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Enzyme Production and Nitrogen Fixation by Free, Immobilized and Coimmobilized Inoculants of *Trichoderma harzianum* and *Azospirillum brasilense* and Their Possible Role in Growth Promotion of Tomato

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Summary

A plant growth-promoting rhizobacterium (*Azospirillum brasilense* strain Az) and a biocontrol fungus (*Trichoderma harzianum* strain T24) have been evaluated for their individual and combined production of hydrolytic enzymes, nitrogen fixation and their possible role in growth promotion of tomato seedlings. The studied organisms were inoculated as free or calcium alginate-encapsulated cells. All freshly prepared macrobeads showed high encapsulation capacity (EC/%) of inocula compared with dry macrobeads. Results of enzyme production did not exhibit consistent pattern of the effect of encapsulation process on enzyme production. Beads entrapping bacterial and/or fungal cells were used successfully in 3 repeated cycles in the presence of fresh sterile culture medium in each growth cycle. Enzyme production by immobilized bacterial and/or fungal cells increased as the growth cycles were repeated. Co-culturing of *A. brasilense* with *T. harzianum* (free or immobilized) in semisolid nitrogen deficient medium (N-free medium) enabled *A. brasilense* to fix nitrogen on pectin, chitin and carboxymethyl cellulose. The activity of nitrogen fixation by *A. brasilense* in the case of single and combined cultures with *Trichoderma* (using dry encapsulated beads) into the sterile soil increased with the addition of carbon source. Most of inoculations with free or alginate macrobead formulations of *T. harzianum* and/or *A. brasilense* showed significant increase in the growth parameters of tomato seedlings. The root system grew more profusely in the case of all seeds treated with *A. brasilense*. The growth parameters of Az/T24-treated seeds using dry coimmobilized macrobeads were higher than those of the untreated control. Moreover, the effect was improved significantly in soil enriched with different C sources. Enhanced tomato seedling growth after the co-inoculation could be due to the synergistic effect of both *Trichoderma* and *Azospirillum.* Finally, co-inoculation with *Azospirillum* and other microorganisms is one of the major frontiers of *Azospirillum* technology and perhaps the main area for future applications.

Key words: coimmobilization, alginate, *Trichoderma harzianum*, *Azospirillum brasilense*, enzyme production, nitrogen fixation, plant growth promotion, tomato growth, pot experiment

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Introduction

Under natural conditions plants continually interact with soil microorganisms. This interaction exists primarily at the root level and may be harmful, neutral, or beneficial. Beneficial effects of the introduction of specific microorganisms on plant growth have been reported for numerous crops, including tomato (*Lycopersicon esculentum* Mill.) grown in the field (*1,2*) or under greenhouse conditions in organic media (*3*). Such beneficial microorganisms referred to as PGPR (plant growth-promoting rhizobacteria) or PGPF (plant growth-promoting fungi) enhance plant growth through numerous mechanisms including the protection of roots against infection by minor and major pathogens (*4,5*), enhancing the availability of nutrients to the host plant, lowering the ethylene level within the plant or enhancing the production of stimulatory compounds, such as plant growth regulators (*6*).

Plant growth-promoting bacteria (PGPB) are commonly used to improve crop yields. In addition to their agricultural usefulness, there are potential benefits in environmental applications. For example, species of *Azospirillum* can improve bioremediation of wastewater by increasing algal proliferation. Additionally, these genera and several other bacterial species may prevent soil erosion in arid zones by improving the growth of desert plants, which in turn leads to reduced dust pollution. Other PGPB promote plants that extract hazardous materials from soil (*7*).

Bacteria belonging to the *Azospirillum* genus have been studied as plant growth-promoting rhizobacteria (*8*). They are known as obligate nitrogen-fixing microsymbionts. Conceptually, PGPR can affect plant growth and development either directly or indirectly. Rhizobacteria may decrease or prevent some effects of phytopathogenic organisms through the production of antibiotics. On the other hand, bacteria either directly provide the plant with different compounds or facilitate their incorporation *via* nitrogen fixation or phosphorus solubilization.

Azospirillum is a plant growth-promoting bacterium, which lives in close association with the roots of many cultivated plants. Exploitation of synergistic interactions between co-inoculated *Azospirillum* and other plant growth-promoting microorganisms to further increase the beneficial effect of *Azospirillum* on plant growth was first discussed by Bashan and Holguin (*9*). Double inoculation studies of *Azospirillum* mostly showed that this had some advantages over single inoculation for grain and plant dry matter production, and N and P uptake, depending on the co-inoculant and the plant used (*9*). Information regarding the effects of combined inoculation of *Azospirillum* and a biocontrol microorganism on plant growth is very limited. Compatible combinations of the inoculated microbes, such as nitrogen-fixing *Azospirillum* bacteria and fungi (like arbuscular mycorrhizal fungi, AMF), may result in an enhanced effect on plant development in various microsymbiont–legume systems (*10*).

Several strains of the genus *Trichoderma* are being tested as alternatives to chemical fungicides (*11*). However, full-scale application of *Trichoderma* for biological control of plant pathogens is not yet widespread. *T. harzianum* fungus is a biological control organism against a wide range of soil-borne pathogens and also has plant growth-promoting capacity (*12*).

According to Punja and Utkhede (*13*), *Trichoderma* spp. are the most widely studied mycoparasitic fungi. The role of extracellular enzymes has been well documented recently by several researchers (*e.g.* proteolytic enzymes (*14,15*), β-1,3-glucanolytic system (*16*) and chitinase (*17,18*)). A complex group of extracellular enzymes has been reported to be a key factor in the lysis of the pathogen cell wall during mycoparasitism (*19,20*).

The ability of *Trichoderma* to recognize and parasitize phytopathogenic fungi in the rhizosphere (*21–23*) has been ascribed to several complex mechanisms, such as nutrient competition, antibiosis, mycoparasitism, induction of systemic resistance, and increased plant nutrient availability (*24,25*). The promotion of plant growth, under axenic conditions and in natural soils (*22*), is among the many reported effects of the plant-fungus interaction (*25,26*).

