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Original article

Effect of inoculation of *Bacillus licheniformis* on tomato and pepper

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Abstract – The effects of inoculation with a strain of *Bacillus licheniformis* on the growth of pepper and tomato were investigated in three experiments, one under seedbed conditions and two under greenhouse production conditions. In the first experiment, the bacterium significantly increased the height of plants and the leaf area in both species and in both cultivars. Effects were greater on pepper than on tomato. In the second experiment, seedlings growing in sand and in hydroponic culture were studied. The number and diameter of tomato fruits produced in sand and in hydroponic medium were increased significantly by inoculation. Treated plants had less disease than non-treated plants. In the third experiment the total weight of pepper harvested from inoculated plants increased significantly with regard to control non-inoculated plants. This strain had considerable colonisation and competitive ability, and it could be used as a biofertiliser or biocontrol agent without altering normal management in greenhouses.

PGPR / tomato / pepper / colonisation / biofertiliser

1. INTRODUCTION

Environmental protection and the need to enhance agricultural output have made research in new sustainable technologies necessary. In recent years, interest in soil microorganisms that can promote plant growth [2] or help prevent the attack of soil-borne plant pathogens has increased [6, 42]. These beneficial bacteria are usually referred to as plant growth-promoting rhizobacteria or PGPRs [22]. Kloepper and Schroth [23] first reported that certain rhizosphere bacteria could promote plant growth. Further, studies with these bacteria (PGPRs) demonstrated their control of soil-borne pathogens [45]. Recently, some PGPRs have shown their capacity to protect plants against pathogens through mechanisms associated with induced systemic resistance [7, 35].

The use of PGPRs to control soil-borne pathogens is a practice with a promising future, because the Montreal Protocol (an international treaty to protect the earth from the detrimental effects of ozone) proposes the elimination of pesticides such as methyl bromide before 2005. This forces us to look for new alternatives to replace them.

A number of different bacteria promote plant growth, including *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas* sp., *Acetobacter* sp., *Burkholderia* sp. and *Bacillus* sp. [3, 5, 21, 22, 30–33, 40]. The principal mechanisms of growth promotion are: production of growth-stimulating phytohormones [1, 13, 14]; mobilisation of phosphate [8, 44]; siderophore production [24, 34]; antibiotic production [37]; inhibition of plant ethylene synthesis [10, 11], and induction of plant systemic resistance to pathogens [35, 47].

The above mechanisms suppose a direct contact between bacteria and the root surface or inner tissues between cells root cortex, sites where there is maximum bacterial activity due to the release of organic components [12, 28]. Root colonisation is therefore considered essential for plant growth promotion by rhizobacteria [17, 20]. Temperature, pH, soil type, plant genotypes and the competence of indigenous microorganisms are some of the factors that can affect the colonisation process [17, 18].

The use of microbial products has certain advantages over conventional chemicals: they are considered safer than many of the chemicals now in use; they do not accumulate in the food chain; the target organisms seldom develop resistance as is the case when chemical agents are used; and properly-developed biocontrol agents are not considered harmful to ecological processes or the environment [38]. On a worldwide level, many microbial products have been developed for agricultural purposes and are rapidly being commercialised [15, 19, 45].

The use of PGPRs does not imply the elimination of pesticides; therefore, it is very important to know the compatibility of PGPRs with the pesticides currently used to ensure that the bacteria survive in the soil.

The aim of this work was to study the effects of one *Bacillus* PGPR on the growth of two tomato and three pepper cultivars. The study was carried out in three experiments. The first experiment was conducted at the same time and under the same

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environmentally controlled conditions as used by seedling production companies before transplanting into greenhouses. The second and third experiments were carried out under normal production conditions, with routine fertilisation and phytosanitation, in a greenhouse in Almería (Spain).

2. MATERIALS AND METHODS

2.1. Bacterial strain

The bacterium used in this study (*Bacillus licheniformis* (B2; CECT 5106)) is deposited in the Spanish Culture Type Bank (CECT). It was isolated from alder and promoted growth of this tree [32], and pine [33]. It also induced systemic resistance in tomato and pepper in greenhouse experiments against *Xanthomonas campestris* (unpublished data).

