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Nitrogen Biofixing Bacteria Compensate for the Yield Loss Caused by Viral Satellite RNA Associated with Cucumber Mosaic Virus in Tomato

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To overcome the problem of the yield reduction due to the viral satellite mediated protection, a culture mix of three nitrogen-fixing bacteria species of the genus *Azospirillum* (*A. brasilienses* N040, *A. brasilienses* SP7, and *A. lipoferum* MRB16), and one strain of cyanobacteria (*Anabena oryzae* Fritsch) were utilized as biofertilizer mixture in both greenhouse and field experiments. When protected plants were treated with biofertilizer mixtures, the fruit yield of biofertilized plants increased by 48% and 40% in a greenhouse and field experiment, respectively, compared to untreated plants inoculated with the protective viral strain alone. Polyacrylamide gel electrophoresis (PAGE) analysis of total nucleic acid (TNA) extracts revealed that biofertilization did not affect the accumulation of the viral satellite RNA (CARNA 5) that is required for plant protection against other destructive viral strains of CMV. The yield increment was a good compensation for the yield loss caused by the use of the protective viral strain associated with CARNA 5.

Keywords : Biofertilizers, Viral satellite RNA, CMV, CARNA 5

Recently, there has been a growing interest in the use of viral satellite RNAs, such as *Cucumber mosaic virus* (CMV)-associated RNA 5 (herein abbreviated CARNA 5), as biological control agents against severe strains of CMV (Gallitelli et al., 1991; Montasser et al., 1991, 2006). However, protected plants with a combination of a mild strain of CMV, as a helper virus, and CARNA 5 as a biological control agent resulted in about 15-20% yield loss (Montasser et al., 1991). To overcome the problem of yield reduction, applications of biofertilizers, consisting of a mixture of nitrogen fixing bacteria of the genus *Azospirillium* and cyanobacteria, were used. Inoculation of plants with *Azospirillum* spp. strains increased the yield of many cereal and vegetable crops (Kapulnik et al., 1983; Kotob et al., 1990; Caballero-Mellado et al., 1993; Thakuria et al., 2004). It was reported that growth-stimulating compounds and nitrogen-fixation are the reasons for the yield increases (Rodgers et al., 1979).

Growth promotion of tomato plants by rhizobacteria (*Azospirillum* sp., *Azotobacter chroococcum*, and *Pseudomonas fluorescens*) and imposition of energy stress via the pathogen *Rhizoctonia solani*, studied by Gupta et al., 1995, also resulted in seedling emergence rate, improved plant growth and potential for biocontrol of *Rhizoctonia* damping-off.

Studies have examined the effect of *Azospirillum* inoculation on several crops. Burris (1977) ran a field trial using nineteen different varieties or species of plants, fourteen of which showed a positive yield response, while the remaining five showed a negative response, to inoculation with *Spirilum lipoferum*. Numerous other studies have also demonstrated that the response to inoculation is extremely variable.

The main objective of this work was to determine the efficacy of biofertilizers on tomato fruit yield in order to compensate for the yield loss caused by the use of satellite-mediated protection (Montasser et al., 1991; 2006). Greenhouse and field experiments were designed to examine the effects of *Azospirillum* and cyanobacteria on number and fresh weight of tomato fruits, nitrogen contents, and dry weight of tomato plants inoculated with and without the protective strain of CMV associated with CARNA 5.

Materials and Methods

Virus source and maintenance. CMV strain containing CARNA 5 used as a biological control agent was maintained and propagated in tomato (*Lycopersicon esculentum* cultivar UC82B) plants. Inoculated tomato plants were kept in 20-cm-diameter pots containing a mixture of soil, peat moss, and vermiculite (2:1:1, v/v) in an insect-proof greenhouse where the temperature ranged from 24 to 35 C.

