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ORIGINAL ARTICLE

Infection with *Micromonospora* strain SB3 promotes in vitro growth of *Lolium multiflorum* plantlets

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Abstract
Cattle breeding is an important economical activity in Argentina, highly dependent on grass production. In the last decades, grasslands zones were reduced and confined to less productive lands due to the advance of agronomical cultures. Therefore, it is important to develop new strategies to improve forage production. New eco-friendly trends in plant growth promotion include the use of microbial endophytes, but the in vitro studies of plant-bioinoculant interactions is limited by the scarce current technological development. In this work, we use a micropropagation protocol for *Lolium multiflorum*, developed in a previous work, to study the effect of bacterization with actinobacterial endophytes, isolated from Argentine native grasses, on the growth of *L. multiflorum* in vitro plantlets. To achieve this objective, *L. multiflorum* plantlets were inoculated with three *Micromonospora* strains (SB3, TW2.1 and TW2.2). The results obtained showed that the effect of actinobacterial inoculation depends on the *Micromonospora* strain used. The inoculation with SB3 promoted plant growth, increasing plant biomass, root length and the rate of plantlets ready to be acclimatized after 4 weeks of in vitro culture. Strain TW2.1 did not show, statistically, differences compared to control treatments, while TW2.2 inhibited plant growth, decreasing plant biomass, root length and the rate of plants ready to acclimatize. Our results showed that *Micromonospora* strain SB3 could be a good candidate to use in breeding programs for *L. multiflorum* and other grasses to increase their yield.

Keywords Actinobacteria · Bacterization · *Lolium multiflorum* · Grass · Micropropagation

Introduction

The Argentine grasslands comprise approximately 160 million ha and are essential to the national economy. These grasslands are spread throughout a large variety of climates, types of soil and vegetation. The majority of cattle breeding in Argentina is carried out on these grasslands, and this practice, compared to intensive breeding (feedlots), has minor ecological impact and less dependent on fossil energy. In the last years, the expansion of the agricultural frontier and the "salinization" of arid and semi-arid zones have resulted

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in a decrease of grasslands and their displacement to less productive non-conventional areas, affecting agricultural yield. This displacement is a worldwide phenomenon that not only affects Argentina. The growing human population also increases food demand; thus, it is fundamental to establish breeding programs that maintain both the productivity and biodiversity of the natural grasslands. Traditionally, pastures from Argentine Pampa and Patagonia have been enhanced by the introduction of improved grasses, such as *Lolium multiflorum* Lam (Soriano 1992). *L. multiflorum*, or Italian ryegrass, is an annual grass considered as one of the most important temperate forage grasses in the world, being a high quality and profitable option to produce forage during the winter and spring (Wang et al. 2016a). Usually, plants have been bred altering their genetic information and selecting those with better agronomical traits such as growth, nutritional quality, pathogen immunity, or stress tolerance (Wei and Jousset 2017).In recent years, plants are starting to be considered as an holobiont: an ecological and evolutionary unit composed by both the host and its associated microbiome (Vandenkoornhuyse et al. 2015). Billions of microorganisms, bacteria and fungi, colonize the inside and outside of the plant tissues and organs (Berendsen et al. 2012). This microbiota plays a fundamental role in plant growth and plant physiology and many, such as endophytes, can affect the agronomical traits of their host (Wei and Jousset 2017). Endophytes are microorganisms that colonize healthy plant tissue inter and/or intracellularly, persisting for the whole or part of the life cycle of the plant without causing disease symptoms (Rodriguez et al. 2009; Wilson 1995). In the last years, the microbiota, especially endophytes, have been increasingly taken into account for their hosts´ breeding (Gopal and Gupta 2016; Sessitsch and Mitter 2015). In this sense, the study of the microbiota associated to native grasses can pave the way towards the isolation of numerous endophytes that have a potential role in the breeding of these and other grasses, such as *L. multiflorum. Bromus auleticus* Trin. is a native grass considered as one of the most valuable grasses of the Southern cone due its excellent agronomical traits: high productivity, palatability, protein abundance, persistence in the field and resistance to drought (Bustamante et al. 2012; Gasser et al. 2005). Recently, our research group studied the microbiota associated with *B. auleticus* roots (Della Mónica et al. 2017). That work focused on the study of endophytic bacteria belonging to Phylum Actinobacteria and the result was the isolation of various strains of the genus *Micromonospora*. The genus *Micromonospora* has recently been of great interest because of its interaction with nitrogen fixing nodules (Trujillo et al. 2015). *Micromonospora* strains have been isolated from nitrogen fixing root nodules of different leguminous and actinorhizal plants (Carro et al. 2012, 2016; Martínez-Hidalgo et al. 2014; García et al. 2010; Trujillo et al. 2006, 2007, 2010; Valdés et al. 2005). Furthermore, some strains were isolated from non-nodulating plants such as rice (Thawai et al. 2016; Thanaboripat et al. 2015; Kittiwongwattana et al. 2015), *Lycium chinense* (Zhao et al. 2016), *Terminalia mucronata* (Kaewkla et al. 2017) and *Parathelypteris beddomei* (Zhao et al. 2017). There are few studies concerning plant growth promotion produced by *Micromonospora* strains, and most were performed in leguminous and actinorhizal plants such as: *Medicago sativa* (Martínez-Hidalgo et al. 2014; Solans et al. 2009), *Trifolium* sp. (Trujillo et al. 2014), *Discaria trinervis* (Solans 2007) and *Ochetoplila trinervs* (Solans et al. 2011). In general, *Micromonospora* sp. promoted plant growth when they were co-inoculated with nodule-forming microorganisms (Trujillo et al. 2014). Nitrogen fixing nodules are not formed in *L. multiflorum* roots or in other grasses; therefore, the effect of *Micromonospora* sp. can only be studied individually without co-inoculation with nodule-forming microorganisms. The recent development of a successful micropropagation protocol for *L. multiflorum* (Regalado et al. 2017) allows the in vitro inoculation of *Micromonospora* sp. in *L. multiflorum* plants and the study of the microbial effect on plant growth.

