Potential use of rhizobacteria from the *Bacillus* genus to stimulate the plant growth of micropropagated banana

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Abstract — Introduction. Soil microbiota communities have demonstrated their crucial role in maintaining the soil ecological balance and therefore the sustainability of either natural ecosystems or agroecosystems. Rhizospheric microbe-plant interactions have a great influence on plant health and soil quality since these root-associated microorganisms are able to help the host plant to deal with drought, nutritional and soil-borne pathogen stress conditions. Plant growth-promoting rhizobacteria (PGPR) can be considered among rhizosphere-beneficial microorganisms. In a micropropagated plant system, bacterial inoculation at the beginning of the acclimatisation phase must also be observed from the perspective of the establishment of the soil microbiota rhizosphere. The objective of this work was to evaluate the effect of a rhizobacteria consortium of *Bacillus* spp. on the first developmental stages of two micropropagated bananas. **Materials and methods**. Two varieties of banana plant cultivars ('Grande Naine' and a banana-derived tetraploid hybrid 'ITC 1297') were inoculated or not with a suspension of *Bacillus* spp. at the beginning of the weaning phase. Six plants were considered per treatment and cultured under greenhouse conditions in a randomised design. For both cultivars, plants were harvested (135 and 185) days after bacterial inoculation and analysed for growth parameters and nutrient contents. **Results**. Concerning plant development, bacterial application induced a positive effect on both cultivars although this effect showed some time differences depending on the banana cultivar. Foliar mineral contents were significantly increased only in 'Grande Naine' plants at 135 days. Our results demonstrated for the first time that the *Bacillus* spp. consortium tested was able to improve banana development (both cultivars) and foliar mineral contents in one of them. **Conclusion**. Therefore, this bacterial consortium can be described as PGPR for banana under these experimental conditions. This biotechnology, adaptable to the hardening phase, thus represents a prospective way to increase plant health and survival rates in commercial nurseries.

Spain / Canary Islands / *Musa* / micropropagation / weaning stage / *Bacillus* / rhizobacteria / growth / mineral content

Utilisation de rhizobactéries du genre *Bacillus* pour stimuler la croissance de bananiers issus de micropropagation.

Résumé — Introduction. Le rôle primordial de certains micro-organismes du sol pour maintenir l'équilibre écologique de sol et la durabilité des écosystèmes naturels ou des agroécosystèmes a déjà été démontré. Les interactions micro-organisme-plante dans la rhizosphère ont une grande influence sur l'état sanitaire des plants et sur la qualité du sol puisque ces micro-organismes associés aux racines peuvent aider la plante hôte à lutter contre les stress dus à la sécheresse, aux problèmes nutritionnels ou aux pathogènes du sol. Les rhizobactéries stimulant la croissance des plantes (PGPR) peuvent être considérées comme des micro-organismes utiles de la rhizosphère. Dans un processus de multiplication des plantes par micropropagation, l'inoculation bactérienne en début de phase d'acclimatation peut contribuer à établir une rhizosphère. Pour cela, l'objectif de notre travail a été d'évaluer l'effet d'un consortium de rhizobactéries constitué de *Bacillus* spp. sur les premières étapes de développement de deux cultivars de banane micropropagés. **Matériel et méthodes**. Deux cultivars de bananiers ('Grande Naine' et un hybride tétraploïde dérivé de la banane 'ITC 1297') en début de phase de sevrage ont été inoculés ou non avec une suspension de *Bacillus* spp. Chaque traitement a été appliqué à six plants cultivés en serre et placés dans un dispositif randomisé. Les plants ont été déracinés à (135 ou 185) jours après inoculation par les bactéries, et analysés par rapport à certains paramètres de croissance et à leurs teneurs en éléments minéraux. **Résultats**. L'inoculation bactérienne a induit un effet positif sur le développement des plants des deux cultivars bien que cet effet ait été un peu décalé dans le temps selon le cultivar de bananier. En revanche, les teneurs en éléments minéraux dans les feuilles n'ont été sensiblement augmentées que pour les plants de 'Grande Naine' 135 jours après leur inoculation. Nos résultats ont démontré pour la première fois que le consortium de *Bacillus* spp. testé pouvait améliorer le développement des deux cultivars de banane utilisés et les teneurs en éléments minéraux des feuilles de l'un d'eux. **Conclusion**. Le consortium de bactéries utilisé peut donc être décrit comme un ensemble de rhizobactéries stimulant la croissance des bananiers dans les conditions expérimentales utilisées. Cette technique de biotechnologie appliquée à la phase d'endurcissement des plants micropropagés est dès lors une méthode prometteuse pour améliorer l'état sanitaire et la survie des plants en pépinières commerciales.