The activity of free-living heterotrophic nitrogen fixers is limited by the deficiency of monomeric compounds. In this relation, the interactions between the diazotrophic bacteria and fungi, which are the main destroyers of plant polymers in terrestrial ecosystems, are very important. The pool of monomeric compounds is formed in the hyphosphere and the surrounding soil as a result of the extracellular hydrolysis of polymers, and this allows the associated bacteria to perform nitrogen fixation (*27*). The positive effect of fungi on the nitrogenase activity of bacteria upon their mutual growth on nutrient media has repeatedly been demonstrated (*28, 29*). However, there are few data about this important interaction between bacteria and fungi for soil conditions *in vivo* (*30*). There is information about significant enrichment of soil incubated under an increased moisture content (often under the conditions of total moisture capacity) with nitrogen after the addition of wheat straw together with the cultures of a nitrogen-fixing bacterium (*Clostridium butyricum*) and cellulolytic fungi (*Penicillium corylophilum* or *T. harzianum*) (*31*).

In recent years the method of immobilizing living cells has gained a wide range of applications (*32,33*). Encapsulation of microbial cells for soil application provides a range of advantages such as ease of application to the soil, reduced off-site drifting, and protection of cells from environmental stress (*34*). In addition, encapsulated preparations possess high cell loading capacity, high retention of cell viability, increased rate of production of microbial products and also act as a reservoir that releases cells at a slow and constant rate (*35*). Microbes contained within polymers provide a convenient inoculum for numerous industrial, environmental, and agricultural applications (*36,37*). For example, the microalga *Chlorella* sp. coimmobilized in a polymer with other microorganisms has been used in biotechnological processes where the microalga provides oxygen for the accompanying microorganism involved in the compound transformation (*38*).

Alginate is the most commonly used polymer for microbial cell encapsulation, also called immobilization

(*39,40*), and used for various industrial microbiological purposes. Bashan and Gonzalez (*41*) reported that *Azospirillum* can survive in dry alginate inoculants for prolonged periods without losing effectiveness. Moreover, El-Katatny *et al.* (*42*) demonstrated that microbial immobilization gives prolonged metabolic activity when microbial cells are reused. Organisms could be immobilized separately or coimmobilized together (*43,44*).

The objective of this study is to evaluate the capacity of free or alginate-encapsulated formulations of *A. brasilense* and *T. harzianum* in single or dual (co-inoculated) cultures to produce polysaccharide-degrading enzymes. The concept of applying *Azospirillum* as a nitrogen-fixing microsymbiont alone or in co-inoculation with *T. harzianum* for the growth promotion of tomato plants has also been studied in pot experiment.

Materials and Methods

Microorganisms

The *Azospirillum brasilense* strain Az used in this study was isolated from the rhizosphere of maize and rice by El-Komy *et al.* (*45*), and identified on the basis of usual phenotypic and genetic properties (G+C content and DNA-DNA homology) according to Tarrand *et al.* (*46*). This bacterial strain was obtained from the culture collection of the Department of Botany and Microbiology, Faculty of Science, Minia University, Egypt and was maintained on nutrient agar (NA) slopes.

Trichoderma harzianum strain T24 was also obtained from the culture collection of the Department of Botany and Microbiology, Faculty of Science, Minia University, Egypt. Strain T24 was identified by DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). The T24 culture was maintained on potato dextrose agar (PDA) at 4 °C.

Production of inocula and macrobead formation

Methods used for the production of inocula and macrobead formation had been described earlier (*42,47*). Single bacterial or fungal inoculum was immobilized and coimmobilization of both inocula was carried out. The population in free cultures always reached a concentration of at least $3 \cdot 10^9$ CFU/mL for *Trichoderma* and $5 \cdot 10^{14}$ CFU/mL for *Azospirillum.* However, cell loading capacity of liquid alginate was 10^9 and 10^{14} for *Trichoderma* and *Azospirillum*, respectively. The population in fresh beads always reached a concentration of at least $0.4 \cdot 10^9$ CFU/g for *Trichoderma* and $0.42 \cdot 10^{14}$ CFU/g for *Azospirillum.* Usually, wet preparations of macrobeads were either used immediately or after overnight storage at (4±1) °C, which did not affect bacterial or fungal counts.

Encapsulation was performed using 2 % alginate to obtain macrobeads. The resulting alginate beads (mean diameter of 2 mm) entrapped the bacterial and/or fungal cells. The viable population size of *A. brasilense* and *T. harzianum* was determined in pellets before their use in batch cultures and pot experiments. A mass of 1 g of fresh beads was dissolved in 10 mL of 0.1 M potassium phosphate buffer (pH=6) by shaking vigorously for 45 min. The suspended cells were serially diluted and total counts were measured by the plate count method on NA plates for *A. brasilense* and PDA for *T. harzianum* and expressed as colony forming units (CFU) per g of alginate beads.

The same mass of fresh beads (1 g) was placed in a thin layer on a filter paper on a Petri dish and dried at (38±1) °C for 48 h. Then the dry macrobeads were collected and stored in hermetically sealed containers with silica gel until usage. Additionally, dry beads were prepared by standard lyophilization (VirTis 5L, VirTis, Gardiner, NY, USA). The same procedures of measuring the population count in fresh beads were performed for the dry beads. Encapsulation capacity (EC/%) of the cells contained in macrobead formulations was calculated and compared with the initial amount of cells used for encapsulation (1 mL of liquid alginate with cell suspension gave 2.2 g of fresh encapsulated macrobeads). The antagonistic activity of T24 and Az was studied using a dual culture plate on PDA.

Enzyme production by free or immobilized inoculation in batch culture fermentation

Batch culture fermentation was carried out in Erlenmeyer flasks (50 mL), each containing 10 mL of minimal synthetic medium (MSM) consisting of (in g/L): KCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, K_2HPO_4 0.9, NH_4NO_3 1.0, $MnSO_4$ 0.002, $FeSO_4 \cdot 7H_2O$ 0.002, and $ZnSO_4$ 0.002. The appropriate carbon source, *i.e.* 0.5 % chitin, laminarin, carboxymethyl cellulose (CMC), xylan or pectin (Sigma-Aldrich, St. Louis, MO, USA) was supplied and the pH was adjusted to 5.5 with 50 mM acetate buffer. Flasks were inoculated with either 1 mL of fungal spore or bacterial cell suspensions (half of the volume of each for mixed inoculant cultures). After inoculation, the flasks were incubated in a shaking incubator (125 rpm and 30 °C) for 7 days. At the end of the incubation period, cultures were separated by filtration and centrifuged at 6000 rpm for 10 min in a cooling centrifuge at 4 °C, and the clear supernatants were used as crude enzyme preparations. Similarly, enzyme production was also carried out using freshly encapsulated beads (2.5 g of *T. harzianum* macrobeads, *Azospirillum* macrobeads or coimmobilized macrobeads).

