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Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato

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Abstract. Recent works suggest that the combination of several PGPRs could be more effective than individual strains as a horticultural product. LS213 is a product formed by a combination of two PGPRs, *Bacillus subtilis* strain GB03 (a growth-promoting agent), *B. amyloliquefaciens* strain IN937a (an inducer of systemic resistance) and chitosan. The aim of this work is to establish if the combination of three PGPR, *B. licheniformis* CECT 5106, *Pseudomonas fluorescens* CECT 5398 and *Chryseobacterium balustinum* CECT 5399 with LS213 would have a synergistic effect on growth promotion and biocontrol on tomato and pepper against *Fusarium* wilt and *Rhizoctonia* damping off. When individual rhizobacterium and the LS213 were put together, the biometric parameters were higher than with individual rhizobacterium both in tomato and pepper, revealing a synergistic effect on growth promotion, being the most effective combination that of *B. licheniformis* and LS213. When *P. fluorescens* CECT 5398 was applied alone, it gave good results, which could be due to the production of siderophores by this strain. Biocontrol results also indicate that those treatments that combined LS213 and each of the bacteria (Treatments: T7 and T8) gave significantly higher percentages of healthy plants for both tomato (T7: 65%) and pepper (T7: 75% and T8: 70%) than the LS213 alone (45% of healthy plants for tomato and 60% for pepper) three weeks after pathogen attack. The effects in pepper were more marked than in tomato. The best treatment in biocontrol was the combination of *P. fluorescens* and LS213. In summary, the combination of microorganisms gives better results probably due to the different mechanisms used.

Key words: *Bacillus*, biocontrol, *Chryseobacterium*, LS213, PGPR (Plant Growth Promotion Rhizobacteria), *Pseudomonas*

Abbreviations: CECT – *Colección Española de Cultivos Tipo*. Spanish Type Cultures Collection; PGPR – Plant Growth Promotion Rhizobacteria; ISR – Induced systemic resistance

Introduction

Plant growth promotion rhizobacteria are universal symbionts of higher plants, which enhance the adaptative potential of their hosts through a number of mechanisms, such as the fixation of molecular nitrogen, the mobilization of recalcitrant soil nutrients and the synthesis of phytohormones and the control of phytopathogens (Van Peer and Schippers, 1989; Lugtenberg et al., 1991; Weller and Thomashow, 1994).

Studies have shown that plant growth-promoting rhizobacteria can be applied to a wide range of plants for the purpose of disease control and growth enhancement (Tuzun and Kloepper, 1994; Van Loon et al., 1998). Induced systemic resistance (ISR) has been reported as one of the mechanisms by which PGPR reduce plant disease, through the manipulation of the host plant's physical and biochemical properties (Pieterse et al., 2002). PGPR elicited-ISR has been demonstrated in many plant species, including *Arabidopsis* spp., bean, carnation, cucumber, radish, tobacco, tomato (Van Loon et al., 1998).

In addition, PGPRs have a number of traits which allow them to act as biocontrol agents. For example, they produce a number of substances such as siderophores, antibiotics and a variety of enzymes that may be used to limit the damage to plants by fungal pathogens. Most approaches for biocontrol of plant diseases and plant growth promotion have used applications of single biocontrol agents as antagonist to a single pathogen (Wilson and Backman, 1999). This may partially account for the reported inconsistent performance by PGPR, because a single biological agent is not likely to be active in all soil environments in which it is applied, neither will it be active against all pathogens that attack the host plant.

Several works suggest that combinations of biocontrol agents could be more effective in controlling soil-borne pathogens than a single agent (Fukui et al., 1994; Pierson and Weller, 1994; Duffy et al., 1996). For example, a biological preparation termed LS213, which contains industrially formulated spores of *B. subtilis* strain GB03 as a growth-promoting agent, *B. amyloliquefaciens* strain IN937a as an induced systemic resistance (ISR) agent and chitosan, was used to enhance the growth of several vegetable transplant systems and it also provided

ISR activity to various foliar pathogens (Reddy et al., 1999; Kenney et al., 1999; Kloepper et al., 1999). The maximum level of plant growth promotion and ISR activity was achieved through the synergy of the three components used compared to the individual components.

The exact mechanisms by which combinations provide increased plant growth promotion, biocontrol of disease and ISR are not fully understood, although a number of hypotheses, including the synergistic action of antifungal metabolites such as antibiotics and hydrolytic enzymes, have been proposed (Paulitz et al., 1990; Duffy and Weller, 1995; Duffy et al., 1996; Pieterse et al., 2002).