Espagne / Canaries (îles) / *Musa* / micropropagation / phase de sevrage / *Bacillus* / rhizobactérie / croissance / teneur en éléments minéraux

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Received 17 July 2003
Accepted 1 December 2003

Fruits, 2004, vol. 59, p. 83–90

DOI: 10.1051/fruits:2004008

***RESUMEN ESPAÑOL**, p. 90*

1. Introduction

Nowadays micropropagation is a successful technique for the mass production of plants for research and commercial purposes [1]. Banana can be included among the crops that have been increasingly propagated by this method. Banana microplants are replacing conventional material for the establishment of new or replacement of existing plantations. Rapid production and high availability of uniform and disease-free plants are the main reasons for using such vegetal material. Nevertheless, micropropagated plants are not free from inconveniences such as more probability of somaclonal variations, weak physiology and the lack of soil microbiota. Consequently, acclimatisation, or the weaning phase, is the most critical period in the micropropagation process [2]. Survival rates of microplants often decrease due to weaning stress during this phase.

Soil microbiota communities have demonstrated their crucial role in maintaining the soil ecological balance and therefore the sustainability of either natural ecosystems or agroecosystems [3, 4]. The root-soil interface, where microorganisms, plant roots and soil constituents interact [5], defines what is known as the rhizosphere [6]; it is the most dynamic environment of microbe-plant interaction since it is the zone of influence of plant roots on the soil microbiota. Rhizospheric microbe-plant interactions have a great influence on plant health and soil quality [7] since these root-associated microorganisms are able to help the host plant to deal with drought, nutritional and soil-borne pathogen stress conditions [8].

In the rhizosphere, plant growth-promoting rhizobacteria (PGPR) [9] can be considered beneficial microorganisms. Their positive effects on plant development or the establishment of seedlings have been described for different crops; either herbaceous ones such as potato [10], bean [11] and soybean [12], or woody ones such as apple trees [13] and citrus [14]. Several mechanisms, which involve phytohormone production [15], mineral solubilisation and availability [16] or biological control of soil-borne pathogens [17], have been suggested to explain bacterial activity. Authors have frequently described as PGPR certain strains of *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter, Enterobacter* and *Serratia* [17].

The benefits provided by these microorganisms during the *ex vitro* phase of some crops have already been described [1, 18]: PGPR can contribute to easing the weaning stress of these plants since they promote vegetal development and nutrition. Bacterial inoculation at the beginning of the acclimatisation phase must also be observed for the establishment of the soil microbiota in the rhizosphere.

The aim of our study was to determine whether certain *Bacillus* strains previously described as PGPR on other crops were able to develop this activity on banana. Particularly, we investigated the potential use and benefits of a *Bacillus* consortium on two micropropagated commercial banana cultivars, during their weaning phase.

2. Materials and methods

2.1. Plant materials

Two micropropagated banana cultivars – *Musa acuminata* Colla AAA, *cv.* 'Grande Naine', and a banana-derived tetraploid hybrid from Nigeria known as TMBx 5295-1 — were provided by CULTESA (*Cultivos Vegetales* in vitro *de Tenerife* S.A.), Tenerife, Spain. TMBx 5295-1 microplants were provided by INIBAP (International Network for improvement of Banana and Plantains) ITC (INIBAP Transit Centre) code 1297 (thus, from now on, this cultivar will be cited as 'ITC 1297'), as proliferating material; they were then multiplied by CULTESA. Plantlets, measuring approximately (8 ± 1) cm in height and with three fully developed leaves, were placed in nutrient agar [19] and subsequently transplanted to 24-L seed trays (20 plantlets / tray and two trays / cultivar), filled with a water steam sterilised substrate mixture (1:1:1= soil:volcanic ash:peat TKS®1-Instant Sphagnum-Torf Klasmann Deilmann GmbH, Germany).