Repeated batch fermentation

The reusability of the immobilized cells was tested in batch cultures three times. Repeated batch fermentation was conducted with the immobilized cells by running the fermentation for 7 days using only three kinds of carbon sources, chitin, laminarin and CMC. At the end of each cycle, the obtained supernatant was used as the enzyme solution. Beads containing immobilized cells were thoroughly washed with sterile distilled water, a fresh culture broth was added and fermentation was continued.

Enzyme activity assays

Chitinase activity was assayed using the colorimetric method described by Molano *et al.* (*48*). The reducing sugars produced were determined by the method of Miller (*49*) using dinitrosalicylic acid (DNS) reagent. The amount of reducing sugars released was calculated

using a standard curve recorded for *N*-acetylglucosamine (GlcNAc).

β-1,3-glucanase was assayed based on the release of reducing sugars from pure laminarin (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. The activity was routinely assayed by incubating 500 μL of 0.5 % (by mass per volume) laminarin in 50 mM acetate buffer (pH=4.8) with 200 μL of crude enzyme preparation at 45 °C for 30 min, and the reducing sugars produced were determined by the method of Miller (*49*) using DNS reagent.

CMCase activity was carried out by mixing 0.5 mL of enzyme sample with 0.5 mL of 1 % CMC (Sigma-Aldrich, St. Louis, MO, USA) at pH=5.0, followed by incubation for 30 min with shaking at 50 °C. Somogyi reagent was added and the released total reducing sugars were determined using the Somogyi-Nelson method (*50, 51*). Filter paper digesting activity (FPase) was assayed by the release of glucose through Whatman No. 1 filter paper as a substrate according to Emtiazi *et al.* (*52*).

Xylanase activity was measured by mixing 0.25 mL of the sample with 0.25 mL of 1 % xylan (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mL of acetate buffer (pH= 4.0) and incubation for 30 min at 40 °C. The reaction was terminated by adding Somogyi reagent, while the reducing sugars were determined using the Somogyi-Nelson method (*50,51*).

Polygalacturonase (PGase) activity was determined by measuring the increase in reducing end groups (*53*).

Enzyme activities were expressed in nanokatal (nkat) or picokatal (pkat), which is equivalent to the release of one nano- or picomole of reducing sugars per second, respectively, under the given conditions.

Nitrogenase activity

Nitrogenase activity was assayed in 5-mL culture samples using the acetylene reduction (AR) assay as described by Turner and Gibson (*54*) and El-Katatny *et al.* (*55*), with gas-liquid chromatography (610 Series F.I.D. gas chromatograph, ATI Unicam, Cambridge, UK) equipped with glass column (1650×460 mm) filled with activated alumina. Briefly, semisolid N-free medium (*56*) in which L-malic acid, colloidal chitin, pectin or CMC was inoculated with free (0.1 mL of bacterial or fungal cells) or encapsulated cells (0.3 g of fresh macrobeads) in glass test tubes and sealed with a cotton plug. The cotton plug was replaced with a rubber stopper after the required incubation period (24 and 48 h). About 10 % of the atmosphere of the test tube was replaced with acetylene by injection, and then incubation was continued for 2 h. Gas samples (1 mL) were removed from the atmosphere of the test tubes using a 1-mL syringe and the ethylene concentration in the gas samples was measured by gas chromatography. Results were expressed as nmol C_2H_4 per h·mL of culture medium. The test was repeated three times.

The potential of nitrogen-fixing activity was evaluated in the presence of glucose, sucrose and CMC in the soil (*57*). The activity of nitrogen fixation in the soil was determined using the AR assay. The role of *T. harzianum* in supporting the nitrogen fixation was studied in a series of tests with the addition (1 %) of glucose, sucrose or CMC into the test tubes containing soil (5 g, 60 % total water capacity) instead of adding the semisolid N-free medium. Tubes were inoculated with dried macrobeads (0.1 g) of *A. brasilense* (Az) or *A. brasilense* coimmobilized with *T. harzianum* (T24), and were incubated for 1–5 days at 30 °C. The same procedures of AR assay were performed every 24 h, and the results were expressed as nmol C_2H_4 per h·g of soil.

Pot experiment

A pot experiment was performed to study the effect of *T. harzianum* and *A. brasilense* on the growth of tomato seedlings (*Lycopersicon esculentum* var. commune cv. Piline). Tomato was grown in surface-sterilized plastic pots (500 mL) filled with steam sterilized mixed soil of sand and clay in a mass ratio of 1:2. Soil used in pot experiment was analyzed according to El-Komy (*58*). Pots, each containing 500 g of soil, were arranged in groups of 3 replications for each treatment. The substrate was saturated with distilled water before sowing. Before being sown, seeds were superficially disinfected by soaking them for 15 min in a 30 % sodium hypochlorite solution containing 0.1 % Triton X-100, followed by two washes with sterile water.

Seed coating with free cells or macrobeads

Seeds were sterilized as previously mentioned and immersed in a solution of 2 % alginate and soaked with either *T. harzianum*, or *A. brasilense* or a mixture of both ($3{\cdot}10^9$ CFU/mL for *Trichoderma* and $5{\cdot}10^{14}$ CFU/mL for *Azospirillum*). Seeds were left in cell suspensions for two hours. Control seeds were soaked in an equal volume of distilled water.

Dry macrobeads were manually mixed with dry seeds of tomato immersed in 2 % alginate. This solution created a sticky surface but did not allow the seeds to stick to one another. Seeds were then coated with the macrobeads (approx. 30–35 per seed). Macrobeads adhering to seeds were counted under stereoscopic microscope. The level of inoculation was 30–35 macrobeads per seed on average, corresponding to $1.5{\cdot}10^8$ CFU per seed for *Trichoderma* macrobeads and $1.5{\cdot}10^{13}$ CFU per seed for *Azospirillum* macrobeads (the same average values for macrobeads of the coimmobilized formulations per seed). An equivalent mass of inoculum of freshly prepared macrobeads (approx. 25–30 per seed) was buried near the germinated seeds.

