83 ± 12.2 a	52.4 ± 10.1 a
	TRQ65	26.84 ± 11.5 a	42.0 ± 6.7 a
	TSO2	43.65 ± 13.8 a	46.2 ± 9.8 a
	TSO9	43.60 ± 7.1 a	50.5 ± 19.8 a
	TSO22	30.27 ± 17.1 a	47.8 ± 11.9 a

Means (n = 15) with the same letter are not significantly different, according to Turkey-Kramer test (P = 0.05)

Table 6 Nucleotide sequences and melting temperature of oligonucleotides amplifying studied genes

Gene	Gene associated to	Oligonucleotide	DNA Sequence	Tm	Reference
Nitrate transporter 1.4 (NRT1.4)	Nitrate transport	Forward	AGCA GCAAGGCGG-AGCAA	65	Guo et al. 2014
		Reverse	CATA CGGACGTACA TGGAAGC		
Glutamyl-tRNA reductase 1 (GluTR)	Chlorophyll synthesis	Forward	CCAG CCTGAATCAT ATTG	52	Stephenson et al. 2011
		Reverse	TCCA CTACTTCTCT AATACC		Designed in this work
Suc:fructan 6-fructosyltransferase (6-SFT1)	Water-soluble carbohydrate synthesis	Forward	GAGA TGGACTCAGC GCACAA	60	Xue et al. 2008
		Reverse	GCCT TCCTTGGTGA GCTTCTTT		
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Housekeeping gen	Forward	GTCC ATGCCATGAC TGCAA	60	Jarošová and Kundu 2010 with a modification in the first nucleotide.
		Reverse	CCAG TGCTGCTTGG AATGATG		Jarošová and Kundu 2010
18S ribosomal RNA (18S rRNA)		Forward	GTGA CGGGTGACG-GAGAATT	60	
		Reverse	GACA CTAATGCGCC CGGTAT		

However, the bacterial ability of establishment in the plant x soil interface is a determinant factor associated with the expected success of microbial inoculants for food production (de Souza et al. 2015). In agro-systems, the establishment ability of bacteria is attributed mainly to i) agricultural practices, ii) type of soil, iii) climate and biotic interactions, and iv) plant genotype (Philippot et al. 2013). Thus, an easy and feasible alternative was developed and validated in this work in order to identify bacterial strains with the ability of establishment in the soil x plant interface as well as to promote plant growth, named Plant-Assisted Selection (PAS) (Fig. 1), under variable abiotic conditions and using wheat as a model plant. In this work, we analyzed 170 bacterial strains isolated from wheat commercial fields in the Yaqui Valley, under conventional agricultural practices by, at least, 40 years (de los Santos Villalobos et al. 2018). It suggests that these strains have the ability to interact with the wheat crop, under intensive agricultural practices, and soil and climate conditions observed in this region.

First of all, PAS did not have a negative effect on wheat seedlings, even when a large number of bacterial strains were co-inoculated [170 strains, belonging to 22 bacterial genera (Table 2), with a final population of 8×10^9 CFU plant^{-1} or 5×10^6 CFU g^{-1} dry soil]. Although wheat growth promotion was not observed in phase I, the co-inoculation of bacterial strains showed a tendency to promote wheat development compared with the un-inoculated treatment (Table 3), which suggests that this co-inoculation to wheat seedlings (under a short-term assay and degraded soil conditions) demanded high amounts of wheat exudates and nutrients (carbohydrates, vitamins, phyto-hormones, etc.) (Weinberg et al. 2003) to increase the bacterial population until 6.8×10^8 CFU g^{-1} dry soil and 4×10^2 CFU g^{-1} fresh plant, which could not be supplied only by the low content of organic matter and major nutrients observed