For many years, it was considered that in vitro cultures should be maintained in complete sterile conditions (Orlikowska et al. 2017), and the presence of microorganisms was hidden in the published manuscripts since it smeared a tissue culture laboratory´s reputation (Orlikowska et al. 2017). However, recently, the growing interest in endophytic microorganisms has also reached in vitro culture. The in vitro microbial inoculation allows the study of the benefits produced by endophytes without depending on environmental conditions. The reports on in vitro bacterization have multiplied in the last years (Quambusch et al. 2016; Larraburu and Llorente 2015; Parray et al. 2015; Bashan et al. 2014; Thomas et al. 2010; Bashan 1998), and the same has occurred for studies where endophytic fungi were inoculated (Wang et al. 2016b; Verma et al. 2015; Prasad et al. 2013; Thomas et al. 2010).

The aim of this work is to study the effects of three *Micromonospora* strains (TW2.1, TW2.2 and SB3) on the growth of in vitro plants of *L. multiflorum* and their potential use as biofertilizers.

Materials and methods

Plant material

In vitro plantlets of *L. multiflorum* were used in this work. The plantlets were obtained from seeds of *L. multiflorum* (Ribeye cultivar) following the protocol developed by our research group (Regalado et al. 2017). The seeds were harvested from a field in INTA-Concepción del Uruguay

Agronomic Experimental Station, Entre Rios province, Argentina. The plants were multiplied in vitro (Regalado et al. 2017) and 120 plants were used for infection with different *Micromonospora* strains.

Identification of Micromonospora strains

Three endophytic *Micromonospora* strains, named TW2.1, TW2.2 and SB3 were evaluated in this work. Briefly, these strains were isolated from surface sterilized (6 min in 6% NaOCl, washed with sterile distilled water, and 4 min in 70% ethanol) healthy roots of *B. auleticus*. The plants were collected in the field growing on a vertisol soil at INTA-Concepción del Uruguay Agronomic Experimental Station, Entre Ríos province, Argentina. Surface sterilized roots were cut into 5 mm fragments and cultured in three different isolation media (SB medium: starch 15 g, yeast extract 4 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, distilled water 1 l, agar 18 g; TWYE medium: yeast extract 0.25 g, K_2HPO_4 0.5 g, tap water 1 l, agar 18 g; GE medium: 0.5% glycerol, yeast extract 2 g, K_2HPO_4 1 g, distilled water 1 l, agar 18 g at 29 °C for 4 weeks. When endophytic bacteria growth was observed, the colonies were placed in fresh International Streptomyces Project 2 medium (ISP2) (Shirling and Gottlieb 1966).

Molecular and morphological identification was done following the protocol described in Solans et al. (2016). DNA was extracted from fresh liquid cultures (4 weeks, 29 °C and 200 rpm incubation in dark) and the region from positions 27 to 1492 of the 16S rRNA gene was amplified with 27f and 1518r. The PCR products were submitted to Macrogen Inc Seoul (Korea) (http://www.macrogen.com) for purification and sequencing of both strands. Both sequences of each isolate were assembled with Vector NTI 10® Software and the consensus sequence was obtained. The consensus sequences of each strain were used for identification by comparison with DNA sequences in the GenBank database using the basic local alignment search tool (BLAST). In addition, a pairwise comparison analysis was done among the isolates with Mega2 program (Kumar et al. 2001).

IAA production by Micromonospora strains

Production of IAA was quantified spectrophotometrically (Glickmann and Dessaux 1995). Erlenmeyers (125 ml), three Erlenmeyers for each Micromonospora strain, with 20 ml of nutritive broth supplemented with 0.2% of tryptophan were inoculated with *Micromonospora* strains and incubated in shaker (120 rpm) at 30 °C in dark for 4 weeks (Khamna et al. 2009). After incubation, cultures were centrifugated at 7000×*g* for 15 min, and supernatants collected for further quantification. 1 ml of each supernatant was mixed with 1 ml of Salkowski reagent and absorbance was measured at 530 nm.

Inoculation of in vitro plantlets with Micromonospora strains

The bacterial inoculum was obtained by culturing the strains in Petri dishes containing 10 ml of ISP2 medium (Shirling and Gottlieb 1966), incubating in dark at 29 °C until mycelium growth. When sporulation was observed, 2 ml sterilized distilled water was added to the dishes and colonies were superficially rubbed to obtain a spore suspension, which was used to obtain a 10^7 CFU ml^{-1} by serial dilution (Hastuti et al. 2012).