Virus purification and inoculum preparation. CMV strain was purified from 7-14 day old infected tomato plants according to Lot et al., 1972 with some modifications. Plant

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tissues were blended together with chloroform and 0.5 M Na-citrate-citric acid buffer (pH 6.5) containing 0.1% thioglycolic acid (TAG) in the ratio of 1g tissue/2 mL chloroform/2 mL buffer. The homogenate was filtered, squeezed through cheesecloth and then clarified by centrifugation at 5900 × g for 10 min. The supernatant volume was measured and stirred with 10% PEG 8000 (w/v) for 15 min at 4°C. The mixture was cooled for 30-40 min in an ice bath followed by centrifugation at 13,200 × g for 20 min. Pellets were resuspended in 0.05 M Na-citrate-citric acid buffer at pH 7 containing 2% Triton X-100, and then submitted to a low speed centrifugation at 5,900 × g for 5 min. The supernatant was centrifuged at 85,000 × g for 4 hours. Pellets were resuspended in water overnight at 4°C, homogenized with a glass rod, and then clarified by a 5 min centrifugation at 5,900 × g. The supernatant was diluted in 0.05 M Na-citrate-citric acid buffer pH 7. To determine the virus purity, absorbance readings were taken at 260 and 280 nm. Viral RNA genome was further extracted from purified virus preparations using the chloroform/phenol extraction method described by White and Kaper, 1989 and reported by Montasser et al., 1999, 2006. The extracted viral RNA was used as an inoculum for the mechanical transmission of the protective strain.

Mechanical inoculation of the protective viral strain and biofertilization. Protective strain was mechanically inoculated on the cotyledonary leaves of tomato seedlings.

Inoculations with extracted viral RNA were made by rubbing, with a cotton swap, the cotyledonary leaves of tomato plants that had previously been dusted with 600-mesh Carborundum (Montasser, 1999). Immediately after inoculation, the leaves were rinsed with distilled sterile water. Test plants were kept in 20-cm diameter pots containing a mixture of soil, peat moss and vermiculite (2:1:1, v/v/v) in a greenhouse where the temperature ranged from 24 to 35C. Biofertilizer mixtures (12 ml culture suspension/test plant) of cyanobacterium and/or *Azospirillum spp.* mix were inoculated into the soil by using a sterile-glass pipette. Each treatment was applied into the soil adjacent to the crown area and to the root-system zone of each test plant. Test plants grown either in pots, in the greenhouse or in the field were treated according to the experimental design for each treatment.

Bacterial strains and biofertilizer inoculum preparations. A culture mix of *Azospirillum brasilliensis* strains N040 and Sp7, and *Azospirillum lipoferum* strain MRB16 (kindly provided by Dr. S. Kotob, USDA, Maryland, USA), was used separately or in combinations with cyanobacterial strain of *Anabaena oryzae* Fritsch (kindly provided by Dr. F. Hashem, University of Maryland, College Park MD, USA) as a biofertilizer inoculum. Biofertilizer treatments were used either separately or in combinations with protected (preinoculated with the protective viral strain containing CARNA 5) and non-protected healthy tomato plants as a control in both greenhouse and field experiments.

Experimental designs and statistical analyses. The effect of biofertilization on the yield of tomato plants inoculated with the protective strain of CMV associated with CARNA 5 was determined by measuring plant growth under both greenhouse and field conditions. Eight different treatment variables were used in both greenhouse and field experiments with healthy/negative control plants (H), plants preinoculated with the protective viral strain (V) to serve as a positive control, cyanobacterium treated plants (C), *Azospirillum* treated plants (A), plants treated with C + A, C + V, A + V, and A + C + V (Fig. 1C), as this set up is shown in Fig. 1.C. Ten plants were tested for each treatment and replicated three times in 2 different sets of greenhouse and field experiments (*i.e.* 10 plants × 8 treatments × 3 replicas × 2 sets = 480 test plants). The effect of biofertilizers on tomato plants grown in the greenhouse was determined by measuring plant growth, fruit yield and nitrogen content. In addition, pH of the tomato fruits was determined in two sets of three replications in greenhouse experiments. Dry weight and nitrogen contents were assessed on a per plant basis. In the field experiment, fruit number and fruit fresh weight (average yield) were measured per ten plants. Average tomato yield was compared to healthy untreated controls by measuring average fruit number and average fruit yield per 10 plants in 3 replicas that were repeated twice as in 2 sets of experiments. A complete randomized block design was used for both greenhouse and field experiments. Fruit yield assessment, fresh and dry weight, nitrogen contents and fruit pH data were statistically analyzed for each treatment. Fruit yield values were calculated for each treatment with the mean of healthy untreated plants as baseline. Data collected at the final reading for each treatment were used for statistical analyses using Student's *t* test to compare treatments and treatments with untreated-control plants. Least significant differences (LSD) were determined for tomato fruit yield and for the number of fruits.