2.2. Bacterial resistance to the pesticides

Bacterial resistance to the pesticides was carried out following the method of McKeen et al. [27]. All pesticides were provided by Almeriplant S.A.T. and all of them are permitted for use in production greenhouses. The resistance of the bacterium was measured with each pesticide at normal, double, and half the concentration recommended by the manufacturer. Holes of 1 cm in diameter were made in nutritive agar (DIFCO) previously sown with *Bacillus licheniformis* (on Petri plates). Each hole was filled with 150 µl of pesticide, one hole per concentration. Plates were incubated for 24 h at 28 ºC, and the appearance of any inhibition area was observed.

2.3. Experiment I: culture chamber conditions

This experiment was carried out in a culture chamber. The conditions were the same as those used by plant-producing companies during the seedbed phase, before transplanting into the production greenhouse. From sowing until germination, trays were kept in darkness at 30 ºC and 70% of relative humidity. When the seeds germinated, the trays were placed in a culture chamber under the following conditions: 35/25 ºC, 16/8 h light/dark cycle, achieving 350 µE m^{-2} s^{-1}.

2.3.1. Plant material

Two *Lycopersicon esculentum* cultivars (Daniela and Brillante), and two *Capsicum annum* cultivars (Roxy and Antonio) were used. Seeds were kindly provided by Almeriplant S.A.T. (Spain).

2.3.2. Bacterial inoculation and plant growth

Bacteria were grown in 100 mL nutritive broth (DIFCO) in a 250-mL Erlenmeyer flask on a shaker (125 rpm) at 28 ºC for 24 h. The culture was centrifuged (350 *g* for 10 min), washed with sterile water and pellets resuspended in sterile water to achieve 10^8 CFU g^{-1} soil. The enumeration and calculations were carried out following the "drop method" [16]. This procedure was the same in experiments II and III, except that in these two experiments, bacteria were grown in 1000 mL nutritive broth in a 2000-mL Erlenmeyer flask.

The substrate in which seeds were germinated and plants grown was inoculated on two occasions: the first at sowing, and the second 15 d after. In both cases, the soil was soaked with a bacterial suspension to give a concentration of 10^8 cfu per gram of soil, prepared as above. The experiment was carried out following a design of random blocks with 3 replicates and 7 repetitions: twenty-one seeds of each species and cultivar were placed in 1 tray with 500 g of peat (Flora Gard) and a layer of vermiculite, and the other two trays were prepared in the same way. Each tray was considered a replicate and each plant a repetition of this replicate. The controls were designed in the same way, but in this case, these trays were not inoculated.

2.3.3. Biometric analysis

Forty days after germination, the foliage of the plants (stems plus leaves) were cut off, and dried between paper towels. For each plant: height (cm), leaf area (cm^2) and foliage dry weight (g) were measured using an image analyser (Delta T, Devices Inc., England) and DIAS software.

2.3.4. Statistical analysis

Unidirectional analysis of variance (ANOVA) was performed on each variable, and when differences were significant, the LSD (least significant difference) test was performed [39]. This procedure was the same in experiments II and III.

2.4. Experiment II: production conditions

The experiment was carried out in production greenhouses in Almeria (Spain). Tomato and pepper plants were grown in sand and hydroponic culture. The three greenhouses chosen had 1 ha each of these plants. Under greenhouse conditions, tomato seedlings were arranged in rows. In each row, seedlings were 1 m apart with 2 m separation between rows. In this case, there was a total of 5 000 plants. Pepper plants were 50 cm apart within rows with 1 m separation between rows. In total there were 20 000 plants.

2.4.1. Plant material

Cv. Daniela of *Lycopersicon esculentum* and cv. Roxy of *Capsicum annum* were grown in soil. In addition, one cv. Portela *Lycopersicon esculentum* was grown in hydroponic culture. The company Almeriplant S.A.T. carried out the management in the greenhouse, taking into consideration the results of bacterial resistance to the pesticides described earlier.

2.4.2. Bacterial inoculation and plant growth

Twenty days after transplanting, two-month-old plants into the production greenhouses, the plants were inoculated in the following experimental design: fifty plants of each species and cultivar were randomly selected in each greenhouse (separate greenhouses were used for each species, cultivar and culture type). In each greenhouse, the plants had similar biometric parameters. Inoculation was carried out with 1 L of bacterial suspension with 10^8 cfu ml^{-1} per plant. Inoculations were repeated every 20 days from 3 October 2000 until 3 December 2000. The plants grew in the greenhouses under normal production conditions. The amount of fertilisers used (in the experiments II and III) during the time of production (1 October until

30 March) in 1 ha was: 335 Kg N, 110 Kg P_2O_5, 420 Kg K_2O, 210 Kg CaO, 62 Kg MgO and 2% of oligoelements mixture.

2.4.3. Harvest

One-and-a-half months and 3 months after the last inoculation, the number and diameter (cm) of tomato and pepper fruits per plant were measured. Due to an error no data were collected on the number of tomatoes in sand culture at the second sampling time.