Plantlets of *L. multiflorum* ready to be recultured (Fig. 1) were immersed for 5 min in a solution with 10^7 CFU ml^{-1} of each actinobacterial strains. Thirty plantlets were inoculated with each *Micromonospora* strain. As control, 30 plantlets were immersed for 5 min in distilled water without actinobacterium. After the inoculation, the plantlets were weighed and the fresh biomass was recorded to be used as initial biomass in the growth tests. The initial biomass of each plantlet was analyzed to discard significant differences between the biomass of the control plantlets and the biomass of the plantlets inoculated with the different *Micromonospora* strains.

Culture of plantlets and in vitro growth tests

After inoculation, the inoculated and control plantlets were cultured in individual test tubes with 10 ml of Regeneration Medium (RM medium) (Regalado et al. 2017), which consists of MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and 0.2 mg l^{-1} kinetin, and incubated for 4 weeks in an incubator model I-291PF (Ingelab) at 25 ± 2 °C under 16:8 h (L:D) photoperiod with a light intensity level of 40 µmol photon m^{-2} s^{-1}. After 4 weeks,

Fig. 1 In vitro plantlets of *L. multiflorum* used in the inoculation assays with different strains of *Micromonospora*

we analyzed the effect of each *Micromonospora* strain in the in vitro growth of the *L. multiflorum* plantlets, especially the effect on root development. In each plantlet we measured biomass, biomass increase, root number and root length, and compared the results obtained with each *Micromonospora* strain inoculated. The biomass increase (BI) of each plantlet was calculated as the difference between the biomass after 4 weeks of culture and the initial biomass. Also, we determined the survival rate and the percentage of plantlets ready to be acclimatized (plantlets with more than four shoots and more than five roots with at least 5 cm of length) in the inoculated and the control plants.

Re-isolation of Micromonospora strains

To confirm the actinobacterial inoculation, root samples taken from ten plantlets inoculated with each strain and controls without bacterial inoculation were crushed aseptically in a sterile mortar with 2 ml distilled sterile water per sample. Then, 100 µl were plated in Petri dishes containing ISP2 medium. Plates were incubated in dark at 29 °C for a month. After this time, the presence of *Micromonospora* strains colonies were checked. The re-isolation of the *Micromonospora* strains indicated the success in the inoculation of *L. multiflorum* plantlets.

Multiplication of plantlets

To increase the number of plantlets inoculated with each *Micromonospora* strain and the control plantlets for acclimatization, the plantlets were subcultured in new test tubes with 10 ml of RM medium. The multiplication consisted in the mechanical division of the plantlets into individual plantlets with shoots and roots. The new tubes were cultured in the same conditions described above (25 ± 2 °C under 16:8 h (L:D) photoperiod with a light intensity level of 40 µmol photon m^{-2} s^{-1}) for 6 weeks.

Acclimatization of plantlets

Twenty-five plantlets inoculated with each *Micromonospora* strain (75 in total) were acclimatized following the protocol developed by Regalado et al. (2017). As control, 25 non-inoculated plantlets were also acclimatized. The plantlets were thoroughly washed with tap water and transplanted to 5×5 cm polyethylene alveolus trays containing a mixture of tyndallized sand:peat:perlite (1:1:1). Plantlet acclimatization was carried out in a culture chamber at 22 °C, 60% relative humidity and 14:10 h (L:D) photoperiod with a light intensity level of 30 µmol photon m^{-2} s^{-1}. The tray with the plantlets was wrapped with plastic film for 2 weeks to maintain high humidity. During the next 2 weeks, holes were made in the plastic film to reduce the humidity down to 60%. Finally, at the end of the fourth week, the plastic wrap was removed, and the acclimatization rate was measured.

Statistical analysis

All data were analyzed using SPSS software package (version 19.0; SPPS INC., Chicago, IL, USA). The initial biomass, the biomass after 4 weeks and the root length of the plantlets inoculated with each *Micromonospora* strain and the control were analyzed by one-way ANOVA. Also, the bacterial IAA production was analyzed by one-way ANOVA. When significant differences were found ($p \leq 0.05$) a HSD-Tukey test in the post-hoc analysis was used for comparisons among groups. The survival rate, the percentage of plantlets ready to be acclimatized and the acclimatization rate for each treatment were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. Pairwise comparisons among groups were performed by Fisher's least significant difference (LSD) test.

Results

Identification of Micromonospora strains

The 16S gene sequence of the strains TW2.1 and TW2.2 presented 99% of similarity with sequences from *Micromonospora halotolerans* (99% similarity, Accession Number NR_132303.1). The sequence of the strain SB3 was similar to *Micromonospora palomenae* (99% similarity, Accession Number NR_136848.1). All sequences were submitted to GenBank database (SB3 Accession Number: MH194972, TW2.1 Accession Number: MH194974; TW2.2 Accession Number: MH194973). The pairwise comparison between strains TW2.1 and TW2.2 was 0, and between SB3 and TW2.1/TW2.2 was 0.014. The colonies were different in color, size and pigment production among different strains in 2 week-old ISP2 agar plates incubated at 29 °C in dark. SB3 showed medium growing colonies (0.72 mm diameter/week) with dark-brown substrate mycelium, intense brown pigment production and aerial mycelium absent; TW2.1 produced low growing colonies (0.36 mm diameter/week) with pale-orange substrate mycelium, aerial mycelium absent and no pigment production; TW2.2 presented fast growing colonies (1.25 mm diameter/week) with orange-brownish substrate mycelium, aerial mycelium absent and presence of light-brown pigmentation.