Polyacrylamide gel electrophoresis and northern hybridization. Purified viral RNA preparations and total nucleic acid extracts (TNA), prepared from random leaf samples taken four times during the season from different treatments, were analyzed by electrophoresis on 6% polyacrylamide (39:1 acrylamide: bis-acrylamide containing 7 M urea and 1X TBE; 90 mM Tris-borate, pH 8.3, 2.5 mM EDTA). After staining, the gels with ethidium bromide and UV-photography, the RNA was electro transferred to nylon

membranes, which were later probed for the presence of CARNA 5 as described by White and Kaper, 1989.

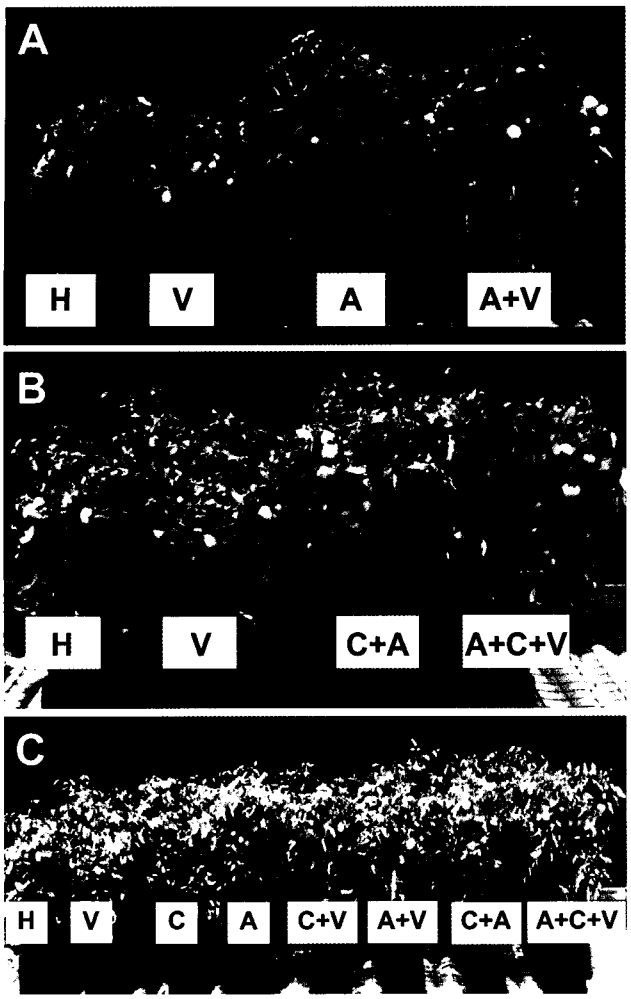

Fig. 1. A) Greenhouse experiment to determine the effects of biofertilizers (*Azospirillum* and cyanobacterium cultures) on the growth of tomato plants (cultivar UC82B) inoculated with a protective viral strain of CMV (cucumber mosaic virus) associated with CARNA 5 (CMV associated RNA 5) as a biological control agent against viral strains, but it causes a yield reduction in tomato plants. To compensate for the growth and the yield reductions biofertilizers were used in the greenhouse experiment that is showing comparisons of healthy control (negative control) tomato plants (H), inoculated tomato with CMV associated with CARNA 5 as protective viral strain and as a positive control (V), *Azospirillum* treated tomato (A) and *Azospirillum* treated and protective viral strain (A+V) tomato plants. **B)** Comparisons of healthy tomato plants (H) with protected (V), cyanobacterium mixed with *Azospirillum* (C+A), and a mixture of *Azospirillum* Cyanobacterium treated tomato that were inoculated with the protective viral strain (A+C+V). **C)** General comparisons of healthy control tomato plants (H) with protected (V), Cyanobacterium (C), *Azospirillum* (A), cyanobacterium and protected (C+V), *Azospirillum* and protected (A+V), cyanobacterium with *Azospirillum* mixture culture (C+A), and *Azospirillum* mixed with cyanobacterium culture on protected (A+C+V) tomato plants.