2.2. Bacterial inoculum materials

Our experiments used the *Bacillus* consortium containing three strains (INR7, T4 and IN 937b) isolated and identified by Dr. Kloepper (Alabama, USA) and kept in TSB (Trytone Soy Broth) with 20% glycerol at the *Institut de Recerca i Tecnologia Agroalimentàries*, IRTA (Spain).

Bacterial inoculum was prepared after culturing the strains on petri dishes with TSA (Tryptone Soy Agar) for 2 weeks. For each culture session, plates were incubated for 48 h at 25 °C. The bacterial inoculum consisted of a sterilised NaCl (0.85%) suspension containing approximately an equal amount of the three *Bacillus* strains. The inoculum concentration was approximately 10^8 CFU (colony-forming units)·mL^{-1} and was determined by using a viable versus absorbance at 600 nm curve for each *Bacillus* strain. Bacterial inoculation was carried out 20 days after transplanting to trays by adding 20 mL of suspension per plant.

2.3. Experimental design and culture conditions

For the plants of each cultivar, there were two treatments – inoculated and non-inoculated treatments – with 15 replicates of each. The hardening phase lasted for 50 days (30 days after bacterial inoculation) while the plantlets were under an acclimatisation tunnel with an ambient temperature of 25 °C and relative humidity of 90%. The plantlets were irrigated with (50 to 75) mL distilled water according to the hydric requirements. Then, the plants were transplanted to individual 3-L pots filled with a sterilised substrate mixture (1:1:1= soil:volcanic ash:peat TKS®1-Instant Sphagnum-Torf Klasmann Deilmann GmbH, Germany). The substrate surface of each plant was covered by a volcanic ash layer to keep the substrate humid. Plants were grown under greenhouse conditions. The experiment was arranged as a randomised factorial design with six banana plants of each group being harvested (135 and 185) days after bacterial inoculation. During the experiment, the plants were fertilised with Wuxal-Ca® (Argos Shering, AgrEvo, S.A. Valencia, Spain) using a 3% product dose (foliar application), twice a month.

2.4. Assessment of variables

Aerial and root fresh weight, aerial dry weight, pseudostem length, diameter, adventitious root length, and foliar surface were measured at each harvest. Foliar surface was determined by using the surface measurer of Li-COR Inc., Lincoln, Nebraska, USA (Mod. Li-3100).

The nitrogen, phosphorus and potassium macroelements were determined on shoots. The pseudostem and leaves of the banana plants were thoroughly washed in a mild detergent, rinsed three times in distilled water avoiding senescent or necrotic tissue, and prepared for foliar analysis. Samples were then dehydrated in a temperature-controlled fan-ventilated oven at 60 °C for 24 hours, ground in a ball mill and digested in wet acid. Analysis was done with a F586-587 Varian Liberty 220 inductively coupled plasma (ICP) emission spectrometer [20]. Two readings were carried out per sample.

2.5. Statistical analysis

All data were analysed by ANOVA. Means were separated by Tukey's multiple range test ($P \leq 0.05$). The analysis was performed using Systat® 7.0.1. (SPSS Inc© 1997).

3. Results

The *Bacillus* spp. consortium significantly promoted plant development although each banana cultivar showed different behaviour (*figure 1*). 'Grande Naine' plants showed the greatest significant effects (71%) at 135 days after bacterial inoculation, whereas in 'ITC 1297', significant effects (36%) were detected at 185 days. However, significant increases for the 'Grande Naine' cultivar were higher than those registered in 'ITC 1297'.