IAA production by Micromonospora strains

All *Micromonospora* strains showed different IAA production ability. SB3 was the strain with highest values (11.31 ± 1.45 µg IAA ml^{-1}), followed by TW2.2

(8 ± 0.55 µg IAA ml^{-1}) and TW2.1 (5.06 ± 0.45 µg IAA ml^{-1}). Strains TW2.1 and TW2.2 did not show significant differences between them. SB3 IAA production was significantly higher than the other strains.

Effect of Micromonospora strains on the in vitro growth and acclimatization of plantlets

In vitro plantlets of *L. multiflorum* used in the inoculation assays with *Micromonospora* strains are shown in Fig. 1. As can be observed in Fig. 2a, there was a great variation in

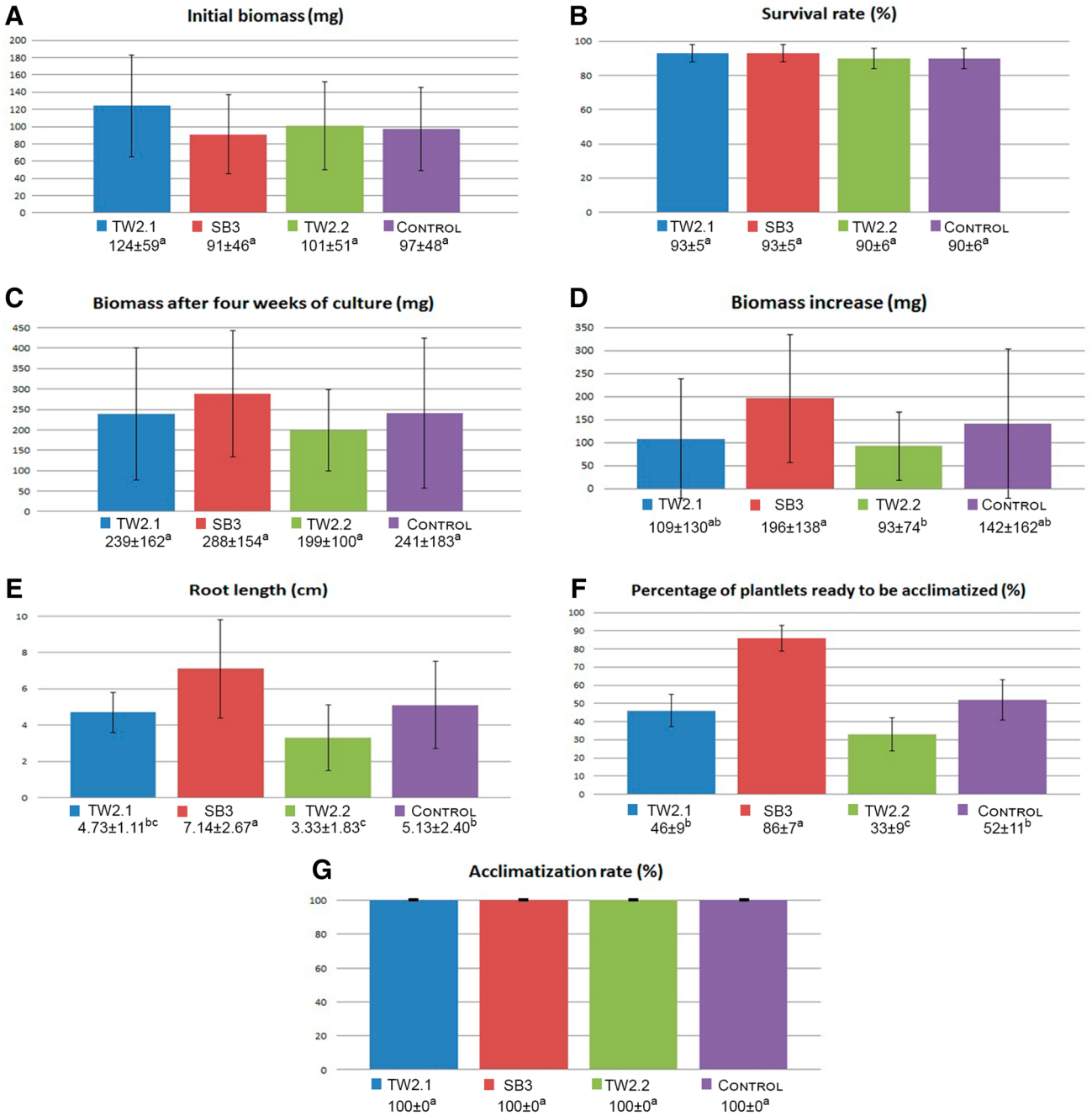

Fig. 2 Effects of the inoculation with three *Micromonospora* strains in plantlets of *L. multiflorum*. **a** Initial biomass before the inoculation (mean ± SD, n = 30). **b** Survival rate (%) after 4 weeks of culture in RM medium (mean ± SD, n = 30). **c** Biomass (mg) after 4 weeks of culture in RM medium (mean ± SD, n = 30). **d** Biomass increase (mg) after 4 weeks of culture in RM medium (mean ± SD, n = 30). **e** Root length (cm) after 4 weeks of culture in RM medium (mean ± SD, n = 30). **f** Percentage of plantlets ready to be acclimatized after 4 weeks of culture in RM medium (mean ± SD, n = 30). **g** Acclimatization rate (%) after 4 weeks of acclimatization (mean ± SD, n = 25)