Plant growth conditions

The experimental design was performed as follows: treatment 1, uninoculated seeds (control seeds); treatment 2, seeds inoculated with free cells of *T. harzianum*; treatment 3, seeds inoculated with free cells of *A. brasilense*; treatment 4, seeds inoculated with free cells of a mixture of both *T. harzianum* and *A. brasilense.* A similar experiment was carried out using the same treatments as above with cells encapsulated in calcium alginate to compare the results of free and immobilized cells (fresh and dry). Three seeds were sown in each pot at 2 cm depth. In the third week after sowing, plants were thinned down to 1 per pot. Distilled water was added only when needed to keep the pots moist without exceeding the water-holding capacity of the soil. Three replications were done for each treatment.

To study the effect of fungal hydrolysis of carbon source polymers on the activity of nitrogen fixation as well as on plant growth promotion, another pot experiment was performed. Seeds were coated with dry macrobeads of *A. brasilense* or coimmobilized beads of *A. brasilense* and *T. harzianum.* Soil was mixed with individually different carbon sources (1 %) such as glucose, sucrose or CMC before sowing with inoculated seeds. Plant growth was compared in soil with and without a carbon source.

After 45 days, standard plant growth parameters such as root length, shoot height, and their respective dry mass were measured separately as responses to inoculation. Dry mass of the stems and roots was determined by placing each into small, pre-weighed aluminum foil and drying them in a forced draught oven at (60±2) °C until constant mass. Stem caliper at the soil line of tomato seedling for all treatments was also recorded.

Seedling root colonization by T. harzianum or A. brasilense

Seedling root colonization by free and immobilized inoculum of *T. harzianum* or *A. brasilense* was estimated as reported earlier (*59*). Root systems of seedlings after 45 days of sowing were rinsed with sterile distilled water to remove soil particles. The collected root samples were cut into small fragments (1 cm long). Surface-disinfested root fragments were transferred onto acidic PDA and NA growth media for determining the fungus and bacterium count, respectively (5 fragments per plate), and incubated at 30 °C for 5 days. The percentage of *Trichoderma* or *Azospirillum* root colonization was recorded from the number of roots yielding at least one colony of the target organism. All results were presented as the mean of at least three replicates. In addition standard deviations were calculated.

Results

Encapsulation capacity

All freshly prepared beads showed a high encapsulation capacity (EC/%; Table 1) compared with dry macrobeads. Dry bead formulations of immobilized *T. harzianum* T24 or *A. brasilense* Az retained 70 % of the total encapsulated cells (T24 or Az), whereas the dried beads of coimmobilized T24 and Az retained 60 and 65 % of the total encapsulated cells of T24 and Az, respectively.

Enzyme production using free or immobilized cells

Results presented in Fig. 1 showed no consistent pattern of the effect of encapsulation process on enzyme production. This result had previously been reported for enzyme production by *T. harzianum* T24 (*42*). Free or encapsulated cells of *T. harzianum* and *A. brasilense* showed no significant difference in chitinase, β-1,3-glucanase and PGase production, while alginate-immobilized cultures of *T. harzianum* and *A. brasilense* produced lower levels of FPase and CMCase than those of the free cultures. On the other hand, significantly higher levels of xylanase were produced by immobilized or coimmobilized cells of *T. harzianum* and/or *A. brasilense.* Interestingly, xylanase and PGase production improved significantly when macrobeads of coimmobilized fungal and bacterial cells were used, compared with the mixture of these cells (free), in contrast to chitinase production obtained with the same formulations. At the same time, *T. harzianum* enhanced PGase production when co-inoculated (free or encapsulated) with *A. brasilense.* However, *A. brasilense* alone produced a small amount of PGase when inoculated free or immobilized into the culture medium.

Table 2 shows the possibility for the reuse of calcium alginate biocatalysts to produce chitinase, β-1,3-glucanase and CMCase in semi-continuous mode. Beads entrapping bacterial and/or fungal cells were used successfully in 3 repetitions in the presence of fresh sterile culture medium in each set. The reusability of immobilized *T. harzianum* cells for chitinase and β-1,3-glucanase production was previously studied (*42*). Results in Table 2 show that enzyme production by immobilized fungal and/or bacterial cells increased with repeated growth cycles. The gradual improvement of enzyme production was significantly higher when *T. harzianum* cells were entrapped alone or co-encapsulated with bacterial cells in all repeated batch sets.

Nitrogenase activity

Nitrogen fixation by microbial cells on different carbon sources

The ability of bacteria to fix dinitrogen was measured based on their acetylene reduction activity (ARA). Although *A. brasilense* was not able to use pectin, chitin or CMC as a carbon source for nitrogen fixation, which was measured for 48 h of incubation (Table 3) in semi-solid N-free medium, co-culturing of this bacterium with *T. harzianum* (free or encapsulated) enabled it to fix nitrogen on these carbon sources. Generally, most of the

Table 1. Encapsulation capacity (EC/%) of cells in fresh and dried bead formulations calculated for each liquid alginate mixture (1 mL) with *T. harzianum* and/or *A. brasilense* cells

Inocula of encapsulation	Cell loading capacity of liquid alginate	Freshly prepared beads		Dried beads	
	CFU/mL	CFU/g fresh beads before dryness	EC/%	CFU/g fresh beads after dryness	EC/%
T. harzianum T24 (immobilized)	$(1\pm0.30)\cdot10^{9}$	$(0.40\pm0.08)\cdot10^{9}$	90	$(0.32\pm0.031)\cdot10^{9}$	70
A. brasilense Az (immobilized)	$(1\pm0.23)\cdot10^{14}$	$(0.42\pm0.09)\cdot10^{14}$	91	$(0.32\pm0.053)\cdot10^{14}$	70
T24 (in coimmobilized beads)	$(0.5\pm0.1)\cdot10^{9}$	$(0.17\pm0.005)\cdot10^{9}$	76	$(0.13\pm0.008)\cdot10^{9}$	60
Az (in coimmobilized beads)	$(0.5\pm0.1)\cdot10^{14}$	$(0.20\pm0.007)\cdot10^{14}$	84	$(0.16\pm0.008)\cdot10^{14}$	65