3.1. 'Grande Naine' at 135 days after *Bacillus* spp. inoculation

3.1.1. Plant development

Bacterial inoculation induced plant development since significant increases in aerial,

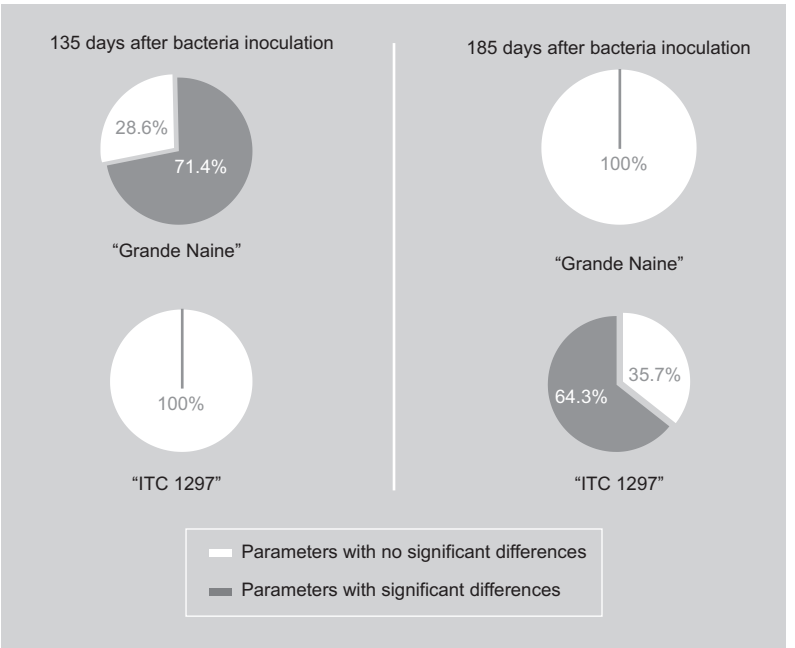

Figure 1.
Effect of *Bacillus* spp. consortium on growth parameters for micropropagated banana 'Grande Naine' and 'ITC 1297' cultivars.

root and total fresh weight were detected (*table I*). Bacterisation increased fresh biomass and aerial dry matter by about (40 and 43)%, respectively. Diameter and foliar surface (*table II*) were also significantly greater (about 20%) in plants treated with *Bacillus* spp. consortium than in control plants. However, no significant differences were registered in pseudostem and adventitious root lengths.

Table I.
Effect of *Bacillus* spp. consortium on micropropagated banana 'Grande Naine' cultivar, 135 days after bacterial inoculation.

Treatment	Fresh weight (g)			Aerial dry weight (g)
	Aerial	Root	Total	
Control	89.73 b	30.62 b	120.19 b	7.81 b
Bacillus spp.	128.47 a	43.64 a	172.10 a	11.19 a
Standard error	7.27	3.15	10.15	0.67
Probability	0.004	0.015	0.005	0.005

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

3.1.2. Foliar mineral content

Bacillus spp. inoculation increased foliar nutrient content in 'Grande Naine' plants (*table III*). Nitrogen, phosphorus and potassium contents were significantly higher in plants previously inoculated with PGPR than in control plants. Those increases were about 34% for nitrogen, 40% for phosphorus and 41% for potassium.

3.2. 'ITC 1297' at 185 days after *Bacillus* spp. inoculation

3.2.1. Plant development

Plants inoculated with PGPR showed a significantly greater biomass content than control plants, except for the aerial dry weight parameter (*table IV*). The significant increase in the total (aerial and root) fresh weight was about (20, 17 and 34)%, respectively. Bacterial inoculation also significantly promoted the pseudostem and adventitious root lengths, which were (11 and 19)% higher, respectively, than control plants (*table V*). Other parameters, such as pseudostem diameter and foliar surface, did not show significant differences between the two treatments.

3.2.2. Foliar mineral content

Despite the improvement detected in plant development after bacterial inoculation, no significant differences were observed in the foliar mineral content of 'ITC 1297' (*table VI*).