each plantlet´s initial biomass, ranging from 30 to 240 mg. Nevertheless, the random distribution of these plantlets among treatments was appropriate, since there were no significant differences between the initial biomass of the plantlets used in the inoculation with each actinobacterium and the non-inoculated control plantlets (Fig. 2a).

Four weeks after inoculation with the strains of *Micromonospora*, the effect of each actinobacterium was studied. First, we studied the survival rate: 90 ± 6% control plantlets survived after 4 weeks of culture on RM medium (Fig. 2b). The survival rate of the inoculated plantlets was very similar to the control: 93 ± 5% in the case of strains TW2.1 and SB3, and 90 ± 6% for TW2.2 (Fig. 2b).

The next aspect studied was the final biomass value of each plantlet. The plantlets used as control showed a mean biomass of 241 ± 183 mg (Fig. 2c). The mean biomass of the plantlets inoculated with strain TW2.1 was very similar to the control (239 ± 162 mg). On the other hand, in plantlets inoculated with the strain SB3, the mean biomass was higher than the control (288 ± 154 mg), and lower than the control in the plantlets inoculated with strain TW2.2 (199 ± 100 mg) (Fig. 2c). These differences were not statistically significant due to the high variability of the plants, as can be observed in the high standard deviations. Nevertheless, these differences were important, since the plantlets inoculated with the strain SB3 showed a mean biomass 20% higher than control plants, while those inoculated with the strain TW2.2 showed a mean biomass 17% lower than control plants.

However, when we analyzed the BI (biomass increase), instead of the final biomass, the differences observed among treatments increased, despite the high data variability (Fig. 2d). Inoculation with strain SB3 caused an average BI of 196 ± 138 mg, significantly higher than the increase produced with strain TW2.2 (93 ± 74 mg). The increase produced in the inoculation with strain TW2.1 (109 ± 130 mg) and in the control plants (142 ± 162 mg) did not present significant differences with SB3 or TW2.2 inoculation. In percentage, the inoculation with strain SB3 caused a BI 38% higher than control plants, while inoculation with TW2.1 and TW2.2 decreased the BI, 23 and 34%, respectively, compared to control plants.

The effects produced by strains SB3 and TW2.2 on the *in vitro* growth of *L. multiflorum* plantlets were even more remarkable in the root length and the percentage of plantlets ready to be acclimatized. The characteristics of the plantlets inoculated with different actinobacterium and control plants after 4 weeks of in vitro culture can be seen in Fig. 3. The mean root length of control plants was