Results

Greenhouse experiments. The dry weight of tomato plants indicated that the plant growth was significantly higher in biofertilized non-protected and protected plants than the untreated control plants (Table 1). The maximum plant dry weight was ranged between 9.08 g/plant and 8.68 g/plant for cyanobacterium treated plants and *Azospirillum* mixed with cyanobacterium treated tomato plants while the dry weight of untreated protected plants was 6.78 g/plant.

Fresh weight of tomato fruits was greater for biofertilized protected and non-protected plants than untreated plants. The greatest fruit weight per plant, 1150 g, was for cyanobacterium treated plants, followed by 1040 g/plant for the cyanobacterium-*Azospirillum* mixture on protected plants and minimum, 610 g/plant, was for protected plants. Biofertilized protected and non-protected plants produced more fruits, except for the cyanobacterium treated protected plants that yielded the lowest fruit number/plant (31 fruits/plant). Maximum fruit number (55 fruits/plant) occurred on *Azospirillum* + cyanobacterium treated plants, in comparison to 37 fruits/plant for cyanobacterium treated protected plants and 35 fruits/plant for healthy control plants as shown in Table 1.

Increased plant growth was observed in plants treated with *Azospirillum* alone and protected plants treated with *Azospirillum* compared to the growth reduction observed in both healthy control and protected plants (Fig. 1. A). Cyanobacterium with *Azospirillum* resulted in a higher growth in both non-protected and protected plants (Fig. 1. B) in comparison to the protected and healthy control plants (Table 1). The maximum dry weight was 9.08 g/plant, for

Table 1. Effect of biofertilizers on plant growth and fruit yield in tomato plants grown in the greenhouse

Treatments[a]	Dry weight (g/plant)	No. of Fruits/plant	Fruit yield (g/plant)
H	7.00 c	35 cb	700 cb
V	6.78 c	31 c	610 c
C	9.08 a	52 a	1150 a
A	7.75 ab	54 a	1020 a
C+A	8.68 ab	55 a	1070 a
C+V	7.87 ab	37 cb	980 a
A+V	8.56 ab	46 ab	950 ab
A+C+V	8.40 ab	44 ab	1040 a

[a] H = healthy tomato UC82B plants (negative control); V = inoculated tomato plants with the protective viral strain of CMV associated with CARNA 5 (positive control); C = cyanobacterium treated tomato plants; A = *Azosperillium* treated tomato plants grown in the greenhouse.
Values followed by the same letter do not differ significantly from each other at probability level $P = 0.05$ according to Student's t test.

cyanobacterium treated tomatoes, followed by biofertilizers (cyanobacterium and *Azospirillum*) alone or in combination with protected plants. No significant differences were detected among these treatments. Minimum dry weight was for control and protected plants, with difference between these treatments. Fruit number in these treatments was greater than the *Azospirillum* inoculated-protected, cyanobacterium plus *Azospirillum* mixture inoculated-protected and non-protected control plants. A minimum of 31 fruits/plant resulted from the untreated protected plants (Table 1). Average fresh weight of fruits, per plant, was at the highest rate in biofertilized protected and non-protected plants but it was at the lowest rate in untreated protected plants (610 g/plant). There was no difference among those treatments, except *Azospirillum* treated and protected plants (950 g/plant) weighed more than the control plants (700 g/plant) and protected plants (610 g/plant).

Nitrogen contents and pH of tomato fruit juice. The nitrogen content was at the highest rate of 223 mg/plant and 2.79% nitrogen in plants treated with cyanobacterium and *Azospirillum* alone without protection, as compared to 140 mg and 2.06% for untreated protected control plants (Table 2).