Evidence suggests that these PGPR could be used and that the efficacy of PGPR could be related to local environmental and geographical conditions. Seedling growth response following inoculation with certain PGPR, can be species specific and could be an impediment, to the development of effective soil or seed inoculants for use in greenhouse nurseries, to produce vegetable transplants for transplantation in the field.

On the other hand, most of the approaches about biocontrol assays, challenge a single pathogen (Wilson et al., 1999) though it is normal that the plant in natural conditions is in contact with several ones. Therefore, the control of a wide range of pathogens by applied antagonists largely remain an unfulfilled goal for biological control. Few works on biocontrol have performed the treatment of soil-borne diseases including more than one pathogen, so challenging with several pathogens is a very relevant issue.

In this study our objective was to test whether a combination of PGPR strains would enhance growth promotion, biological control and ISR activity on tomato and pepper transplants under greenhouse conditions, against a mix of pathogens. Therefore, in this study, we decided to combine three different PGPR strains with the biological preparation LS213, which has been shown to enhance plant growth and offer ISR activity, to see if beneficial effects were enhanced as compared to the strains alone or LS213 alone, on pepper and tomato transplants and using the combination of two different soil-borne pathogens (*Rhizoctonia solani* and *Fusarium oxysporum*).

Materials and methods

PGPR strains and inoculum preparation

Three PGPR strains and the biological product LS213 were used. PGPR strains were: *B. licheniformis* CECT 5106 which was isolated

from the rhizosphere of *Alnus glutinosa* (Probanza et al., 1996) and is known to produce high levels of indolacetic acid (IAA) and gibberellins (GAs) (Gutierrez-Mañero et al., 2001), *P. fluorescens* CECT 5398 able to produce IAA and siderophores, and *C. balustinum* CECT 5399, IAA producer, both isolated from the rhizosphere of *Lupinus albus* (Gutierrez-Mañero et al., 2003). These strains were maintained at −80 °C in tryptic soy broth (TSB) amended with 20% glycerol. Inoculum was prepared by streaking strains from −80 °C onto tryptic soy agar (TSA) plates, incubating plates at 28 °C for 24 to 30 h, and scraping bacterial cells off plates in sterile distilled water to yield 10^9 colony forming units (cfu)/ml. The biological preparation LS213 was produced by Gustafson Inc., Dallas, Texas. It contains industrially formulated endospores of *B. subtillis* strain GBO3 and *B. amyloliquefaciens* strain IN937a and industrial grade chitosan as formulation carrier, and stored at room temperature prior to use. In this formulation, density of each strain was 10^{11} cfu/g.

Fungal pathogen and inoculum preparation

Two fungal pathogens *Fusarium oxysporum* f. sp. *radicis-lycopersici* isolate AU-TF1 and *Rhizoctonia solani* (AG-2) isolate AU-TR1, from the culture collection of the Department of Plant Pathology, Auburn University, Alabama, USA were used. These cultures were maintained on potato dextrose agar (PDA) prior to use. For the production of fungal inoculum, these pathogens were grown on PDA for 5–6 days at 28 °C, five to six plates of each were mixed with sterile distilled water in a Waring blender for 2 min. *Fusarium oxysporum* inoculum was adjusted to reach 10^{6-7} conidia per ml. *Rhizoctonia solani* inoculum containing mycelial fungus was adjusted to reach 10^{6-7} mycelial fragments per ml. Both measurements were made using a hemocytometer under light microscopy.

Growth promotion assays

Growth promotion assays were set-up on tomato *cv. Jupiter* and pepper cv *Rutgers*. For each crop, there were 8 treatments defined as follows: T1: Nontreated control; T2: *C. balustinum* CECT 5399; T3: *P. fluorescens* CECT 5398; T4: *B. licheniformis* CECT 5106; T5: LS213; T6: *C. balustinum* CECT 5399 + LS213; T7: *P. fluorescens* CECT 5398 + LS213; T8: *B. licheniformis* CECT 5106 + LS213. Seeds were sown in Styrofoam trays with 128 cavities per tray (Speedling Inc., Bushnel, FL). One tray per treatment was filled with soil-less Speedling-mix