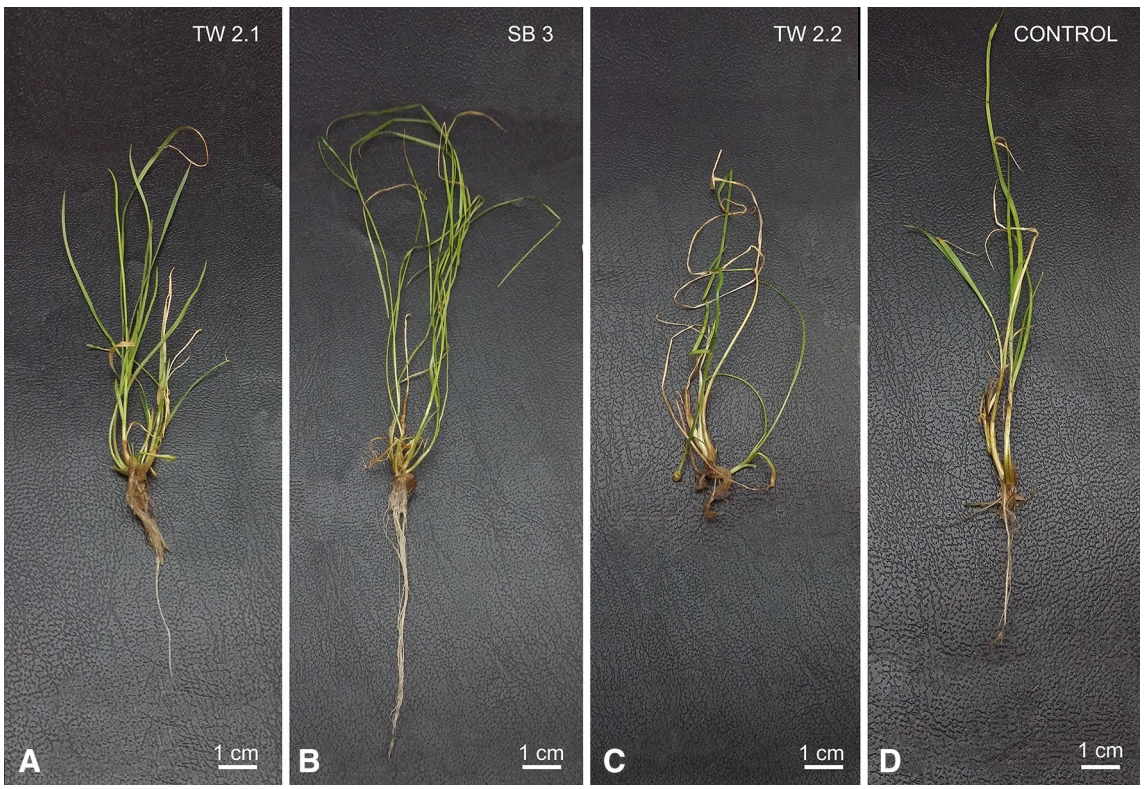

Fig. 3 Plantlets of *L. multiflorum* inoculated with three *Micromonospora* strains and cultured on RM medium for 4 weeks. **a** Plantlets inoculated with strain TW2.1. **b** Plantlets inoculated with strain SB3. **c** Plantlets inoculated with strain TW2.2. **d** Plantlets used as control without inoculation

5.13 ± 2.40 cm (Fig. 2e). The infection with strain SB3 caused a statistically significant increase in root length, which reached a mean of 7.14 ± 2.67 cm, while infection with strain TW2.2 caused a statistically significant decrease in root length (3.33 ± 1.83 cm). Finally, there were no statistically significant differences between the root length of the plantlets inoculated with strain TW2.1 (4.73 ± 1.11) and the control (5.13 ± 2.40 cm).

The greatest difference observed, as a result of microbial inoculation, was in the percentage of plantlets ready to be acclimatized (Fig. 2f). As stated in "Materials and methods", we considered that the plantlets ready to be acclimatized were those that presented more than four well-developed shoots and more than five roots with at least 5 cm of length (e.g. plantlets in Fig. 3b). Thus, the percentage of plantlets ready to be acclimatized also indicated the vigor of the plants inoculated with each *Micromonospora* strain. Approximately half of the control plantlets were ready to be acclimatized (52 ± 11%). The inoculation with strain SB3 significantly increased this percentage to almost 90% (86 ± 7%), while inoculation with TW2.2 reduced it to a third of the initial plants (33 ± 9%). Strain TW2.1 did not have a significant effect in the percentage of plantlets ready to be acclimatized (46 ± 9%) compared to non-inoculated plantlets (52 ± 11%).

The conditions selected to consider a plantlet ready for acclimatization were correct, since all plantlets selected were successfully acclimatized, independently of inoculation with actinobacterium (Fig. 2g). This result indicated that once the ideal conditions for acclimatization are achieved, the different actinobacterium do not influence the acclimatization process.

Re-isolation of Micromonospora strains

Micromonospora strains were re-isolated from roots of inoculated plantlets (Fig. 4). Strain TW2.2 was re-isolated from 100% of the ten in vitro plantlets inoculated with this strain, while strain TW2.1 was re-isolated from 80% of the in vitro plantlets and SB3 from 50%. No actinobacterium were recovered from ten control plants (without inoculation) studied. Strain TW2.1 presented a high CFU amount per root sample, followed by strain TW2.2 (high-moderate CFU per root sample) and SB3 (low CFU per root sample) (Fig. 4).