4. Discussion

Under our experimental conditions, bacterial inoculation significantly induced plant development for both cultivars and increased foliar mineral content in 'Grande Naine' microplants. These results must be compared with those obtained in other crops since no reference has been found for the use of PGPR to promote banana growth and/or nutrition. Results in other crops often showed greater development and increases in yield or nutritional content than those obtained in our work [16, 21–23]. Among these previous studies, several mechanisms,

such as indol-acetic acid (IAA) production [21], have been proposed to explain this positive effect on plants. It is well known that IAA promotes root development: elongation and stimulation of lateral and primary roots have been described in maize and pea after inoculation with IAA-producing bacteria [24, 25].

However, depending on the banana cultivar, some differences in timing and magnitude of response to bacterial inoculation were detected. This can be explained according to the genetic and physiological differences between the two cultivars, i.e., variability of both *Musa* genotypes in the root system and/or in the root exudate composition. Variations in the root systems of ten *Musa* spp. cultivars were registered under hydroponics by Sweenen *et al.* [26]: total root length; relative proportions of adventitious, first and second order roots; length of first, second order and lateral roots, and proportion of adventitious roots covered by lateral ones. Other authors have also confirmed these hypotheses under field conditions [27]. Concerning root exudates, it is demonstrated in other crops that bacterial colonisation ability could be affected by affinity with exudate compounds. Such an affinity could determine the rate and location of colonisation [28] and it has also been proved that not all plant compounds have equally important effects on root-colonising bacteria [29]. Consequently, quantitative differences in root colonisation between four wheat genotypes have been described [30].

The significant improvements in the mineral contents of the 'Grande Naine' cultivar registered in our experiment confirm results obtained by other authors. Increases in N contents in lentil seedlings after bacterisation with a *Bacillus megaterium* suspension have been reported [31]. Another experiment also described certain *Bacillus* strains as N-fixing strains [32]. However, *Bacillus* spp. are most commonly cited as soil P-solubilising bacteria [16]. In this way, these organisms would be able to increase the availability of P in soil, which can be detected in P vegetal contents [16, 31, 33].

The results of this experiment allow us to conclude the viability of using PGPR during the weaning phase of microplants [1, 18, 34].

Table II.
Effect of *Bacillus* spp. consortium on micropropagated banana 'Grande Naine' cultivar, 135 days after bacterial inoculation.

Treatment	Diameter (cm)	Foliar surface (cm^2)	Pseudostem length (cm)	Adventitious root length (cm)
Control	2.39 b	975.44 b	50.40 a	15.21 a
Bacillus spp.	2.92 a	1174.70 a	54.20 a	15.26 a
Standard error	0.11	49.43	4.62	0.74
Probability	0.006	0.017	0.573	0.961

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

Table III.
Effect of *Bacillus* spp. consortium on foliar mineral content on micropropagated banana 'Grande Naine' cultivar, 135 days after bacterial inoculation.

Treatment	Mineral contents ($mg \cdot plant^{-1}$)		
	N	P	K
Control	240.43 b	20.52 b	583.61 b
Bacillus spp.	322.73 a	28.64 a	820.81 a
Standard error	19.14	1.61	73.76
Probability	0.012	0.005	0.046

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

Table IV.
Effect of *Bacillus* spp. consortium on micropropagated banana 'ITC 1297' cultivar, 185 days after bacterial inoculation.

Treatment	Fresh weight (g)			Aerial dry weight (g)
	Aerial	Root	Total	
Control	347.33 b	82.67 b	430.33 b	27.33 a
Bacillus spp.	406.33 a	110.50 a	516.33 a	29.55 a
Standard error	14.99	4.14	17.82	1.23
Probability	0.019	0.001	0.007	0.229

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

Table V.
Effect of *Bacillus* spp. consortium on micropropagated banana 'ITC 1297' cultivar, 185 days after bacterial inoculation.