growth media (Speedling Inc., Bushnel, FL) and tomato and pepper seeds were sown on each tray (64 seeds for tomato and 64 seeds for pepper) with one seed per cavity. For treatments containing LS213, it was mixed with soil-less media at a ratio of 1:40 (v/v) prior to filling trays. For all other treatments, PGPR were applied as a seed drench method immediately after seeding (1 ml/seed of 10^{8-9} cfu/ml). Treatments were arranged in a greenhouse and maintained with natural light at 25 ± 2 °C and 20 ± 2 °C at night. During the growing period, 20–10–10 (20% N, 10% P, 10% K) High Nitro Special Peters fertilizer (Scotts. Inc.) was used two weeks after seeding once a week, and plants were watered regularly. Three weeks after seeding for tomato and 4 weeks after seeding for pepper, 10 randomly selected seedlings per treatment were harvested, and seedling height, calliper and shoot fresh weight were measured. Data were analyzed using JMP software (SAS Institute INC., Cary, NC). When analysis of variance showed significant treatment effects, the least significant differences test (LSD) was applied to make comparisons among the means (Sokal and Rohlf, 1979).

Biocontrol experiments

Treatments for biocontrol assays including control with no pathogen were T1: Healthy control (Control without PGPR bacteria and without pathogen); T1a: Pathogen control (control without PGPR bacteria and with a mix of pathogens). Treatments T2–T8 were the same as described for *Growth promotion assays* but in all cases controls without pathogen were used.

Seeds were grown in the greenhouse in Speedling-Mix soil, planting 4 seeds per pot (9 cm × 9 cm). The biological preparation, LS213 (described above) was mixed into Speedling-mix at a ratio of 1:40 (v/v). Five pots were used per treatment. Pathogen inocula (*Fusarium* + *Rhizoctonia*), obtained as explained before, was spread on the seeds. Bacterial inocula were applied as seed drench treatment (1 ml/seed; 10^{9}–10^{10} cfu/ml). Results are expressed as percentage of healthy plants. Healthy plants are those which did not suffer from damping off and maintained rigidity. Data were analyzed using JMP software (SAS Institute INC., Cary, NC). When analysis of variance showed significant effects, the least significant difference test (LSD) was used to make comparisons among the means (Sokal and Rohlf, 1979).

Results

Growth promotion results

Tomato plants grow earlier than pepper plants, so the growth promotion assays were harvested 3 weeks after inoculation for tomato and 4 weeks after inoculation for pepper.

Growth promotion results for tomato appear in Table 1. All treatments that combined LS213 plus one of the bacteria tested were significantly different from the controls, in the three biometric parameters. LS213 alone also showed significant differences, but in all cases combinations performed better than individual bacteria. Among them, the best treatment was the combination of LS213 plus *B. licheniformis* CECT 5106 (T8) which showed significant increases comparing with the rest of the treatments, in all the biometric parameters except for height (see Table 1), in which it did not present significant differences with treatment T7 (LS213 + *P. fluorescens* CECT 5398). On the other hand, *C. balustinum* CECT 5399 (T2) and *B. licheniformis* CECT 5106 (T4) applied alone caused significant increases in caliper compared with the control, while *P. fluorescens* CECT 5398 (T3) significantly increased also height and weight.

Plant growth promotion results for pepper appear in Table 2. Results show that in all cases, significant increases were found when

Table 1. Effect of PGPR on growth of tomato cv. Rutgers transplant plugs 3 weeks after seeding under greenhouse conditions ($n = 10$)

Treatment	Height (cm)	Caliper (mm)	Shoot fresh weight (g)
T1: Nontreated control	4.41^{a}	1.59^{a}	0.24^{a}
T2: *Chryseobacterium balustinum* CECT 5399	4.88^{ab}	1.85^{b}	0.24^{a}
T3: *Pseudomonas fluorescens* CECT 5398	5.71^{b}	2.13^{c}	0.42^{a}
T4: *Bacillus licheniformis* CECT 5106	5.19^{ab}	1.86^{b}	0.34^{a}
T5: LS213	7.50^{c}	2.21^{c}	0.78^{b}
T6: LS213 + *C. balustinum* CECT 5399	10.64^{d}	2.65^{d}	1.54^{d}
T7: LS213 + *P. fluorescens* CECT 5398	12.34^{e}	3.01^{e}	1.16^{c}
T8: LS213 + *B. licheniformis* CECT 5106	12.23^{e}	3.33^{f}	1.89^{e}
LSD ($p = 0.05$)	0.83	0.19	0.19

Different letters indicate significant differences.

