American Journal of Agricultural and Biological Sciences 6 (2): 189-195, 2011
ISSN 1557-4989

Biocontrol of Soil Fungi in Tomato with Microencapsulates Containing *Bacillus subtilis*

[1]Marcela Hernández-Suárez, [1]Francisco Daniel Hernández-Castillo, [1]Gabriel Gallegos-Morales, [2]R. Hugo Lira-Saldivar, [3]Raul Rodríguez-Herrera and [3]Cristóbal N. Aguilar
[1]Departament of Agricultural Parasitology,
Universidad Autonoma Agraria Antonio Narro, Buenavista, 25315,
[2]Department of Plastics in the Agriculture, Centro de Investigación en Química Aplicada,
[3]Departament of Food Science and Technology, School of Chemistry,
Universidad Autonoma de Coahuila, 25280,
Saltillo, Coahuila, 25253, Mexico

Abstract: Problem statement: An option to reduce pollution by synthetic agro-chemical in root plant disease management is the use of antagonist rhizobacteria belonging to *Bacillus* genus, because their inhibitory properties, stimulation of plant growth and crop yield increase. **Approach:** This study was carried out in order to evaluate if *Bacillus subtilis* strains could play an antagonists role of plant pathogens and if they can be microencapsulated inside a biopolymer matrix. It was adapted an equipment and evaluated a technique for microcapsules elaboration, in order to incorporate *B. subtilis* strains and to analyze their potential as biocontrol agents by determining their antagonistic effect against pathogenic soil fungi; in addition, it was analyzed their effect on tomato plant growth promotion under greenhouse conditions. *B. subtilis* strains identified as B1, J1, M2 and their mixture were used; microcapsules containing bacterial strains were inoculated to tomato seeds cv. Floradade. When seedlings emerged, a second application of microcapsules containing *B. subtilis* was performed on the pots, which previously were inoculated with the fungi *Rhizoctonia solani* and *Fusarium oxysporum*. Response variables were: Incidence and disease severity, plant growth, aerial and root dry weight, leaf area and fruit yield. **Results:** The outcome showed that the equipment designed and adapted for microcapsules elaboration was useful to obtain microcapsules containing the bacterial strains. *B. subtilis* strains exerted apparent biocontrol, since incidence and disease severity was reduced and for that reason inhibited the infective activity of the inoculated plant pathogens, also microcapsules containing *Bacillus* strains stimulated tomato growth and fruit yield. **Conclusion:** Microcapsules containing *B. subtilis* strains could be effective biocontrol agents against soil fungi plant pathogens and could have a potential biofertilizer effect, since they stimulated growth and yield of tomato plants compared to control.

Key words: *Rhizoctonia solani*, *fusarium oxysporum*, biological control, microcapsules elaboration, biocontrol agents, *bacillus subtilis* strains, Plant Growth Promoting Bacteria (PGPR), *F. oxysporum*, antagonist rhizobacteria, *R. solani*

INTRODUCTION

An option for reducing pollution caused by the use of synthetic agrochemical in tomato disease management is biocontrol by using of antagonist rhizobacteria belonging to the *Bacillus* genus, because they are considered the most efficient for their inhibitory properties (Hernández-Castillo *et al*., 2008, Mahadtanapuk, *et al*., 2007), stimulation of plant growth and crop yield enhancer (Wahyudi *et al*., 2011). Plant Growth Promoting Bacteria (PGPR) also help in solubilization of mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates and improve soil structure and organic matter content (Al-Taweil *et al*., 2009; Shah *et al*., 2006). PGPR retain more soil organic N and other nutrients in the plant-soil system, thus reducing the need for fertilizer N and P and enhancing release of the nutrients (Hayat *et al*., 2010; Baset *et al*., 2010). Therefore, the inhibitory effect of biocontrol agent and

Corresponding Author: Francisco Daniel Hernández-Castillo, Department of Agricultural Parasitology, Universidad Autónoma Agraria Antonio Narro, Buenavista, 25315, Saltillo, Coahuila, Mexico Tel: +52 844 411 03 26 Fax: +52 844 411 02 26

beneficial bacteria on crop diseases and their use as biofertilizers is considered a sustainable and efficient technique for eco friendly farming (De Curtis *et al*., 2010; Haghighi *et al*., 2010).