Values are means±S.D. of three replications

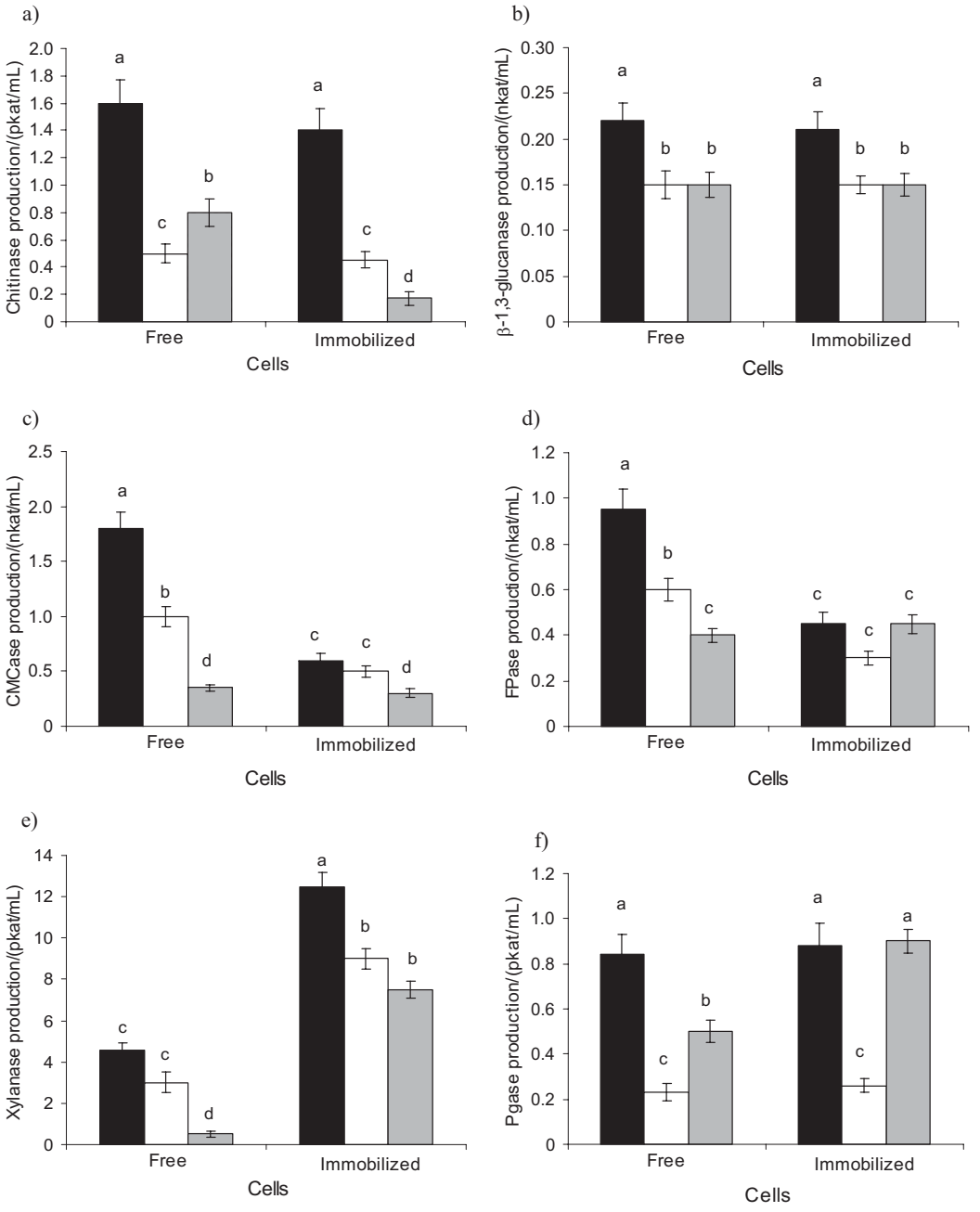

Fig. 1. Enzyme production in single or co-inoculated cultures of free and immobilized cells of *T. harzianum* and *A. brasilense* in the presence of the appropriate C source (0.5 %). a) chitinase, b) β-1,3-glucanase, c) carboxymethyl cellulase (CMCase), d) filter paper digestion (FPase), e) xylanase, f) polygalacturonase (PGase). The values represent the mean value, and the error bars indicate the standard deviation. Different letters for each parameter mean significant differences for $p \leq 0.05$. ■ *T. harzianum* T24, □ *A. brasilense* Az, ▩ T24+Az

co-inoculated culture media (free or encapsulated) gave positive results for nitrogen fixation in the presence of any applied C source. *Azospirillum* showed a positive effect on nitrogen fixation in semisolid N-free medium inoculated either with free or encapsulated formulations, and the activity was significantly higher in the presence of L-malic acid. *T. harzianum* alone failed to fix nitrogen in all AR assay measurements.

The nitrogen fixation activity of pure bacterial cultures or cultures combined with fungus (using dry encapsulated beads) in the sterile soil increased with the addition of different carbon sources (Figs. 2 and 3). The activity of nitrogen fixation was 3 times higher after two days of incubation when *A. brasilense* (as the only inoculant) was inoculated into the soil enriched with glucose than that in the unamended soil (Fig. 2). The increase of nitrogen fixation activity to the maximum values took place on the second day of incubation. A smaller increase in the nitrogen fixation activity was observed upon the inoculation of these cultures into the soil with sucrose or CMC, and all peaked at the fourth day of incubation.

Table 2. Chitinase, β-1,3-glucanase and CMCase production by entrapped inocula of *T. harzianum* and/or *A. brasilense* during the repeated use of these formulations for three sets

Entrapped inoculum for enzyme production	1st set of use	2nd set of reuse	3rd set of reuse
Chitinase/(pkat/mL)			
T. harzianum (T24)	1.55±0.20	3.50±0.4	4.4±0.5
A. brasilense (Az)	0.58±0.10	0.88±0.1	1.2±0.1
T24+Az	0.25±0.07	4.80±0.5	5.7±0.5
β-1,3-glucanase/(nkat/mL)			
T. harzianum (T24)	0.21±0.08	0.35±0.09	1.2±0.10
A. brasilense (Az)	0.15±0.04	0.18±0.06	0.3±0.07
T24+Az	0.16±0.04	0.50±0.04	1.5±0.20
CMCase/(nkat/mL)			
T. harzianum (T24)	0.58±0.10	1.51±0.2	2.4±0.4
A. brasilense (Az)	0.40±0.10	1.00±0.1	1.2±0.1
T24+Az	0.30±0.08	1.80±0.2	3.2±0.3

Values are means±S.D. of three replications

The activity of nitrogen fixation was higher when *A. brasilense* was inoculated into the soil enriched with different C sources (glucose, sucrose or CMC) together with *T. harzianum* than in the case without *Trichoderma* or a C source (Fig. 3). The increase in the nitrogenase activity of the soil when combined cultures of *A. brasilense* and *T. harzianum* were used with the addition of CMC was higher on the third day of incubation, in comparison with the soil inoculated only with *A. brasilense*. This can be explained by the high CMCase activity of *T. harzianum*. In all treatments of the soil enriched with C sources, nitrogen fixation peaked earlier, on the third day of incubation, whereas the unamended soil peaked with low activity later, on the fourth day (Fig. 3).