There is no any significant differences regarding pH values in all treatments compared with the healthy control plants. pH of tomatoes, determined in two sets of three replications, was at the highest rate in cyanobacterium treated and protected plants, showing a value of pH 4.48. pH was minimum in fruits of protected plants inoculated with the cyanobacterium and *Azospirillum* mixture, showing a value of pH 4.11 as shown in Table 2. Maximum nitrogen contents resulted from the cyanobacterium and *Azospirillum* treated plants, at 223 and 216 mg, respectively; these values were greater than the other biofertilizer treatments of protected and non-protected plants. The lowest value occurred for protected and non-protected control plants. Table 1 showed that the maximum nitrogen percentage (2.79%) occurred in *Azospirillum* treated plants; the value was greater than those of cyanobacterium inoculated and protected plants, followed by biofertilizer treated and protected plants, and non-protected plants, and then the non-protected control plants. The lowest value (2.06% nitrogen) resulted from the protected control treatment. Number of fruits calculated per ten plants was not different among cyanobacterium plus *Azospirillum*, *Azospirillum* alone and cyanobacterium alone treated non-protected plants.

Field experiments. Tomato fruit yield of field grown tomato plants treated with biofertilizers was determined and compared to untreated control plants. Fresh weight and fruit number were greater for protected biofertilized plants than the non-protected biofertilized plants. Maximum number of fruits resulted from the cyanobacterium and *Azospirillum* mixture treated protected plants was in average of 204.7 ± 7.0 fruits per 10 plants. The mean value for the number of fruits yielded from healthy control plants was 124.7 fruits/10 plants, and for protected plants was 115 fruits as shown

Table 2. Effect of biofertilizers on nitrogen content and fruit pH in tomato plants grown in the greenhouse

Treatments[a]	Nitrogen (%)	Nitrogen (mg/plant)	Fruit Juice pH
H	2.16 de	152 c	4.20 a
V	2.06 e	140 c	4.30 a
C	2.46 cb	223 a	4.41 a
A	2.79 a	216 a	4.37 a
C+A	2.45 cb	212 ab	4.25 a
C+V	2.60 ab	205 ab	4.48 a
A+V	2.38 cb	204 ab	4.11 a
A+C+V	2.51 cb	211 ab	4.11 a

[a] H = healthy tomato UC82B plants (negative control); V = inoculated tomato plants with the protective viral strain of CMV associated with CARNA 5 (positive control); C = cyanobacterium treated tomato plants; A = *Azosperillium* treated tomato plants grown in the field.
Values followed by the same letter do not differ significantly from each other at the probability level $P = 0.05$ according to Student's t test.

Table 3. Effect of biofertilizer applications on number of fruits and their fresh weight in field grown tomato plants

Treatments[a]	Fruit Number[b]	Average yield[c]
	No./10 plants	Kg /10 plants
H	124.7 a	27.4 a
V	115.0 b	22.5 b
C	136.3 c	25.7 c
A	130.0 c	26.8 d
C + A	130.7 c	28.7 e
C + V	148.7 d	39.5 f
A + V	200.0 e	34.0 g
A+C+V	204.7 e	40.0 h

[a] H = healthy tomato UC82B plants (negative control); V = inoculated tomato plants with the protective viral strain of CMV associated with CARNA 5 (positive control); C = cyanobacterium treated tomato plants; A = *Azospirillum* treated tomato plants grown in the field.
[b] Average number of tomato fruits per 10 plants. Three replicas for each treatment of 10 plants each were repeated twice in 2 different sets of experiments.
[c] Average yield in kg/10 plants, calculated as average of all 60 plants in each treatment.
Values followed by the same letter do not differ significantly from each other at probability level $P = 0.05$ according to the Student's t test.