In the world market, tomato (*Lycopersicon esculentum* Mill.) is placed in the third place because its volume of production. In addition, it is the vegetable more consumed around the world. This commodity is cultivated under greenhouse and field conditions, reaching the fruits of this vegetable crop high price in the international market during some seasons; however this crop is attacked by many pathogens (De Curtis *et al*., 2010). Crown root rot is caused by *F. oxysporum* which reduce yield to 50%; besides, this fungus causes severe root rot and extensive necrotic lesions in the stalk base, provoking a rot in secondary roots, wither and finally plant death (De Araujo *et al*., 2009; Nihorimbere *et al*., 2010). Prevention and control of this severe disease has been based on different strategies, mainly on the use of synthetic agrochemicals which had not been successful to eradicate the pathogen, causing pathogen resistance to this kind of pesticides (Siddiqui and Akhtar, 2009). Based on that background and the severe environmental problems caused by synthetic agrochemicals around the world, novel organic and biological options have been evaluated in order to prevent and control diverse plant pathogens, given that these options are more eco-friendly. By this reason, the use of antagonist microorganisms for biological control of pathogenic fungi like *R. solani, F. solani, F. oxysporum, Phytophtora infestans, Botrytis cineraea* and many others is a viable option. Therefore, this organic approach may represent an effective solution where other chemical options had been unsuccessful (Baniasadi *et al*., 2009).

On the other hand, excessive utilization of synthetic fertilizers results in high production costs and pollution of soils and water (Kachari and Korla, 2009; Lana, 2009). In the last two decades, one of the study areas that are positively impacting sustainable agriculture is the application of biofertilizers and other organic compounds (Farahvash *et al*., 2010; Haghighi *et al*., 2010) Most of the biofertilizers are based on bacteria and fungi that are living in a symbiotic way with plants; this is a positive way to organic fertilization of different crops like leguminous, grains and others (Pathak and Godika, 2010; Al-Taweil *et al*., 2009). Microencapsulation in a biodegradable matrix based on chitosan, guar gum, arabic gum, sodium alginate and other biopolymers could be an effective way to carry beneficial microorganisms (Puoci *et al*., 2008; Bashan *et al*., 2002); these bio compounds encapsulate live cells and protect them against environmental stress. Additionally, when biopolymers are degraded, microorganisms are released in a gradual way (Petrus *et al*., 2009; Aguilar *et al*., 2008). Consequently microcapsules (MIC) represent some advantages because MIC can be stored to room temperature for long periods, could offers constant quality, a better environment for microorganisms and can be easily manipulate (Chong *et al*., 2009; Bashan *et al*., 2002).

The microbial cells immobilization concept is to store up beneficial microorganisms inside a matrix or microsphere (Licy *et al*., 2010). This technique can produce many useful compounds for pharmaceutical, agricultural, food industries and for application of organic acids, amino acids, enzymes and for biodegradation of toxic materials (bioremediation) during an extensive period of time (Adeyemi, 2009). MIC are a special form of packing, where a material of reduced size is covered in an individual way; in the microencapsulation term are included MIC, micro particles, nanocapsules and bioactive substances trapped or soaked in a polymeric matrix (Rochmadi *et al*., 2010). Formulations with encapsulated bacteria for agricultural uses have at least two objectives: (a) to protect in a temporal way, encapsulated microorganisms against adverse soil a biotic factors (Mirzakhani *et al*., 2009; Ghaly *et al*., 2007) and b) progressively to release the trapped microorganisms in a way that efficiently colonizes crop roots (Fery *et al*., 2004; Yoon and Kinam, 2004). Based on the need of having biological options for prevention and control of plant diseases, the present study was carried out under laboratory and greenhouse conditions with the following objectives: (a) to design an evaluate the efficiency of an equipment for microencapsulation, (b) to analyze the antagonistic effect against *R. solani* and *F. oxysporum* of MIC containing three *B. subtilis* strains and a mixture of them. In addition we analyzed the stimulating effect of MIC on tomato growth and yield.