2.4.4. Pathologies

At the end of experiment, we observed the percentage of sick plants in each species and the growing system was noted, together with their symptoms.

2.5. Experiment III

The experiment was carried out in production greenhouses in Almeria (Spain). Pepper plants cv. Capino were grown in sand. The greenhouse chosen had 1 ha of these plants. Under greenhouse conditions, pepper seedlings were arranged in rows. In each row, seedlings were 50 cm apart with 1 m separation between rows. In this case, there was a total of 20 000 plants.

2.5.1. Bacterial inoculation and plant growth

To inoculate the plants, 11 litres of bacterial suspension, prepared as we described above, were mixed with 1050 L of water. This mixture was put in a tank joined to an automatic water system "drop to drop".

Twenty days after transplanting, two-month-old plants into the production greenhouses, the plants were inoculated with the mixture described above, in the following experimental design: seven hundred plants were inoculated with 1.5 L of bacterial suspension (amount controlled by computer). Inoculations were repeated every 20 days from 26 October 2000 until 16 March 2000. The plants grew in the greenhouses under normal production conditions.

2.5.2. Harvest

On 18 December, 7 January, 22 January, 9 February, 24 February, 13 March and 27 March, the total weight of pepper harvesting of the seven hundred inoculated plants and the other seven hundred non-inoculated plants was measured.

3. RESULTS

The experiment to assess pesticide resistance showed that *Bacillus licheniformis* is highly resistant to the usual pesticides. The bacterium was resistant to some product in all groups of pesticides (Tab. I), defined by the pathology which they are used against, except in the case of that used for mildew (Ridomil) and for caterpillar (Clorpirifos and Flufenoxuron) (Tab. I).

3.1. Experiment I

Increases in foliage dry weight (g), height of plants (cm) and leaf area (cm^2) in both cultivars of pepper inoculated with

Table I. Pesticide resistance of *Bacillus licheniformis*. +: resistant, –: non-resistant.

Use	Commercial name	2C	C	½ C	Values of C
Wide spectrum	Mancofol	-	-	-	2 g l^{-1}
	Metiltiofanato	+	+	+	1.5 g l^{-1}
	Cuprosan 311	-	-	-	4 g l^{-1}
Aphids	Pirimicard 50%	+	+	+	1 g l^{-1}
Caterpillar	Clorpirifos	-	-	-	1 ml l^{-1}
	Flufenoxuron	-	-	-	0.5 ml l^{-1}
White fly	Metomilo	+	+	+	1.5 g l^{-1}
	Piridaben	+	+	+	0.75 g l^{-1}
	Bufuprezin	+	+	+	0.75 g l^{-1}
	Piriproxifen	-	-	+	0.5 ml l^{-1}
	Clopirifos	-	-	-	1 ml l^{-1}
	Endosulfan	-	-	-	2 ml l^{-1}
Trips	Metomilo	+	+	+	1.5 g l^{-1}
	Metamidofos	+	+	+	1.5 ml l^{-1}
Mildew	Ridomil	-	-	-	2 g l^{-1}
Botrytis	Sumico	+	+	+	1 g l^{-1}
Red Spider	Abamectina	+	+	+	0.5 ml l^{-1}
Fungi of soil	Propamocard	-	-	-	0.5 ml l^{-1}
	Himexazol	+	+	+	0.5 ml l^{-1}
	Pencicluron	+	+	+	0.5 ml l^{-1}

+: bacterial growth unaffected.
–: bacterial growth inhibited.