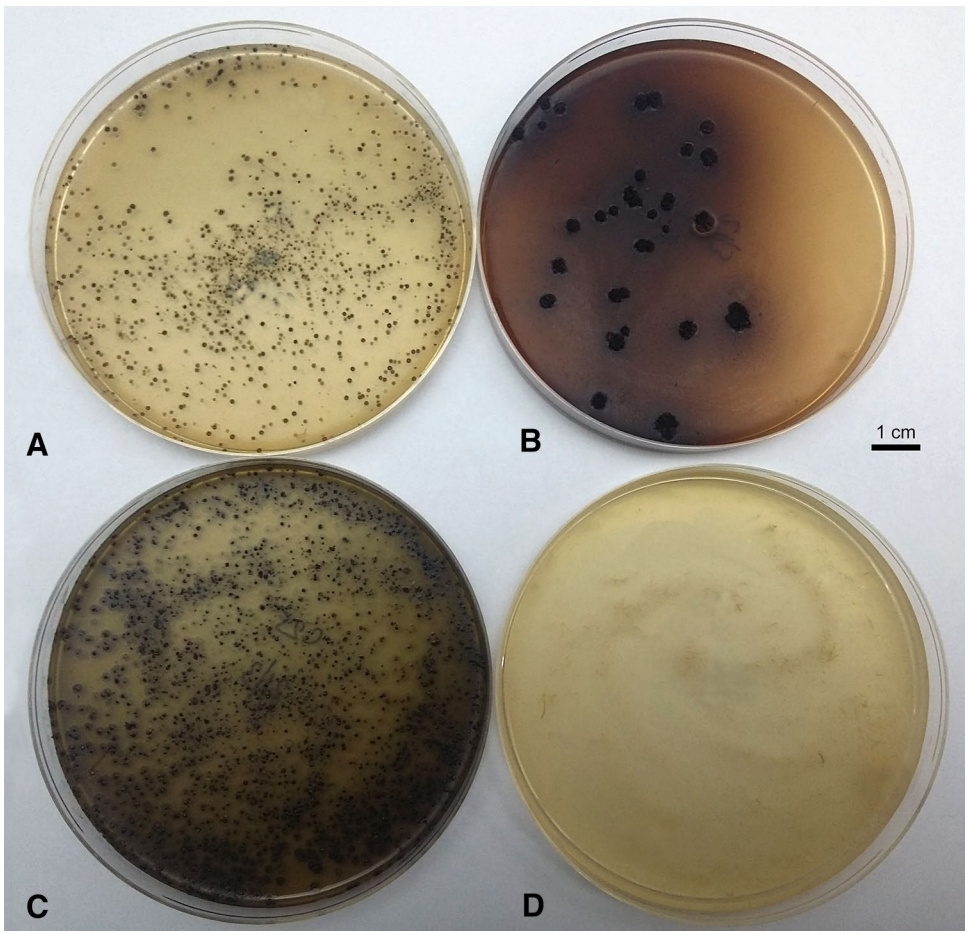

Fig. 4 *Micromonospora* strains re-isolated from *L. multiflorum* roots from *in vitro* plantlets inoculated with three *Micromonospora* strains. **a** Colonies re-isolated from plantlets inoculated with strain TW2.1. **b** Colonies re-isolated from plantlets inoculated with strain SB3. **c** Colonies re-isolated from plantlets inoculated with strain TW2.2. **d** Colonies re-isolated from control plantlets without inoculation

The morphology of re-isolated actinobacterial colonies was consistent with the inoculated strains in each treatment.

Discussion

Since Ørskov (1923) first described the actinobacterium *Micromonospora* in 1923, *Micromonospora* strains have been isolated from many different ecosystems, such as marine, aquatic sediments or mangrove. However, soil is the most frequent source of isolation (Trujillo et al. 2015). In the last years, the nitrogen fixing root nodules of different leguminous and actinorhizal plants (Carro et al. 2012, 2016; Martínez-Hidalgo et al. 2014; García et al. 2010; Trujillo et al. 2006, 2007, 2010; Valdés et al. 2005), as well as the roots of other plants, have been described as new niches for *Micromonospora* sp. (Kaewkla et al. 2017; Zhao et al. 2016, 2017; Thawai et al. 2016). Recently, researchers in our group have isolated different actinobacterium strains from the roots of the argentine pasture *B. auleticus* (Della Mónica et al. 2017). The three *Micromonospora* strains used in this work (TW2.1, SB3, TW2.2) derive from these isolations. The molecular characterization of these strains revealed a remarkable similarity with two species of *Micromonospora*: TW2.1 and TW2.2 presented a 99% sequence similitude with *M. halotolerans* and SB3 a 99% sequence similitude with *M. palomenae*. However, different *Micromonospora* strains that presented a percentage of molecular similarity higher than 99% with other *Micromonospora* sp. have been described as new *Micromonospora* species (Kaewkla et al. 2017; Zhao et al. 2017; Carro et al. 2016). Furthermore, the colonies of the strains TW2.1 and TW2.2 showed different color, size and pigmentation. Therefore, additional assays are necessary to accurately determine the identity of *Micromonospora* strains TW2.1, TW2.2 and SB3. For the time being, we will disregard the species and consider these strains within the genus *Micromonospora*. The objective of this work was to study the effect of these *Micromonospora* strains on the growth of micropropagated *L. multiflorum* plantlets.

As stated in the introduction, the reports on *in vitro* bacterization have been consistently increasing in the last years (Quambusch et al. 2016; Larraburu and Llorente 2015; Parray et al. 2015; Bashan et al. 2014; Thomas et al. 2010; Bashan 1998). These in vitro bacterizations enable the study of microbial effects on plant growth. In particular, grasses are considered among the most recalcitrant crop species for in vitro culture (Giri and Praveena 2015) and there are no protocols describing *B. auleticus* micropropagation, thus restricting the study of *Micromonospora* inoculation in the plant species from which they were isolated. Recently, our research group developed a new protocol for the micropropagation of *L. multiflorum* (Regalado et al. 2017), allowing the *in vitro* bacterization of this specie.

The initial size and biomass of the in vitro plantlets of *L. multiflorum* were both highly variable (Figs. 1, 2a). After 4 weeks of culture, the variability in the biomass within each inoculation treatment and the control was even larger (Fig. 2c, d). This made it difficult to register statistically significant differences in the final biomass and the BI among the plantlets inoculated with each strain and the control ones, even so differences in these measures were registered (Fig. 2c, d). Furthermore, the differences produced by the *Micromonospora* strains on the other parameters such as the root length (Fig. 2e) and the percentage of plantlets ready to be acclimatized (Fig. 2f), were statistically significant.

We re-isolated the *Micromonospora* strains to confirm that the *Micromonospora* caused the differences observed in the inoculated plantlets and the ability of these endophytic bacteria to colonize *L. multiflorum* plantlets in vitro. In this re-isolation we used only a small part of the roots of each plant; thus, not re-isolating the microorganism did not imply absence of the *Micromonospora* in the un-analyzed roots. The percentage of plants from which the *Micromonospora* strains were isolated varied for each strain between 50% (SB3) and 100% (TW2.2). These percentages ensured a high inoculation index of the *Micromonospora* strains (at least 50%). Therefore, the inoculation protocol was effective, allowing us to associate the changes observed in the plants with the presence of these *Micromonospora* strains.