MATERIALS AND METHODS

Obtaining of MIC containing *B. subtilis* strains was performed using equipment designed based on a prototype originally designed and reported (Organisation for Economic Co-operation and Development, 1997), but some adaptations were implemented for the present study.

Microencapsulates preparation: Three strains of *B. subtilis* which were coded as B1, J1, M2 and a mixture of all of them (B1J1M2) were used in this study. The bacterial strains were isolated from agricultural

commercial field. The strains were growth on nutritive agar and then they were placed on nutritive broth and incubated during five days at 37°C with agitation to 200 rpm. Bacterial spores were counted in order to obtain 1×10^9 spores mL^{-1}. Finally, 20 mL of the adjusted suspension were mixed with 80 mL of sterile sodium alginate (2%). Then, this solution was pour into the microencapsulation container and the spraying was started. Microencapsulates were formed after the contact of alginate mixture and *Bacillus* spores with $CaCl_2$ which was added previously in the bottom of a stainless steel tray. After that, microcapsules formed were separated and placed on Petri dishes which had filter paper and then, microcapsules were dried at 40 °C for 72 h. Total amount of bacterial spores were counted using a Neubauer chamber, for this was necessary to dissolve the MIC in a solution containing sodium bicarbonate at 4% concentration, spores were counted under light microscope.

Seed inoculation with microencapsulates: Tomato seeds cv. Floradade previously washed and dried to room temperature were inoculated by hand; seeds were watered with a solution of glue solution (resistol 0.5 %) and mixed with MIC (Bashan *et al*., 2002) in order to secure their adherence to tomato seeds (Fig. 1).

Seedling production and inoculation of plant pathogens: Seeds inoculated with *B. subtilis* MIC were planted in polystyrene trays with 200 cells; peat-moss and perlite were used as substrate (50:50 p/p). Once seedling reached a height of 10 cm were transplanted in plastic pots of 10L of capacity, which contained 5Kg of soil, which previously was infested with 400,000 propagules mL^{-1} of *R. solani* and the same amount of spores for *F. oxysporum*. Pots were placed under greenhouse conditions for 90 days, diurnal temperature inside the greenhouse averaged 24-26°C; pots were distributed under a complete randomized design with twelve replications. In total, six treatment were tested, each bacterial strain was evaluated individually (B1, J1, M2) and a mixture (B1J1M2) of these bacterial strains was also evaluated. Two control treatments were included; (1) a chemical treatment (TQ) tiabendazol, using the concentration recommended by the manufacturer and (2) an absolute control (TA) (without addition of microcapsules). Inoculation of *B. subtilis* MIC to pots was performed before transplanting by adding 10 mg (1×10^9 spores mL^{-1} approximately) in a small hole done previously in the center of the pot which previous was inoculated with *R. solani* and *F. oxysporum* fungi.

Fig. 1: Tomato seed treated and covered with an outside layer containing strains of *Bacillus subtilis* microcapsules.

Plant response variable: Thirty and sixty days after transplanting (dat), the follow parameters were evaluated on four plants per experimental unit: Plant height, fresh and dry stalk, leaves and root weight and foliar area. At harvest (90 dat), all mentioned response variables were measured again. In addition were evaluated fruit weight and diameter, disease incidence and severity caused by soil pathogens. Plant were evaluated as diseased if they showed the stalk typical symptoms like necrotic lesions, wither, obscure and stalk bark and root central cylinder destruction. The proportion of roots damaged was expressed as percentage. In order to establish the level of damage severity by plant pathogens root weight was determined in all treatments. MIC treatments were compared with the controls. Damage severity was determined according to the scale proposed by Engelhard (1986) which establish a range from 1 to 5, which is related to leaf necrotic symptoms; damaged stalk vascular tubes; bleached leaves, stunted and dead plants.