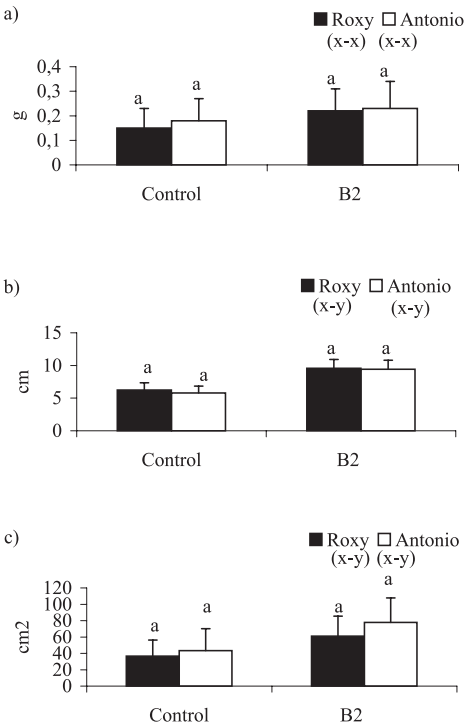

Figure 1. Data of non-inoculated plants (control) and inoculated plants (B2) on: (a) foliage dry weight (stems plus leaves) (g), (b) height of plants (cm), (c) leaf area (cm^2) of two pepper cultivars (Roxy and Antonio). Letters a, b indicate differences between cultivars. Letters x, y indicate differences between control and inoculated plants in each cultivar.

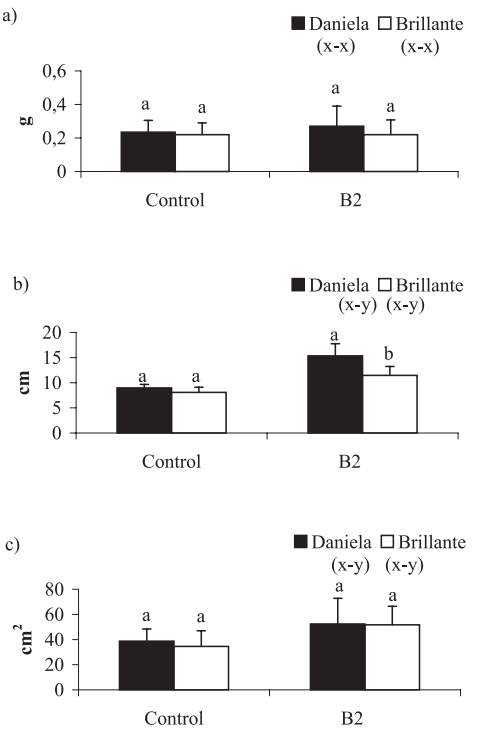

Figure 2. Data of non-inoculated plants (control) and inoculated plants (B2) on: (a) foliage dry weight (stems plus leaves) (g), (b) height of plants (cm), (c) leaf area (cm^2) of two tomato cultivars (Daniela and Brillante). Asterisk indicates differences with regard to control. Letters a, b indicate differences between cultivars. Letters x, y indicate differences between control and inoculated plants in each cultivar.

Bacillus licheniformis vis-a-vis the control plants are shown in Figure 1. There were some growth-promoting effects resulting from inoculation (Fig. 1a–c). All parameters increased with regard to control, but only increases in height of plants (cm) and foliage dry weight (g) were statistically significant (Fig. 1b, c), and increases in these parameters were greater in cv. Antonio than in Roxy. No other significant differences were found.

There were also some increases with inoculated tomato plants (Fig. 2a–c). The height of plants (cm) and leaf area (cm^2) increased significantly with regard to control but not the foliage dry weight (g). The incresases in height of plants (cm) and stem plus leaf dry weight (g), were greater for cv. Daniela than cv. Brillante.

3.2. Experiment II

Under greenhouse conditions and a standard management regime, *Bacillus licheniformis* affected the parameters measured. At one-and-a-half months, the number of fruits per plant and the diameter of fruits (cm) of treated Daniela plants growing in soil were significantly greater than in the control (Tab. II); likewise for cv. Portela in hydroponic conditions (Tab. II). In pepper cv. Roxy, only fruit diameter (cm) was significantly different (Tab. II).

At 3 months, fruit diameter (cm) in tomato cv. Daniela growing in soil and treated with *B. licheniformis* was significantly larger than the control (Tab. III). The same was true for the number of fruits and diameter of treated pepper growing in soil. (Tab. III). However, fruit diameter was significantly decreased compared with control in tomato cv. Portella growing in hydroponic culture (Tab. III).

Figure 3 shows the percentage of sick plants at the end of the experiment. In all cases, there were fewer treated plants with disease than control plants (soft fruits, later turning black).