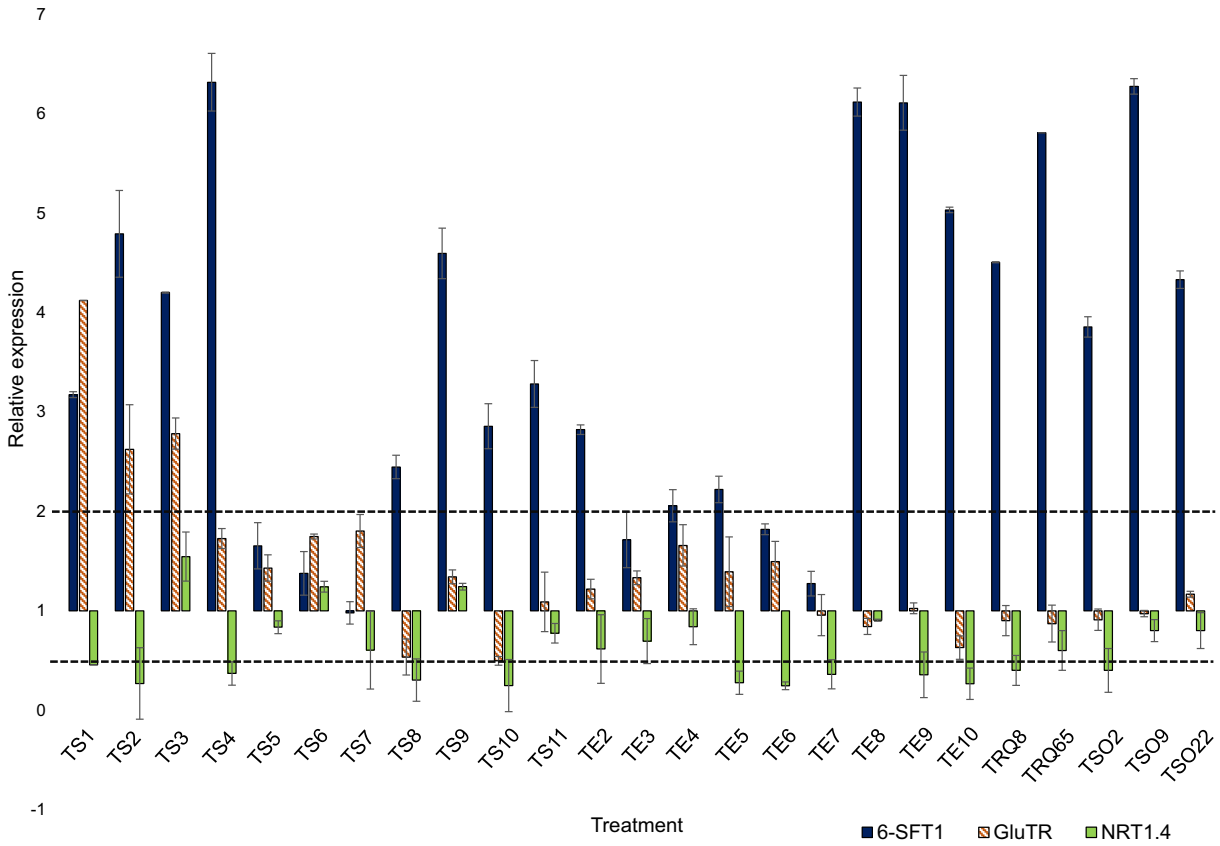

Fig. 3 Expression levels of *6-SFT1*, *GluTR*, and *NRT1.4* genes in wheat plants by inoculation of bacterial strains obtained by Plant-Assisted Selection (PAS) or Metabolic Traits Selection (MTS). Data are showed as relative expression of studied genes in treatments, normalized to GAPDH and 18S rRNA genes and the uninoculated (control) treatment. Error bars, representing standard deviations, are not visible if they are smaller than the symbol size. Horizontal dashed lines indicate the upper and lower limit of relative quantification (RQ) considered as up-regulated and down-regulated genes, RQ = 2.0 and RQ = 0.5, respectively

in the used soil (Table 1). Later, a total of 20 strains were isolated by PAS, 11 rhizospheric and 9 endophytes bacteria, even when all 170 co-inoculated strains were originally isolated from the wheat rhizosphere, which would suggests a complete establishment of the whole inoculated microbiota, however the environment conditions (soil, climate, plant phenology, etc.) under PAS modulated the co-inoculated microbial population and diversity, whose eco-systemic services remains difficult to measure and predict. On the other hand, the isolation of endophytes by PAS indicates that those strains have the ability colonize roots (original source of bacterial strains used in this work), and then plant tissues. This bacterial behavior is a promising PGPB trait, due to endophytes are able to communicate and interact with plants more efficiently than rhizosphere bacteria (Santoyo et al. 2016). According to the molecular identification (sequencing the *16S rRNA* gene) of bacterial strains identified by PAS (Fig. 4), 45% and 36% belong to the genus *Bacillus* and *Paenibacillus*, respectively, which are ubiquitous soil bacterial genera, founded as rhizosphere or epiphytes/ endophytes strains associated to several crops, including wheat, due to their ability to form endospores and produce antimicrobial substances that inhibit other microbial competitors (Cherif-silini et al. 2016; Dal Cortivo et al. 2017; Villarreal-Delgado et al. 2018).