RESULTS

Plant growth and leaf area: Enhancement of plants growth and leaf area of tomato plants by treatments applied compared to the control (Table 1) were statistical significant ($p<0.01$). Moreover the mixture of all strains (B1J1M2) demonstrated to be more effective on stimulating plant growth, since the highest plants attained 121.05 cm when they received this treatment. Although, we detected that this result was statistically similar than the outcome when we used B1 and J1 strains. Plants treated with MIC were 18.2% taller than plants from control pots, as well than those receiving the TQ treatment. In our study, we confirmed a positive effect on leaf expansion when MIC were applied, since tomato plants produced more foliage and growth by comparison to controls (TQ and TA) which reported 48.4 and 44.6% less foliar area respectively.

Table 1: Height and leaf area of tomato plants cv. Floradade subjected to different treatments with microencapsulated strains of *Bacillus subtilis*

Treatments	Height (cm)	Leaf area (cm^2)
Bacillus B1	119.47 a	6857.01 b
Bacillus J1	118.65 a	7762.92 a
Bacillus M2	102.95 b	5393.32 b
Mixture B1J1M2	121.05 a	7022.90 a
*TQ	99.05 b	4007.51 c
**TA	98.9 b	4302.63 bc
CV (%)	3.11	9.29

*: Chemical control treatment; **: Absolute control treatment, Values with same letters are not statistically different (Tukey, p≤0.01)

Table 2: Biomass (g) production of tomato plants cv. Floradade subjected to different treatments with microcapsules containing strains of *Bacillus subtilis*

Treatments	Leaves	Stems	Roots
Bacillus B1	116.41 a	30.31 ab	31.28 c
Bacillus J1	107.57 a	31.92 ab	38.76 b
Bacillus M2	87.67 b	27.90 b	32.04 c
Mixture B1J1M2	94.51 b	34.69 a	96.63 a
*TQ	82.41 c	28.06 b	33.38 bc
**TA	26.21 d	14.57 c	13.92 d
CV (%)	6.54	8.32	6.97

*: Chemical control treatment; **: Absolute control treatment, Values with same letters are not statistically different (Tukey, p≤0.01)

Table 3: Disease incidence and severity at harvest time of tomato plants cv. Floradade subjected to different treatments with microcapsules containing strains of *Bacillus subtilis*

Treatments	Incidence (%)	Severity
Bacillus B1	0.0 d	0.0 c
Bacillus J1	0.0 d	0.0 c
Bacillus M2	12.0 c	1.5 c
Mixture B1J1M2	0.0 d	0.0 c
*TQ	27.0 b	3.5 b
**TA	75.0 a	5.0 a
CV (%)	10.4	1.2

*: Chemical control treatment; **: Absolute control treatment, Values with same letters are not statistically different (Tukey, p≤0.01)

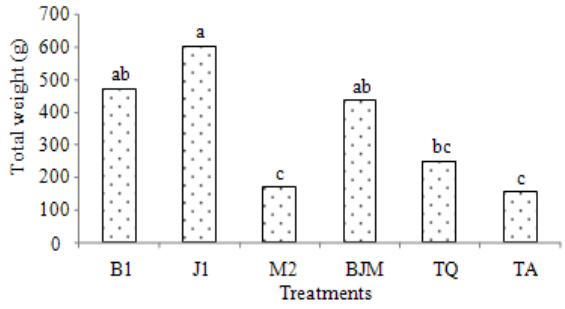

Fig. 2: Average fruit yield of tomato plants cv. Floradade under greenhouse conditions subjected to different treatments with microcapsules containing strains of *Bacillus subtilis*, a chemical control (TQ) and a blank (TA)