Table 2. Effect of PGPR on growth of pepper cv. *Jupiter* transplant plugs 4 weeks after seeding under greenhouse conditions ($n = 10$)

Treatment	Height (cm)	Caliper (mm)	Shoot fresh weight (g)
T1: Nontreated control	4.84^{b}	1.58^{b}	0.350^{b}
T2: *Chryseobacterium balustinum* CECT 5399	3.38^{a}	1.24^{a}	0.150^{a}
T3: *Pseudomonas fluorescens* CECT 5398	4.08^{a}	1.31^{a}	0.190^{a}
T4: *Bacillus licheniformis* CECT 5106	4.03^{a}	1.33^{a}	0.210^{a}
T5: LS213	5.60^{c}	1.83^{c}	0.520^{c}
T6: LS213 + *C.balustinum* CECT 5399	7.55^{e}	2.25^{d}	0.920^{d}
T7: LS213 + *P. fluorescens* CECT 5398	6.65^{d}	2.15^{d}	0.730^{e}
T8: LS213 + *B. licheniformis* CECT 5106	8.75^{f}	2.62^{e}	1.330^{f}
LSD (P = 0.05)	0.76	0.17	0.120

Different letters indicate significant differences.

the soil was treated with LS213, alone, or plus each of the three bacteria. However, as in the case of tomato, coinoculations of each bacteria with LS213 showed the best biometric parameters, indicating a synergistic effect.

In pepper, the best treatment is T8, like in tomato, and also it caused significant increases comparing with all other treatments in the three biometrical parameters tested (see Table 2).

Biocontrol results

Results of biocontrol assays in tomato and pepper appear in Figures 1 and 2 respectively. Data were collected in both experiments 2 and 3 weeks after pathogen challenge, respectively. Both for tomato and pepper, there were marked differences between the healthy and pathogen control. Three weeks after pathogen challenge, the control showed 90 and 85% healthy plants for tomato and pepper, respectively, while in the pathogen control only 30 % healthy plants were registered in both plants.

For tomato, the best results were obtained with the combination of *P. fluorescens* CECT 5398 and LS213 (T7) where significant differences were found compared with the pathogen control (T1a 50% and 30% healthy plants), being the percentage of healthy plants 70% and 65%, 2 and 3 weeks after pathogen challenge, respectively. In general, the combination of LS213 with either of the three bacterial strains increased the percentage of healthy plants.

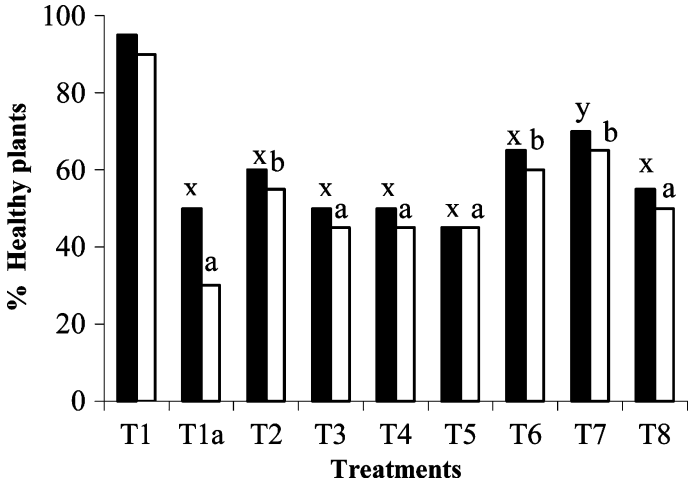

Figure 1. Biocontrol of tomato seedlings grown in *Fusarium* and *Rhizoctonia* infested soil-less mix. T1: Healthy control; T1a: Pathogen Control; T2: *C. balustinum* CECT 5399; T3: *P. fluorescens* CECT 5398; T4: *B. licheniformis* CECT 5106; T5: LS213; T6: *C. balustinum* CECT 5399 + LS213; T7: *P. fluorescens* CECT 5398 + LS213; T8: *B. licheniformis* CECT 5106 + LS213. Different letters indicate significant differences with the pathogen control T1a. (*x*, *y*: 2 weeks after pathogen attack; *a*, *b*: 3 weeks after pathogen attack; $n = 20$) . ■ Percentage of healthy plants after 2 weeks of pathogen attack. □ Percentage of healthy plants after 3 weeks of pathogen attack.