Wheat x PGPB (obtained by PAS), under a greenhouse assay, showed that 10 bacterial strains showed the establishment ability, triggering growth promotion of wheat by regulating the stem diameter and leaf number (Table 4), observing a positive correlation between those parameters for all inoculated bacterial strains ($R2 = 0.866$, $p < 0.01$, Fig. 2). These biometric effects by inoculated bacterial strains are an interesting plant growth promotion mechanism, due to a

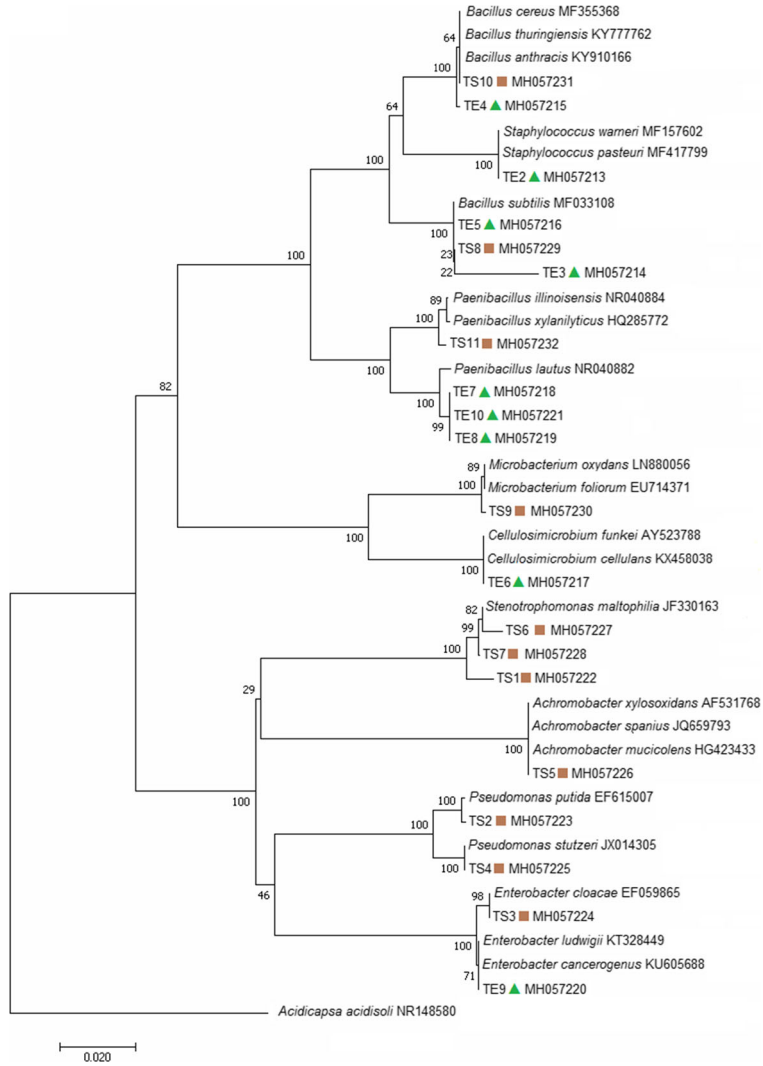

Fig. 4 Phylogenetic relationships of bacterial strains obtained by Plant-Assisted Selection (PAS) inferred by neighbor-joining analysis of *16S rRNA* sequences. *Acidicapsa acidisoli* (Accession No. NR148580) was used as the out-group. The numbers given over selected branches indicate the percentage of 1000 bootstrap re-sampled data sets supporting the clade to the right of the branch. Colors indicate the origin of isolates, brown square: rhizosphere, and green triangle: plant tissue

major number of leaves has been associated with higher plant photosynthetic efficiency, increasing the carbohydrates bio-synthesis (Chang et al. 2015), and larger stem diameter is associated with a higher content of reserve carbohydrates in plants (Xue et al. 2008), which are mobilized and converted into grains (Ruuska et al. 2006). On the other hand, only two of the five strains obtained by MTS showed wheat growth promotion in, at least, one parameter, such as: stem diameter and/or leaves numbers (Table 4). These effects in plants have been associated with the phyto-hormones production and nutrients solubilization by PGPB (de Souza et al. 2015). However, these results showed that bacterial strains obtained by PAS or MTS, even when their metabolites production associated to plant growth promotion have been characterized (Table 7), the beneficial effect in plants is uncertain. Thus, an additional approach was carried out in order to determine the wheat growth promotion by PAS or MTS strains, such as the quantification of the relative expression of genes *NRT1.4*, *GluTR*, and *6-SFT1* (Fig. 4). The *NRT1.4* gene expression was slightly down-regulated by 32% of bacterial strains evaluated [Relative Quantification (RQ) from 0.45 to 0.24] (Fig. 3), it could explains the observed low level of total N content in wheat plants (Table 5), due to this gene is expressed predominantly in the leaf petiole, and involved in petiole NO^{-3} accumulation and remobilization (Chiu et al. 2004). The deficiency or down-regulation of this gene result in significant changes of NO^{-3} content in leaf petiole and the lamina, which can alter leaf development (Hu et al. 2014). Additionally,