Total plant biomass production and tomato yield: The application of *Bacillus* MIC clearly influenced the stimulation of plant biomass (Table 2). The maximum values (116.41 and 107.57 g $plant^{-1}$) were detected when B1 and J1 strains were used, these values were statistically different ($p<0.01$) to those attained with the other treatments studied. The difference (26.21 g) between the dry biomass produced by plants treated with B1 strain compared to the absolute control was remarkable, since this value represents a difference of 77.5%. Therefore plant biomass was improved with the addition of *Bacillus* MIC. The use of the B1J1M2 mixture of strains reported an increase of 34.7 g $plant^{-1}$, being this result statistically significant ($p<0.01$) than that obtained with the two control treatments, since they reported 28.06 and 14.57 g $plant^{-1}$ for TQ and TA treatments, respectively. Microcapsules containing *B. subtilis* strains also promoted an increase on root biomass, given that this parameter reported statistical differences ($p<0.01$) by comparison to treatments where MIC were not applied. Strains mixture influenced positively root growth, because B1J1M2 treatment produced 96.6 g $plant^{-1}$, while plants from TA treatment generated only 13.9 g $plant^{-1}$, this difference represents 85.6% less root biomass among plants with and without adding of MIC. On the other hand, the lowest root biomass value (33.4 g $plant^{-1}$) was attained from TQ plants grown with application of tiabendazole; this difference represents a decline of 65% in comparison to plants treated with the mixture of *Bacillus* strains. Statistical analysis showed that treatments with *Bacillus* MIC resulted in significant total yield increases ($p<0.01$). The addition of strain J1 allowed reaching a value of 600 g $plant^{-1}$ of fruit (Fig. 2), while the control (TA) only reported 180 g $plant^{-1}$; this difference represents 70% decline of total fruit yield per plant.

Disease incidence and severity on tomato plants: A very encouraging result of our work was the nil incidence of root damage of tomato plants at the end of the season by the fungi *R. solani* and *F. oxysporum* (Table 3), since no symptoms of injure was detected in all treatments that received applications of MIC containing the *Bacillus* strains B1 and J1, as well their mixture (B1J1M2). However, disease incidence of control plants was clearly evident since they reported 75% of total plants with symptoms of the disease. In addition, the control treatment also presented a significant severity of the disease aggravated by the attack of the phytopathogenic fungi, implicating a partial or total death of tomato plants.

DISCUSSION

The application of MIC containing the strains of *B. subtilis*, affected positively plant growth, leaf area, biomass, tomato yield; on the other hand they reduced *R. solani* and *F. oxysporum* wilt damages. In a comparable study (Nihorimbere *et al*., 2010), it was reported the biocontrol potential of *B. subtilis* S499 on seeds, since this strain significantly increased growth and yield of tomato plants and also provided a high level of protection against the disease caused by *Fusarium* pathogen. The growth of tomato plants after being treated with *B. subtilis* increased fresh weight of the shoot and production of fruits, but reduced the fresh weight of roots when compared with the control (De Araujo *et al*., 2009). In the same experiment, also was observed an increase on the number of ripe fruits in the treatment with rhizobacteria, therefore it was suggested that *B. Subtilis* served as growth bioregulator of tomatoes. In other horticultural crops such as banana, the inoculation with rhizobacteria also had demonstrated that affect positively some growth attributes like; leaf area, chlorophyll content and consequently total biomass (Baset *et al*., 2010). *B. licheniformis* N1, previously developed as a biofungicide formulation to gray mold disease of tomato and strawberry plants, was investigated and the findings indicate that purified cyclic lipopeptides were the responsible agent for the antimicrobial activity of the N1 strain. Compounds applied at 500 mμ g/ml concentration controlled tomato gray mold, tomato late blight and pepper anthracnose effectively with over 70% disease control value.