3.3. Experiment III

Under greenhouse conditions and a standard management regime, *Bacillus licheniformis* affected the parameters measured. In four of the seven harvests carried out, the total weight of the peppers of inoculated plants was significantly greater than in non-inoculated plants. The total weight of the seven harvests also showed significant differences (Fig. 4).

4. DISCUSSION

Disinfected soil or soil-less substrates such as peat, sand or rockwool, commonly used in greenhouses, lack the microbial diversity and biological buffering present in natural soil. In this biological vacuum, soil-borne pathogens such as *Pythium* and

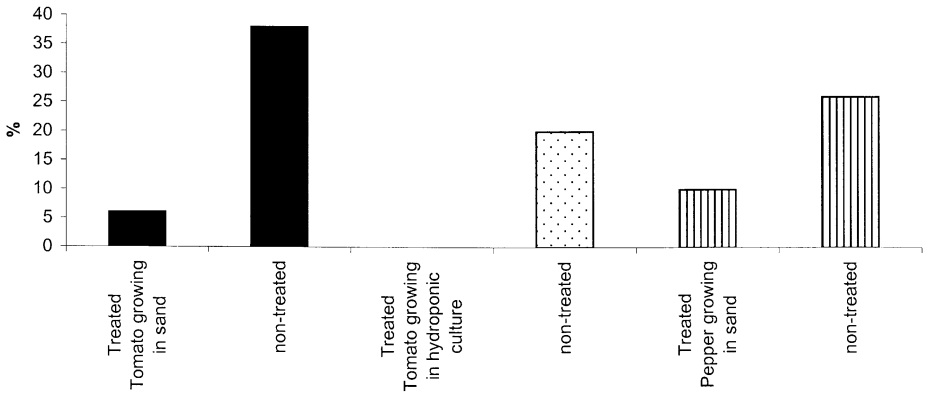

Figure 3. Percentage of sick plants in treated (inoculated with *B. licheniformis*) and non-treated plants under production conditions.

Table II. Results obtained under production conditions at the first sampling time (one-and-a-half months after the last inoculation). Letters a and b show statistical differences between treated and non-treated plants in each parameter.

	Number of fruits	Diameter of fruits (cm)
Tomato growing in sand		
Treated (inoculated with PGPR)	34.26 ± 1.01 a	7.52 ± 0.27 a
Non-treated	27.90 ± 0.58 b	6.23 ± 0.05 b
Pepper growing in sand		
Treated (inoculated with PGPR)	15.20 ± 0.59 a	24.02 ± 0.19 a
Non-treated	12.68 ± 0.50 a	22.66 ± 0.19 b
Tomato growing in hydroponic medium		
Treated (inoculated with PGPR)	29.35 ± 0.67 a	5.92 ± 0.04 a
Non-treated	25.58 ± 0.76 b	5.44 ± 0.05 b

Rhizoctonia can quickly grow and spread. In addition, the life stages of plants most commonly found in greenhouse nurseries are seeds, seedlings and young transplants, all especially susceptible to many pathogens that attack juvenile tissue [31].

Inoculation with the strain of *Bacillus* used in this study has been carried out because of its capacity to induce systemic resistance (ISR) against *Xanthomonas campestris* in tomato and pepper in a greenhouse experiment (unpublished data) and because of its capacity to produce hormones such as auxins or gibberellins in culture media [13, 14]. In previous studies, this strain created sinks of consumption in the aerial system (probably due to translocation of these hormones throughout the plant's vascular system) or improved plant nutrition [33, 36].

Under seedbed conditions inoculated bacteria have to compete with the natural microflora of the peat, more complex than that found in sand or hydroponic production. In these conditions, in both tomato and pepper, increases in the aerial parameters were found (Figs. 1 and 2). The enhancement of foliage dry weight (g) was only significant in pepper plants. The success of the bacterium in seedbeds suggests important competitive and colonisation capacities, necessary characteristics for the success of that bacterium under production greenhouse conditions.

No significant differences were found between tomato cultivars in the studied parameters (Fig. 1). In pepper, significant differences between cultivars appeared only in height of plants (Fig. 2). This indicates that the bacterium does not distinguish between cultivars of the same species; however, bacterium have a greater effect on pepper than on tomato. The establishment of inoculated PGPRs in the root system, showing a closer interaction between the bacteria and pepper roots, is a precondition for beneficial plant growth-promoting effects [17, 25, 26, 46]. Characteristic quantifiers and qualifiers of root exudation play a fundamental role in the colonisation, as do the root structure/architecture and dynamics (e.g. flat rooting versus deep rooting) [4, 29, 41]. In this respect, the root system of pepper presents more surface contact than the root system of tomato, which develops a main root with fewer branches.