The results obtained in this work indicated that the inoculation with the SB3 *Micromonospora* strain promoted in vitro plant growth, especially root elongation. In comparison, inoculation with strain TW2.2 had the opposite effect, inhibiting plant growth, and strain TW2.1 did not affect the measured parameters with respect to the control plants. Root elongation and plant growth promotion have been previously reported in the actinobacterial genus *Streptomyces* (Sathya et al. 2016; Sreevidya et al. 2016; Gopalakrishnan et al. 2015; Palaniyandi et al. 2014; Goudjal et al. 2013; Yandigeri et al. 2012) and *Cellulosimicrobium* (Nabti et al. 2014). This promotion effect appears to be related to the synthesis of plant growth regulators such as IAA (indole-3-acetic acid). Indeed, in strain SB3 IAA production was significantly higher than in the other two *Micromonospora* strains evaluated. The plant growth promotion produced by actinobacterial strains from *Micromonospora* genus has also been studied in leguminous and actinorhizal plants (Carro et al. 2012, 2013), but the present study constitutes the first report on the in vitro effects of *Micromonospora* on grasses.

Micromonospora strain MM18 promoted plant growth when it was inoculated in plants of *Ochetophila trinervis* (= *Discaria trinervis*) (Solans 2007; Solans et al. 2011) and *Medicago sativa* (Solans et al. 2009). This strain produces several plant hormones such as zeatin, IAA,

and gibberellic acid (Solans et al. 2009), explaining this effect. Even so, plant growth promotion was higher when *Micromonospora* strain MM18 was co-inoculated with the nitrogen-fixing bacterium *Sinorhizobium meliloti* in *M. sativa* (Solans et al. 2009) and *Frankia* in *O. trinervis* (Solans 2007; Solans et al. 2011). *M. lupini* strain Lupac 08 enhanced *Trifolium* sp. growth but, as in the previous cases, this effect was much higher when the *Micromonospora* strain was inoculated together with a nitrogen-fixing microorganism (*Rhizobium* sp. E11) (Trujillo et al. 2014). Moreover, Martinez-Hidalgo et al. (2014) studied the effect produced by 15 *Micromonospora* strains in *M. sativa* growth promotion. Each of the strains tested in *M. sativa* had a different effect, and this is in agreement with our results for *L. multiflorum*. Strains AL16, ALFb1 and ALFb7 did not produce changes in the biomass of *M. sativa* plants, strains ALFb5 and ALFr5 caused an increase of 19% and 35% respectively and strain AL4 produced a decrease of 20%. These percentages are comparable to those obtained for *L. multiflorum* in this work. Strain SB3 produced a BI (biomass increase) 38% higher than the control plants, while TW2.1 and TW2.2 decreased it 23 and 32% respectively. The most common effect of plant growth-promoting rhizobacteria (PGPR) on plants is the formation of larger root systems (Vacheron et al. 2013). Our results showed that strain SB3 increased the root length of *L. multiflorum* in vitro plants, while the *Micromonospora* strains used by Martinez-Hidalgo et al. (2014) did not induce larger root systems in *M. sativa*.

Martinez-Hidalgo et al. (2014) also co-inoculated 15 *Micromonospora strains* with a nitrogen-fixing microorganism (*Ensifer meliloti 1021*) and observed that only the strains ALFb5 and ALFpr18c improved the biomass increment and number of nitrogen fixing nodules compared to plants only inoculated with *E. meliloti 1021*. Interestingly, the differences were much larger than those produced by the inoculation alone of the *Micromonospora* strains, suggesting a synergic interaction between actinobacterium and nitrogen-fixing microorganisms but, as no microorganisms are capable of forming nitrogen-fixing nodules in *L. multiflorum*, co-inoculation assays with nitrogen fixers cannot be performed.

In conclusion, this work constitutes the first study concerning plant growth promotion produced by endophytic *Micromonospora* strains in micropropagated *L. multiflorum*, a non-leguminous plant. Our results show that this promotion depends on the inoculated *Micromonospora* strain, which can either promote (strain SB3), inhibit (strain TW2.2) or not affect (strain TW2.1) growth. The most prominent feature affected was root development. In 4 weeks, strain SB3 produced a BI (biomass increase) 38% higher and roots that were 2 cm longer than control plants. *Micromonospora* strain SB3 is a good candidate to use in the breeding programs for *L. multiflorum* and other grasses to increase their yield.

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Author contributions DMIF designed and executed the in vitro experiments, isolated and identified the actinobacterial strains used and collaborated in the writing of the manuscript. NMV performed the statistical analysis, provided the plant material and collaborated in the improvement of the manuscript. ILJ realized the strains molecular pairwise comparison, provided the plant material and critically review the manuscript and improve the text. GQ did the IAA quantification, analyzing the statistic differences among strains and collaborate in the manuscript improvement. SJM did the molecular identification of the actinobacteria and critically review the manuscript. PASI designed the in vitro experiments, revised the manuscript critically for important intellectual content and reviewed the English of the manuscript. RJJ designed and executed the in vitro experiments and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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