Table 7 Biochemical and stress tolerance traits of studied bacterial strains

Strategy of PGPB identification	Strain	Phosphate solubilization (%)	Siderophore production (%)	Indoles production (ppm)	Stress condition		
					Thermal	Saline	Hydric
PAS	TS1	54.1 ± 2.1	0.0 ± 0.0	2.2 ± 0.7	–	+	+
	TS2	9.9 ± 1.4	4.3 ± 0.5	2.2 ± 0.5	+	+	+
	TS3	8.0 ± 1.0	4.3 ± 0.2	67.6 ± 1.2	–	+	+
	TS4	80.1 ± 2.2	49.5 ± 1.3	0.8 ± 0.1	+	+	+
	TS5	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.4	–	+	+
	TS6	35.3 ± 0.8	0.0 ± 0.0	5.8 ± 0..3	–	–	+
	TS7	29.0 ± 1.3	0.0 ± 0.0	4.9 ± 0.4	–	–	+
	TS8	23.5 ± 0.7	0.0 ± 0.0	0.5 ± 0.2	+	–	+
	TS9	38.4 ± 1.9	0.0 ± 0.0	5.7 ± 0.8	–	+	+
	TS10	33.5 ± 0.6	0.0 ± 0.0	4.6 ± 0.6	+	+	+
	TS11	31.2 ± 0.4	0.0 ± 0.0	6.2 ± 0.5	–	+	+
	TE2	6.5 ± 0.1	0.0 ± 0.0	10.1 ± 1.0	–	+	+
	TE3	43.2 ± 1.7	0.0 ± 0.0	1.4 ± 0.1	+	+	+
	TE4	30.4 ± 0.4	0.0 ± 0.0	5.6 ± 0.2	+	+	+
	TE5	40.4 ± 1.3	0.0 ± 0.0	5.6 ± 0.1	+	+	+
	TE6	8.7 ± 0.3	4.3 ± 0.7	5.8 ± 0.4	+	+	+
	TE7	20.0 ± 0.6	11.2 ± 0.9	1.1 ± 0.1	+	–	+
	TE8	6.2 ± 0.9	0.0 ± 0.0	3.8 ± 0.1	+	+	+
	TE9	0.0 ± 0.1	0.0 ± 0.0	5.2 ± 0.1	+	+	+
	TE10	45.5 ± 2.0	0.0 ± 0.0	9.6 ± 0.5	+	+	+
MTS	TRQ8	38.0 ± 0.9	8.1 ± 0.8	12.0 ± 0.4	+	+	+
	TRQ65	0.0 ± 0.0	0.0 ± 0.0	28.8 ± 0.9	+	+	+
	TSO2	40.0 ± 1.2	0.0 ± 0.0	5.4 ± 0.1	+	+	+
	TSO9	54.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	+	+	+
	TSO22	36.0 ± 0.9	0.0 ± 0.0	56.3 ± 1.3	+	+	+

+positive

-negative

biometric and molecular parameters associated with the total N content were measured, in order to associate changes in the relative gene expression (the *GluTR* gene) with the chlorophyll content in wheat plants. The *GluTR* gene –associated to the Chlorophyll synthesis- was up-regulated only by three PGPB obtained by PAS (TS1, TS2, and TS3) (Fig. 3); however, even when none statistical differences were found in the chlorophyll content or total N content compared to un-inoculated wheat plants (control), two of these PGPB showed positive effects on wheat tiller number (TS3), stem diameter (TS1 and TS3), and leaf number (TS1 and TS3) (Tables 4 and 5). These findings showed that metabolic processes involved in N and chlorophyll accumulation and remobilization cannot be completely associated to wheat growth promotion mechanisms used by PGPB, because these processes are very changing due to N is a key macronutrient representing a limiting factor for plant growth and development, which regulates the chlorophyll biosynthesis (Curci 2017). Thus, the expression of the *6-SFT1* gene –associated to the water soluble carbohydrate (fructose and sucrose) synthesis- was studied in order to explain the high correlation ($R^2 = 0.866$, $p < 0.01$, Fig. 2) between stem diameter and the number of leaves in wheat plant inoculated by PAS or MTS bacteria.