Studying the effect of four biofertilizers on growth and yield of cauliflower during two years, Kachari and Korla (2009), reported that even though the treatments performed differently during both years, nitrogen and potassium gave consistent results during both the years with respect to growth and yield attributing characters. Biocontrol of soil pathogenic fungi on tomato by means of delivering antagonistic bacteria through a drip irrigation system also reported a significant increase on growth and yield of tomato plants, promoting a 23% increase over the control plants (De Curtis *et al*., 2010). After performing several studies with growth promoting bacteria on lettuce under greenhouse and field conditions (Vargas *et al*., 2001) it was found that *Hafnia alvei* P-3 strain increased seed germination by 36.5% in comparison to the control, while under field conditions, the same strain increased plant dry weight and root volume by 371% and 300% respectively. In addition, this strain promoted an increment of leaf area by 240%. Application of antagonistic fungi and plant growth-promoting rhizobacteria (*B. subtilis*, *Paenibacillus polymyxa* and *Burkholderia cepacia*) caused a significant ($p<0.05$) increase in tomato growth (based on shoot dry weight), both with and without nematodes (Shah *et al*., 2006). These results clearly show a stimulating effect of *Bacillus* strains, similar to that produced by other rhizobacteria with biofertilizer activity (Wahyudi, 2011; Farzana and Radizah, 2005). For the particular case of tomato, there are some reports demonstrating the beneficial effects of *B. subtilis* on field grown tomato; also the practical applications of bacterial biofertilizers which stimulates root biomass production had been demonstrated (Mahadtanapuk *et al*., 2007).

According to our results, *B. subtilis* strains reduced disease incidence and severity of *R. solani* and *F. oxysporum* on tomato plants. Similar findings had been reported when two commercial biofungicides based on *B. subtilis* (BSF4) and *Trichoderma asperellum* were applied to the soil and evaluated on tomato plants (De Curtis *et al*., 2010) these bacteria significantly reduced both incidence and severity of the diseases caused by *Sclerotium rolfsii* or *Rizoctonia solani*, the isolate T1A-2B reduced up to 58.33% and up to 63.8% the severity of the diseases caused by *S. rolfsii* and *R. solani* respectively; whereas isolate T4B-2A gave reduction of *S. rolfsii* and *R. solani* diseases severity up to 73.2% and up to 62.7%, respectively.

Based on the above results, we consider that the application of *Bacillus* strains incorporated into microcapsules and tested in this trial may be a potential alternative tool for use in an integrated management program for fungi diseases on tomato crop. Our results provided insight for the development of microcapsules which incorporates in their core bacterial isolates, that could represents a suitable method for a more efficient distribution of antagonists in the field, in order to have a better fungal control of root rot on horticultural crops.

CONCLUSION

The use of microencapsulated spores of *B. subtilis* as plant growth promoter or biofertilizer represents an attractive option for sustainable agriculture, because their stimulating effects on plant growth, biomass production and their potential for increasing tomato fruit yield. Our results demonstrated a clear and efficient biocontrol of phytopathogenic fungi, since a low disease incidence and severity of tomato roots damage was evidenced. For that reason this study suggests that *B. subtilis* strains incorporated into MIC can be used as an effective biocontrol agent and as biofertilizer. However, further studies are needed in order to evaluate MIC efficiency on other horticultural crops under greenhouse and field conditions.

ACKNOWLEDGEMENT

Researchers want to thank the financial support of the National Council of Science and Technology (CONACYT) Mexico, through the grant: 138837-PROINNOVA-C0003-2010-02 given to Agro Biological Control S. A. de C. V., to develop this project in the frame of a collaborative program with the Universidad Autónoma Agraria Antonio Narro and the Universidad Autónoma de Coahuila. Also authors want to thank to CEMAP for all technical support.

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