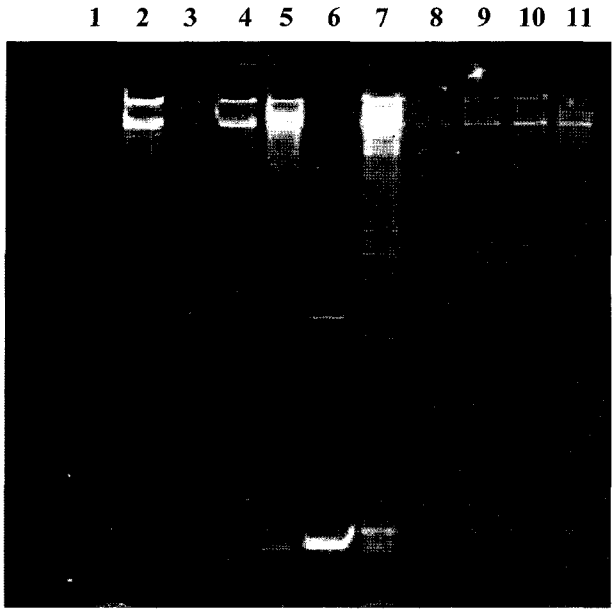

Fig. 2. Polyacrylamide gel (6%) showing the presence of CARNA 5 bands in biofertilizer treatments on protected tomato plants. Lanes 1-5 = Samples extracted from one set of replications (A); Lanes 7-11 = Samples extracted from a second set of replications (B). Lane 1 = Healthy control plant tissues; Lane 2 = Protected tomato; Lane 3 = Cyanobacterium on protected plant samples; Lane 4 = *Azospirillum* treatment on protected plant sample; Lane 5 = Cyanobacterium mixed with *Azospirillum* culture on protected plant sample contained CARNA 5; Lane 6 = Standard CARNA 5; Lane 7 = Cyanobacterium and *Azospirillum* mixture culture treatment on protected plant sample contained CARNA 5; Lane 8 = Protected tomato sample; Lane 9 = Cyanobacterium treatment on protected plant sample; Lane 10 = *Azospirillum* treatment on protected plant sample; Lane 11 = Healthy control. Arrows indicate the presence of CARNA 5 bands.

in Table 3. The highest average of fruit yield (40 Kg/10 plants) resulted from protected plants treated with both cyanobacterium and *Azospirillum* mixture, and the minimum (22.5 Kg/10 plants) resulted from the untreated protected control plants. In general, the fresh weight for biofertilized protected plants was greater than any other treatments. The yield was significantly different than the yield from untreated control plants as shown in Table 3.

Polyacrylamide gel electrophoretic analyses. Tomato samples collected from all treatments were tested for the presence of CARNA 5 associated with the protective strain of CMV. CARNA 5 bands were observed on 6% polyacrylamide gels at the same molecular weight level of the standard control sample in lane 6, Fig. 2. CARNA 5 bands were observed in extracts of all protected plants, including those treated with cyanobacterium and *Azospirillum* as shown in Fig. 2. Northern blotting hybridization revealed positive and radioactive signals, indicating the presence of the CARNA 5 bands from treated plant extracts.

Discussion

Biological control of CMV using satellite-mediated protection in tomato was a previous research work that we already conducted (Montasser et al., 1991, 2006). This resulted in a good protection but with a yield loss of about 20% in protected tomato. The main objective of this current manuscript is to use the biofertilization and nitrogen fixation to compensate for the yield loss caused by the use of satellite-mediated protection.

The genus *Azospirillum* contains symbiotic nitrogen fixing bacteria that have been used successfully in biofertilization (Kapulnik et al., 1983). *Azospirillum* strains thrive in root zones and are able to supply biologically fixed nitrogen, which is absorbed by the plants and can lead to improved plant growth (Thakuria et al., 2004). Crop plant root exudates provide nutrients for survival and multiplication of the bacteria (Hamdi, 1982; Kapolunik et al., 1981; Tien et al., 1979; Vlassak et al., 1981). Our work shows a novel application of these biofertilizers, in that they can allow utilization of an antiviral vaccination.

Cyanobacteria are also used in biofertilization, again, because they fix and supply nitrogen to the plants (Muralikrishna et al., 1985; Venkaturaman, 1979). Commonly used species of cyanobacteria are: *Anabaena*, *Nostoc* and *Tolypothrix*. Cyanobacteria occur naturally and do well under moist conditions, generally growing on the soil surface as they are photosynthetic and, as such, require light (Muralikrishna et al., 1985).