For pepper, biocontrol assay results were better than in tomato. Here, significant differences were found in treatments T2, T4, T5, T6, T7 and T8, 3 weeks after pathogen attack. Again, the best treatment was LS213 plus *P. fluorescens* CECT 5398 (T7) with 85% and 75% of healthy plants, 2 and 3 weeks after pathogen challenge, respectively. Treatments T6, (LS213 and *B. licheniformis* CECT 5106) and T8, (*C. balustinum* CECT 5399) presented 75% and 70% of healthy plants 3 weeks after pathogen attack. With LS213 (T5) results were, 70 % and 60 % of healthy plants two and three weeks after pathogen challenge, respectively. On the other hand, treatments T2, T3 and T4 are performing better than the pathogen treated control, but the number of healthy plants are slightly lower than in the treatments combining LS213 with the rhizobacteria.

Discussion

Since the discovery of the PGPR bacteria by Kloepper et al., (1980), many studies have been made to evaluate the mechanisms by which

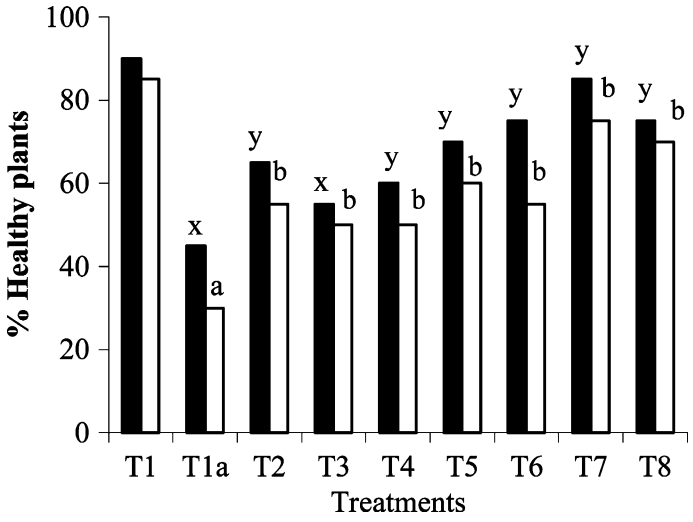

Figure 2. Biocontrol of pepper seedlings grown in *Fusarium* and *Rhizoctonia* infested soil-less mix. T1: Healthy control; T1a: Pathogen Control; T2: *C. balustinum* CECT 5399; T3: *P. fluorescens* CECT 5398; T4: *B. licheniformis* CECT 5106; T5: LS213; T6: *C. balustinum* CECT 5399 + LS213; T7: *P. fluorescens* CECT 5398 + LS213; T8: *B. licheniformis* CECT 5106 + LS213. Different letters indicate significant differences with the pathogen control T1a. (*x*, *y*: at 2 weeks after pathogen attack; *a*, *b*: at 3 weeks after pathogen attack; $n = 20$). ■ Percentage of healthy plants after 2 weeks of pathogen attack. □ Percentage of healthy plants after 3 weeks of pathogen attack.

these bacteria produce these benefits in the plant (Van Loon et al., 1998; Bowen and Rovira, 1999; Kravchenko et al., 2002). Many studies have been carried out in order to obtain an adequate and suitable biological product that could be used for agricultural and horticultural purposes, instead of the chemical ones that also have diverse negative effects on nature and human beings.

Fusarium wilts and take-all by *Rhizoctonia* are severe diseases of many plant species. Many researchers have attempted the development of biocontrol agents against soil-borne plant pathogens. On that last point, the studies of Duffy et al., (1996) and the most recent studies of Raupach and Kloepper (1998), have demonstrated that the use of more than one biocontrol bacterial agent could be more effective than the use of a single one. Resulting from these studies, the commercial product LS213 has been formulated, as it enhances growth of various vegetable transplants plugs and suppresses plant disease (Hallman et al., 1999; Kloepper et al., 1999; Reddy et al., 1999).

Continuing with the labour of improving the effectiveness of the LS213, in this study we have tried to elucidate if the combination of

this product with three PGPR bacteria increases the growth promoting effect and also the capacity of biocontrol and ISR of the new combination and thus, if a synergistic effect is produced.

The use of a combination of biocontrol agents intends to achieve better results based on the fact that each biocontrol agent may use a different mechanism to fight the pathogen, thus better results would be achieved than with a single one (de Boer et al., 1999). In addition, the innovation in these experiments is using a combination of more than one pathogen in more than one plant species, since most approaches for biocontrol of plant diseases have used single biocontrol agents as antagonist to a single pathogen (Wilson et al., 1999) but single biocontrol agents are not likely to be active in all soil environments in which they are applied or against all pathogens that attack the host plant.