Treatment	Diameter (cm)	Foliar surface (cm^2)	Pseudostem length (cm)	Adventitious root length (cm)
Control	3.73 a	2600.30 a	116.17 b	19.23 b
Bacillus spp.	4.05 a	2375.47 a	129.00 a	22.84 a
Standard error	0.12	121.29	3.67	0.55
Probability	0.090	0.219	0.033	0.001

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

Table VI.
Effect of *Bacillus* spp. consortium on foliar mineral content on micropropagated banana 'ITC 1297' cultivar, 185 days after bacterial inoculation.

Treatment	Mineral contents ($mg \cdot plant^{-1}$)		
	N	P	K
Control	754.59 a	63.00 a	1826.85 a
Bacillus spp.	607.58 a	60.84 a	1808.99 a
Standard error	75.76	1.92	78.19
Probability	0.200	0.444	0.875

Within the same column, means followed by the same letter are not statistically different according to Tukey's test ($P \leq 0.05$) ($n = 6$).

Apart from their contribution to plant development and health, these microorganisms are crucial for maintaining the rhizospheric environment of plants [35].

Acknowledgements

The authors would like to thank A.R. Socorro Monzón from Dpto. Suelos y Riegos, ICIA, Spain, for foliar mineral analyses. This work was economically supported by the European Union (INCO-DC ERBIC 18*CT970208).

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Utilización de rizobacterias del género *Bacillus* para estimular el crecimiento de plataneras procedentes de micropropagación.

Resumen — Introducción. Está demostrado el papel primordial de algunos microorganismos para mantener el equilibrio ecológico de suelo y la sostenibilidad de los ecosistemas naturales o de los agroecosistemas. Las interacciones microorganismo-planta en la rizosfera tienen una gran influencia sobre el estado sanitario de las plantas y sobre la calidad del suelo ya que estos microorganismos asociados a las raíces pueden ayudar a la planta huésped a luchar contra los estrés ocasionados por sequía, problemas nutricionales o por patógenos del suelo. Las rizobacterias que estimulan el crecimiento vegetal (PGPR) pueden considerarse como microorganismos útiles de la rizosfera. En un proceso de multiplicación de las plantas mediante micropropagación, la inoculación bacteriana al inicio de la fase de aclimatación puede contribuir a establecer una microbiota rizosférica. Por ello, el objetivo de nuestro trabajo consistió en evaluar el efecto de un consorcio de rizobacterias compuesto por *Bacillus* spp. en las primeras etapas de desarrollo de dos cultivares de plataneras (bananos para América Latina) micropropagadas. **Material y métodos**. Dos cultivares de plataneras ('Gran Enana' y un híbrido tetraploide derivado de plátano 'ITC 1297') al principio de la fase de separación fueron inoculados o no con una suspensión de *Bacillus* spp. Cada tratamiento fue aplicado a seis plantas cultivadas en invernadero y dispuestas en diseño aleatorio. En ambos cultivares, las plantas fueron estudiadas (135 o 185) días después de su inoculación con las bacterias, y analizadas basándose en ciertos parámetros de crecimiento y en sus contenidos de elementos minerales. **Resultados**. La inoculación bacteriana indujo un efecto positivo en el desarrollo de las plantas de los dos cultivares aunque con un ligero desfase temporal según el cultivar de banano. En cambio, los contenidos de elementos minerales en las hojas sólo se incrementaron notablemente en las plantas 'Gran Enana' 135 días después de su inoculación. Nuestros resultados han demostrado, por primera vez, que el consorcio de *Bacillus* spp. probado podía mejorar el desarrollo de los dos cultivares de platanera utilizados y los contenidos de elementos minerales de las hojas de uno de ellos. **Conclusión**. Podemos, por tanto, describir el consorcio de bacterias empleado como un conjunto de rizobacterias que estimula el crecimiento de las plataneras en las condiciones experimentales utilizadas. Esta biotecnología aplicada a la fase de endurecimiento de las plantas micropropagadas es un método prometedor para mejorar el estado sanitario y la supervivencia de las plantas en viveros comerciales.

España / Canarias / *Musa* / micropropagación / fase de adimatación / *Bacillus* / rizobacterias / desarrollo / contenido nutricional