The up-regulation of the *6-SFT1* gene has showed a positive correlation between fructan (water-soluble carbohydrates) content in stem and yield for wheat

(Xue et al. 2008). Sixty-five percent and 100 % of the PGPB obtained by PAS or MTS, respectively, were able to up-regulate the expression of the *6-SFT* gene. This result indicates that those strains influence positively the content of water-soluble carbohydrates in wheat, which can be used to promote its growth (Table 4) and grain filling (Fischer 2011). However, only six strains of those showed wheat growth promotion by regulating the stem diameter and number of leaves, which suggests that these strains are promising PGPB, i.e. TS3, TS9, TS10, TE8 and TE10 obtained by PAS, and TSO22 obtained by MTS. In some cases, the studied bacterial strains showed gene overexpression but it was not supported by biometric parameters, i.e. TS2, TS4, TS11, TE2, TE5, TE9, TRQ8, TRQ65, and TSO2, which could suggests that i) a long term assays is required to observe physiological changes in wheat plants, or ii) negative interactions between wheat x studied PGPB was observed, due to (under conditions of degraded soil) microorganisms compete with plants for nutrients and/or use plant reserve metabolites for their growth, until 40% of photosynthates (Asmelash et al. 2016). On the other hand, a few bacterial strains showed positive biometric parameters regulation, but not gene overexpression was detected, which could be consequence of the physiological traits regulation involves a lot of changes in the expression of several genes; thus, a more robust molecular analysis is crucial to associate physiological traits with changes in gene expression, such as plant transcriptome studies.

Regarding the metabolic characterization of obtained strains by PAS or MTS, 70 % and 100 % of strains, respectively, showed promising metabolic traits (at least, medium level of phosphate solubilization, siderophore production, or indoles production), of which 36% and 60% were not able to promote wheat growth, respectively, i.e. TS4, TS11, TE3, TE4, and TE5 (obtained by PAS), and TRQ8, TRQ65, and TSO2 (obtained by MTS) (Tables 4 and 7). These findings may be associated with ecological events during the plant x PGPB interaction, such as: the microbial establishment, plant and soil colonization by inoculated strains, and microbial biosynthesis of metabolites involved in the plant growth regulation, under variable soil and climate conditions (Bhattacharyya and Jha 2012). On the other hand, 15% of the strains obtained by PAS (TS5, TE6, and TE8) did not show promising metabolic traits, however, those caused wheat growth promotion in the greenhouse assay (Tables 4 and 7), which suggests that these three strains were able to promote growth in the host by others mechanisms of action not studied in this work. It means that, under conventional selection by MTS, those strains would not have been selected, even when their events in wheat were positives. In addition, several factors negatively affect the PGPB x plant interaction, such as abiotic stress conditions, i.e. temperature, pH, and water content, which inhibit the establishment and colonization of promising bacterial strains (Santoyo et al. 2016). In these terms, all strains selected by PAS showed a high tolerance to abiotic (thermal, hydric, or saline) stresses, at least in one of those conditions, this finding is supported by their origin or source of isolation, i.e. the Yaqui Valley [(saline (> 4 dS m-1), and alkaline (pH 8.1) soils (Alvarado et al. 2014; Lares-orozco et al. 2016)].

Conclusion

Sixty percent of bacteria (12 strains) obtained by PAS (20 strains from 170 strains initially co-inoculated to wheat plants), showed the ability to promote wheat growth mainly by the stem development and the number of leaves. In addition, thirteen strains up-regulated the *6-SFT1* gene, and three strains up-regulated the *GluTR* gen. Thus, the strains *Enterobacter cloacae* TS3, *Microbacterium foliorum* TS9, *Bacillus cereus* TS10, *Paenibacillus lautus* TE8, and *Paenibacillus lautus* TE10 were identified as promising PGPB, which showed strong wheat growth promotion events (biometric, metabolic, and molecular traits) compared with those strains obtained by MTS.

Plant-Assisted Selection (PAS) is an easy and feasible strategy for the identification of wheat growth promoting bacteria, under specific soil and climate conditions. This strategy could be used under different abiotic and biotic conditions, even changing the plant host. Finally, the strains obtained by PAS need a number of issues to be addressed in order to be used as active ingredients for microbial inoculant formulations, such as: a comprehensive evaluation of their human health risks, and the effectiveness in the field, determining (among others) their establishment in soil, plant colonization, biosynthesis of active metabolites involved in plant growth promotion, and feasible/economic industrial production.

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