In the case of tomato the combination of LS213 and *P. fluorescens* CECT 5398 increased all parameters studied (caliper, fresh weight and height) achieving the highest values. The second best treatment, was the combination of LS213 plus *B. licheniformis* CECT 5106, and in the third place, the combination of LS213 and *C. balustinum* cect 5399. Even though LS213 also produced significant increases compared with the control, the results were not so good as in the treatments where it was combined with the PGPR tested. Significant differences were also produced by *P. fluorescens* CECT 5398 alone, consistent with the best results obtained with this bacteria and LS213.

The results obtained for pepper were very similar to those obtained for tomato, and here, also the treatments that showed significant differences with the control were those that combined LS213 and each of the bacteria, though here the best combination was LS213 plus *B. licheniformis* CECT 5106. However, few differences were found when compared with the other treatments that combined LS213 with the other PGPR.

Earlier studies showed the ability of *B. licheniformis* CECT 5106 to release auxin-like compounds (Gutierrez Mañero et al., 1996) and gibberellins (Gutierrez Mañero et al., 2001) in culture media. Production of auxin-like compounds could increase root parameters proportionally to an increase in height and calliper (Selvadurai et al., 1991), since auxins affect root growth, as well as root growth patterns, increasing root–soil surface, therefore improving nutrient and water absorption potential (Germida and Walley, 1996). This improvement can strongly influence plant growth capacity. However, the high increase in the aerial parameters could be due to the gibberellins produced by this strain (Gutierrez Mañero et al., 2001). It is probably the synergistic effect of both hormones that results in such an increase

in growth, which is higher in the combination LS213 + *B. licheniformis* CECT 5106 than in the other combinations tested, since gibberellin production is a very rare feature in bacterial systems (Gutierrez Mañero et al., 2001).

However, and independently of the magnitude of the effect, a synergistic effect seems to be evident between LS213 and the PGPR strains. This may point out a common mechanism of action, or it may be an additive, complementary or even synergistic mechanism between the bacterial strains, in which plant growth regulators must play an important role. Chitosan itself has been reported to elicit ISR in some circumstances. To clarify the roles of chitosan and the two bacterial strains in LS213, the response of plants to chitosan alone was compared to the response to LS213 (Kloepper et al., 2004). In this study, tomato, cucumber, pepper, and tobacco treated with LS213 had significantly greater height, shoot fresh weight, numbers of leaves per plant, and leaf surface area, compared to plants treated with chitosan alone (Kloepper et al., 2004). Further, ISR was elicited by LS213 but not by chitosan alone on tomato against bacterial spot caused by *Axonopodis campestris* pv. *vesicatoria* (Kloepper et al., 2004).

As regards to biocontrol activity, effects were different depending on the plant. While percentage of healthy plants significantly increased under the influence of all treatments in pepper, it was only affected by *C. balustinum* and the combination of *C. balustinum* and LS213 and *P. fluorescens* and LS213 in tomato. This difference in response is likely due to specificity in the type of pathogen that is antagonized by the bacteria used in the study. Hence, some specificity between the plant species and the bacterial strain is evidenced by this difference, consistent with the results reported by other authors regarding the growth promoting effect of certain bacterial species (Wiehe and Hoflich, 1995). This is also in agreement with the results obtained for growth promotion and supports the notion of the different bacterial mechanisms. Our results show that *B. licheniformis* has the greatest effect on growth probably due to the gibberellin effect, while the other bacterial strains must use other mechanisms which seem to be related to biocontrol or ISR. Consistent with this hypothesis, *P. fluorescens* CECT 5398 has the ability to produce siderophores *in vitro* (Lucas Garcia et al., 2003) which is an ISR and biocontrol related trait (Van Loon et al., 1998), this could explain its good performance with LS213 in tomato and pepper. However, *C. balustinum* CECT 5399 must use other mechanisms since it achieves the best results either alone or combined with LS213 in both plant species.

Results presented here indicate that the plant growth promotion and biocontrol can be enhanced via combination of LS213 and other PGPR, and that a synergy of components occurred such that the maximum level of plant growth promotion (LS213 + *B. licheniformis* CECT 5106) and the consistency of this and biological control was maximum in some of the treatments (LS213 + *P. fluorescens* CECT 5398). This process takes place not only in one single plant species but as it is demonstrated here, in tomato and pepper. It can be said that PGPR and biocontrol effects are increased significantly and that the results are highly satisfactory. So it can be concluded that the effectiveness of LS213 can be improved when combined with either of these bacteria. However, more experiments need to be done to check if these synergistic effects are also produced under field conditions.

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