The use of cyanobacteria as a soil amendment in agriculture has been well documented (Muralikrishna et al., 1985). Cyanobacteria are non-symbiotic nitrogen fixers and can thus remove dinitrogen gas from the atmosphere and reduce it to ammonium. Cyanobacteria have been inoculated into rice paddy soils for hundreds of years as a means of providing nitrogen to rice (Venkaturaman, 1979). The effect of the inoculation of three treatments of nitrogen fixing cyanobacteria (*Aulosira fertilissima*, *Nostoc muscarum*, or a mixture of the two) was investigated by Saha and Mandal (1980). These authors found that inoculation increased rice (*Oryza sativa*) grain and straw yield, and nitrogen uptake into the grain. The efficiency of inoculation, however, gradually decreased with the increase in levels of nitrogen in the paddy soils.

Because inoculation of rice with cyanobacteria has clearly been shown to be a beneficial process, researchers have wondered whether inoculation of soils directly could increase the yield of vegetable crops. To examine this question (Rodgers et al., 1979) demonstrated that

inoculation of soils containing radish or tomato plants with algal suspensions increased growth rates of both plants and increased their overall yield. Alternatively, Tiedemann et al. (1980) studied the effect of applying a commercial cyanobaterial inoculant, either live or killed, to soils in a greenhouse pot experiment using orchard grass (*Dactylis glomerata*), pine grass (*Equisetum arvense*), douglas fir (*Pseudotsuga menziesii*), and ponderosa pine (*Pinus ponderosa*). It was observed that inoculated plants have greater biomass yields than the uninoculated control. It was found, however, that the "killed" and living inoculants had effects that were not different from each other. The response to the inoculant, compared to the control, thus appeared to be a result of the addition of nutrients present in the inoculant stock solution.

Biofertilizers are ecofriendly inputs and are less damaging to the environment than inputs such as chemical fertilizers (Kannaiyan et al., 2004; Nuttall, 2006). In the present investigation inoculation with cyanobacteria and/or azospirillum showed increased fresh weight of fruits and dry weight of protected tomato plants. Overall, the plant growth, fruit yield, and nitrogen contents were increased by biofertilizer inoculations, in comparison to non-protected and protected control plants without biofertilizer inoculations. This result is in agreement with the reports by Rao and Charyulu (2005) who showed increased plant height, dry weight of shoot and root, and total nitrogen content of shoot, root and grain by inoculating foxtail millet with three strains of *Azospirillum* either alone or in combination with nitrogen fertilizer. Growth-stimulating compounds or nitrogen fixation organisms are the reasons for yield increase. Okon and Labandera-Gonzalez (1994) concluded that various strains of *A. brasilense* and *A. lipoferum* are capable of promoting the yield of agriculturally important crops in a range of soil types and climatic conditions. Use of multiple inoculations can enhance total seasonal N_2 fixation, P uptake, and mineral nutrition in general, but they can also help in controlling plant pathogens. These bio input can allow reductions in chemical inputs, such as fertilizers and pesticides, that are expensive and environmentally unsound. If populations of the inoculated organisms can be established in agricultural soils, the interval between biofertilizer applications could be increased and costs further lowered (Rai et al., 2000). Bashan and Holguin (1997) have reviewed several examples of co-inoculation with *Azospirillum* and *Rhizobium*, *Azotobacter*, *Arthrobacter*, *Enterobacter*, or *Klebsiella*.

Our studies indicated the presence of CARNA 5 in biofertilizered protected plants and the northern blot hybridization results confirmed the gel electrophoresis analyses (White and Kaper, 1989). This proved that biofertilization did not affect the accumulation of viral satellite RNA required for the plant protection. At the same time, the biofertilization compensated for the potential negative effects of inoculation, in the absence of CMV. Thus, viral satellite CARNA 5 can be used as a biological control agent along with nitrogen biofixing agents (both cyanobacteria and *Azospirillum* spp.). This work shows that a set of two biological inputs, together, can be used to provide good crop growth and high quality fruits, allowing reductions in chemical fertilizer inputs. The biofertilization used was a success, and the tomato fruit yield was significantly increased compared to untreated plants inoculated with the protective viral strain associated with CARNA 5 alone.

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