Plant growth promotion

The chemical analysis of the soil used in pot experiment revealed the following data: pH=8.0, organic C 0.15 %, total N 0.014 %, P 4.0 ppm, K 88.0 ppm, NH_4^+ 3.0 ppm and NO_3^- 0.3 ppm. Plant growth promotion by free or encapsulated cells of *T. harzianum* T24 and/or *A. brasilense* Az was examined in pot experiment (using sterile soil) with tomato (*Lycopersicon esculentum* var. commune cv. Piline) seedlings 45 days after sowing. Alginate macrobeads free of inoculant did not exhibit any improvement of plant growth when compared to

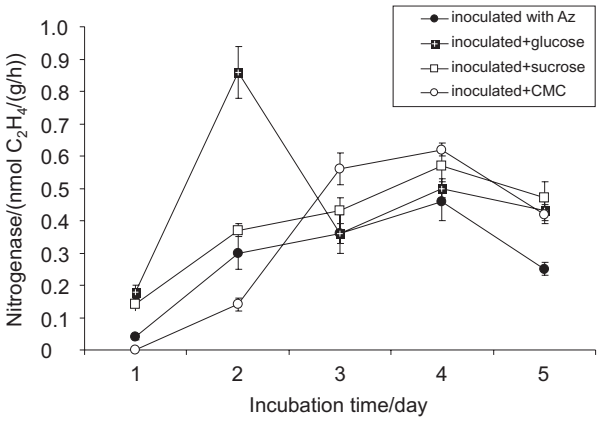

Fig. 2. Nitrogen fixation in sterile soil inoculated with *A. brasilense* and supplemented with different C sources. Values given in the figure represent the mean value, and the error bars indicate the standard deviation

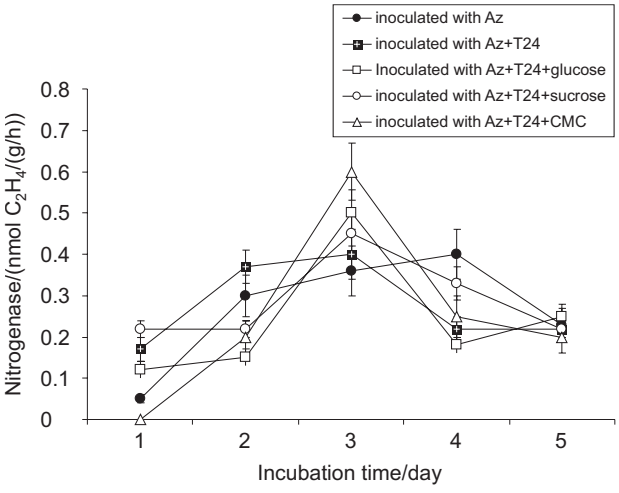

Fig. 3. Nitrogen fixation in sterile soil inoculated with coimmobilized *A. brasilense* and *T. harzianum* in the presence of different C sources. Values given in the figure represent the mean value, and the error bars indicate the standard deviation

Table 3. Nitrogen fixation by free and encapsulated cultures in the presence of different carbon sources when incubated up to two days

	Carbon source	*T. harzianum* T24		*A. brasilense* Az		T24+Az	
		t/h		*t*/h		*t*/h	
		24	48	24	48	24	48
Nitrogen fixation by free cultures	Malic acid	–	–	+++	++	++	+
	Chitin	–	–	–	–	+++	++
	CMC	–	–	–	–	+	++
	Pectin	–	–	–	–	+++	++
Nitrogen fixation by immobilized cultures	Malic acid	–	–	++	+	+	+
	Chitin	–	–	–	–	–	+
	CMC	–	–	–	–	–	+
	Pectin	–	–	–	–	++	++

– no nitrogen fixation; + low, ++ moderate, +++ high acetylene reduction activity

uninoculated controls or to heat-killed bacterium and fungus controls (data not shown). However, inoculation of tomato seeds with free or encapsulated *A. brasilense* formulations significantly increased seedling growth parameters (Tables 4–6, Fig. 4) when compared to untreated controls. Similar results were obtained with the inoculation of the biocontrol fungus *T. harzianum;* there was a slight increase in seedling growth parameters with the exception of seeds treated with fresh macrobeads (Tables 4 and 5, Fig. 4).

Plant seeds inoculated with fresh macrobeads of *A. brasilense* developed better than the control plants (Fig. 4a), as evidenced by 2.2-fold increase in shoot height, 2.75-fold increase in stem caliper, 4.25-fold increase in root length, 4-fold increase in fresh mass of root and 4.5-fold increase in fresh mass of stem. The root system grew more profusely in all treatments of seeds with *A. brasilense* (free, immobilized or coimmobilized cells) (Figs. 4a and b). Seeds inoculated with dry coimmobilized inoculant (T24+Az) macrobeads showed a significantly higher growth parameters compared with untreated seeds (Table 5b, Fig. 4b).

The growth parameters of seeds treated with *A. brasilense* Az in combination with *T. harzianum* T24 using dry coimmobilized macrobeads were higher than those of the untreated controls, and the effect was improved significantly in the soil enriched with different C sources (Table 6). Compared with control treatments, (Az+T24)--treated seeds showed 200 and 456 % increase in seedling shoot height and root length, respectively, when the soil was enriched with glucose before sowing.