Before beginning the same experiment under production conditions, we tested the sensitivity of *B. licheniformis* to the pesticides normally used in these conditions (Tab. I). This testing is very important because the farmer must use only those pesticides compatible with the bacterium. In this experiment we observed that *B. licheniformis* is highly resistant to the pesticides normally used, even at double concentration, indicating that the use of this bacterium does not radically alter the treatments carried out.

The bacterium assayed not only affects the biomass production under seedbed conditions, but also the fructification process and development of the fruit under production conditions. All of the data suggest a hormonal effect. Gibberellins can play

Table III. Results obtained under production conditions at the second sampling time (three months after the last inoculation). Letters a and b show statistical differences between treated and non-treated plants in each parameter.

	Number of fruits	Diameter of fruits (cm)
Tomato growing in sand		
Treated (inoculated with PGPR)		6.18 ± 0.05 a
Non-treated		6.03 ± 0.05 b
Pepper growing in sand		
Treated (inoculated with PGPR)	14.87 ± 0.83 a	7.70 ± 0.11 a
Non-treated	9.00 ± 0.61 b	6.34 ± 0.12 b
Tomato growing in hydroponic medium		
Treated (inoculated with PGPR)	34.27 ± 1.39 a	5.29 ± 0.05 a
Non-treated	32.08 ± 1.49 a	5.97 ± 0.07 b

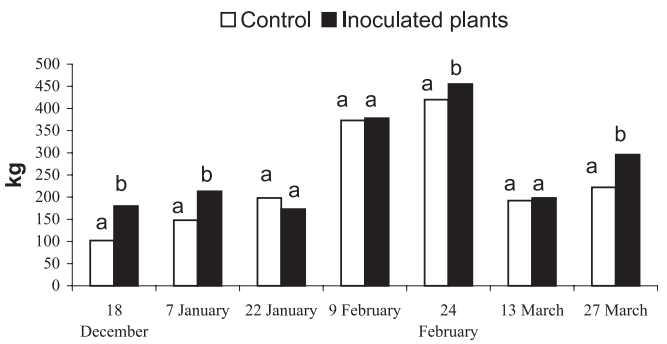

Figure 4. Pepper production under greenhouse conditions at the seven sampling times. Data shown are the total weight of peppers of the seven hundred plants inoculated and the other seven hundred plants non-inoculated. Letters a, b indicate differences between control (non-inoculated plants) and inoculated plants. Total weight (kg) in inoculated plants: 1893.5 a. Total weight (kg) in non-inoculated plants: 1655.5 b.

a very important role because these hormones have a decisive effect on the fructification process [9].

In addition, we noted down the pathologies of the plants throughout the experiment (Fig. 3). There were strikingly fewer pathologies in treated plants. The pathology found in all cases occurred in the fruits, probably due to *Botritys* infection (data pending confirmation). These data suggest that *B. licheniformis* induces some mechanism of systemic resistance (ISR) [42] because the inoculation is at root level and the organ affected by pathologies is the fruit [35, 43]. Another important finding is that we did not detect pathologies in the vascular system of inoculated plants caused by pathogenic fungi (*Phytophthora* and *Fusarium* sp.), which usually produce them. Nevertheless, in control plants, these pathologies affected about 5% of plants, and in all cases, affected plants were changed for healthy ones. These data should be confirmed in further experiments.

The experiments performed show the possibility of using this bacterium in the nursery phase. Normally, plants are kept in the nursery for around 40 days before transplanting. Increases produced by PGPRs in the 40 days of the experiment show that it is possible to shorten the maturation period. However, we must bear in mind the possibility of maintaining the production time to obtain stronger plants with a high adaptative vigour. The experiment carried out in the production greenhouse shows that the PGPR has great colonisation and competitive capacities, which produce very significant effects on plants and on fruit production. These results show the possibility of using this bacterium in production conditions as a biofertiliser or biocontrol agent without altering the normal management. Even more important is the partial or total elimination of some organic pesticides which are very harmful to the environment and to human health.

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