Table 4. Effect of inoculation with a single microbe and combined cultures using free cells on tomato seedling growth (after 45 days of inoculation)

Treatments	Shoot height	Stem caliper	Root length	Fresh mass/g		Dry mass/g	
	cm	mm	cm	Shoot	Root	Shoot	Root
Control (uninoculated)	05.5±0.7	0.7±0.4	2.0±0.3	0.3±0.09	0.05±0.01	0.02±0.01	0.005±0.002
T. harzianum T24	08.0±0.9	1.5±0.6	4.0±0.6	0.5±0.09	0.11±0.04	0.07±0.01	0.020±0.007
A. brasilense Az	12.0±0.8	3.0±0.8	8.0±0.5	1.2±0.20	0.30±0.06	0.12±0.04	0.060±0.008
T24+Az	09.0±0.8	2.0±0.3	7.0±0.7	0.8±0.11	0.12±0.03	0.10±0.03	0.025±0.007

Values are means±S.D. of three replications

Table 5. Effect of inoculation with a single microbe and combined cultures using encapsulated cells of fresh beads and dried beads on tomato seedling growth (after 45 days of inoculation)

Treatments	Shoot height	Stem caliper	Root length	Fresh mass/g		Dry mass/g	
	cm	mm	cm	Shoot	Root	Shoot	Root
Fresh beads							
Control (uninoculated)	06.0±0.7	0.8±0.2	2.0±0.4	0.20±0.07	0.05±0.01	0.02±0.01	0.005±0.002
T. harzianum T24	06.5±0.9	1.5±0.4	2.5±0.2	0.38±0.10	0.07±0.02	0.02±0.01	0.008±0.002
A. brasilense Az	13.0±1.1	2.2±0.7	8.5±1.2	0.90±0.20	0.20±0.06	0.05±0.01	0.060±0.010
T24+Az	09.0±0.8	1.1±0.2	4.0±0.8	0.32±0.10	0.08±0.03	0.02±0.01	0.020±0.007
Dried beads							
Control (uninoculated)	6.0±0.6	0.8±0.2	2.0±0.4	0.25±0.07	0.06±0.01	0.02±0.01	0.005±0.002
T. harzianum T24	9.0±0.9	1.5±0.3	3.0±0.5	0.37±0.07	0.11±0.02	0.03±0.01	0.020±0.002
A. brasilense Az	8.5±0.9	1.5±0.3	4.5±0.8	0.35±0.08	0.10±0.05	0.03±0.01	0.030±0.010
T24+Az	10.0±1.4	2.0±0.4	5.0±0.8	0.60±0.10	0.11±0.04	0.04±0.01	0.031±0.010

Values are means±S.D. of three replications

Table 6. Effect of inoculation with combined cultures of bacterium with fungus using encapsulated cells (dry beads) on tomato seedling growth in the presence of different C sources in the soil (after 45 days of inoculation)

Treatments	Shoot height	Root length	Fresh mass/g		Dry mass/g	
	cm	cm	Shoot	Root	Shoot	Root
Control (uninoculated)	06.0±0.7	1.8±0.4	0.3±0.07	0.06±0.01	0.02±0.01	0.005±0.002
Inoculated only	08.5±0.9	5.1±0.2	0.8±0.11	0.12±0.05	0.05±0.01	0.040±0.002
Inoculated+glucose	12.0±1.1	8.2±1.2	1.4±0.30	0.16±0.06	0.08±0.02	0.042±0.002
Inoculated+sucrose	11.5±0.8	7.5±0.8	1.4±0.32	0.14±0.05	0.09±0.02	0.040±0.002
Inoculated+CMC	09.5±0.8	7.1±0.8	1.0±0.11	0.13±0.05	0.07±0.10	0.035±0.002

Values are means±S.D. of three replications

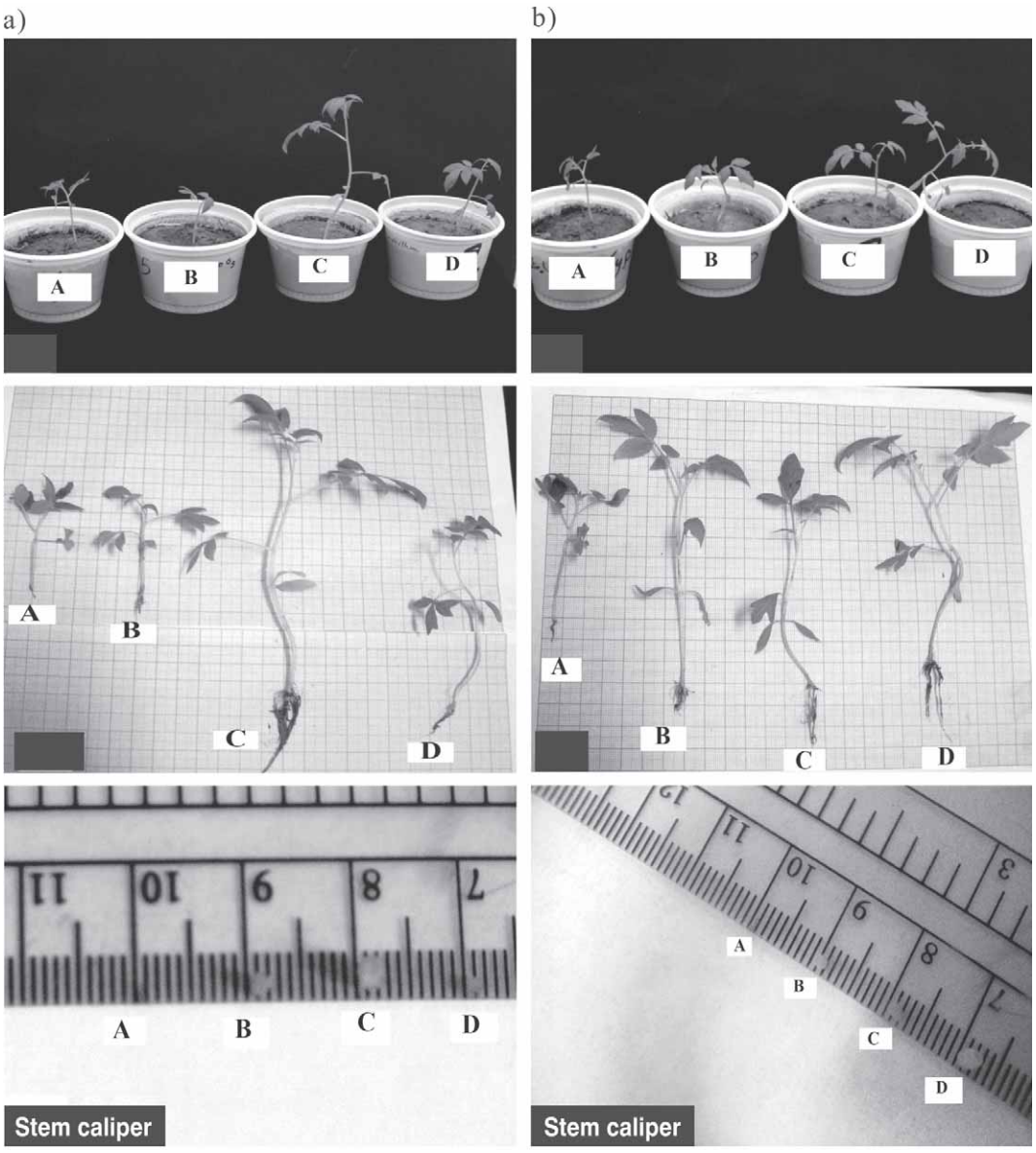

Fig. 4. Pot experiment showing shoot and root development of tomato seedlings when inoculated with: (a) fresh or (b) dry macrobeads of *Trichoderma* (B), *Azospirillum* (C), or combined inoculants of *Trichoderma* and *Azospirillum* (D) compared to uninoculated control (A)

Seedling root colonization by T. harzianum or A. brasilense

There was no difference among the formulations of each organism in colonizing roots of tomato seedling 45 days after sowing (Table 7). Root colonization by *T. harzianum* free cells, fresh macrobeads and dry macrobeads was 86, 95 and 88 % respectively, while the colonization by *A. brasilense* was 88, 100 and 100 % for free cells, fresh macrobeads and dry macrobeads, respectively. Root colonization by free or coimmobilized (fresh and dry) inoculants showed the same pattern of results (Table 7).

Table 7. Percentage of root colonization of tomato seedlings (after 45 days of sowing) by *T. harzianum* and/or *A. brasilense* isolates

Isolate of inoculation	Colonization using free inoculum/%		Colonization using encapsulated inoculum/%			
			Fresh beads		Dry beads	
	T24	Az	T24	Az	T24	Az
Control (uninoculated)	0	0	0	0	0	0
T. harzianum T24	86	0	95	0	88	0
A. brasilense Az	0	88	0	100	0	100
T24+Az	90	83	95	100	100	90

Discussion

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (*60,61*).

The main advantages of alginate preparations are their nontoxic nature, degradation in the soil, their slow release of microorganisms into the soil (*36*) and almost unlimited shelf life (*41*). Several alginate-based preparations were evaluated for agricultural purposes including the encapsulation of biocontrol agents against soil-borne pathogens (*62,63*) and the encapsulation of phosphate--solubilizing bacteria (*64*). This technology was also employed to encapsulate the PGPBs *A. brasilense* and *Pseudomonas fluorescens* (*34*), which were successfully used to inoculate wheat plants under field conditions (*65*). Calcium alginate was found to be an effective and suitable matrix for cell immobilization for higher alkaline protease productivity compared to the other matrices studied (*66*).

Viscosities of different types of alginates had previously been evaluated by Yabur *et al.* (*67*), who reported that the suitable fit and uniform viscosity of alginate was found to be 2 %. In the present study, 2 % alginate was used for all preparations of immobilized or co-immobilized cells of *T. harzianum* and *A. brasilense*. The two studied organisms (T24 and Az) had no antagonistic activity against each other (data not shown).

The most common experimental formulation for bacterial inoculants is macrobeads with a diameter of 1–4 mm either for agricultural or environmental use (*34,36,68*). Nevertheless, their relatively large size is disadvantageous for agricultural uses (*69*). To produce smaller size beads, there are two possibilities: (*i*) to mechanically crush large beads or solid alginate sheets and then sieve them to desired size, or (*ii*) to produce microbeads directly using an appropriate technology. In this study, encapsulating a sufficient amount of cells in beads and production of a powder-like formulation (dry beads) by drying or lyophylization of macrobeads was used, as well as fresh beads for enzyme production or for seed coating for plant growth promotion. It was reported that the bacteria are inactive but alive in the dry agricultural preparation, which is preferable since their activity is needed only after seed germination and the decomposition of beads (*70*).

Results of this study showed no consistent pattern in comparing free with immobilized cells for enzyme production. Previously, high level of alkaline protease enzyme production had been observed in the immobilized cultures of *Conidiobolus* spp. and *Aspergillus flavus* (*71*). El-Ahwany and Youssef (*72*) reported that cells immobilized in 2 % alginate showed a 2.6-fold increase in xylanase-specific activity compared to free cells. The immobilized cells of *Aspergillus* sp. in calcium alginate doubled the production of endoPG in comparison with free cells (*73*), while alginate entrapment of *Aspergillus niger* showed a relatively low β-mannanase activity compared to free culture (*74*).

In the present study, alginate encapsulation of *Trichoderma* and/or *Azospirillum* prolonged the durability of the inoculum and retained, or in most cases even increased the enzyme production during three repetition cycles. This result is consistent with xylanase production by immobilized *Trichoderma reesei* SAF3 in Ca-alginate beads, which increased gradually up to the fourth repeated batch cycle (*75*). During the early repeated cycles, increased amount of xylanase production was noticed and this may be due to the proper adaptation of the fungal cells to the microenvironment and appropriate growth of cells in the beads (*75*). For all C sources used, productivity of enzymes gradually increased up to the third batch, which could be related to the growth of cells. Thus, Ca-alginate is a suitable and robust carrier system for immobilization of these fungal or bacterial strains.

Plant growth-promoting bacteria (PGPB) are defined as free-living soil, rhizosphere, rhizoplane, and phylosphere bacteria that are beneficial for plants under some conditions. PGPB promote plant growth in two different ways (Fig. 5). First, they directly affect the metabolism of the plants by providing substances that are usually in short supply. These bacteria are capable of fixing atmospheric nitrogen, or solubilizing phosphorus and iron, and producing plant hormones such as auxins, gibberellins, cytokinins, indoleacetic acid (IAA) and ethylene. Additionally, they improve the stress tolerance in plants such as drought, high salinity, metal toxicity, and pesticide load. One or more of these mechanisms may contribute to the increases obtained in plant growth and development that are higher than normal for plants grown under standard cultivation conditions. However, these bacteria do not enhance the genetic capacity of the plant, as genetic material is not transferred. A second group of PGPB, referred to as biocontrol PGPB, indirectly promote plant growth by preventing the deleterious effects of phytopathogenic microorganisms (bacteria, fungi, and viruses) (Fig. 5). They produce substances (siderophores, β-1,3-glucanases, chitinases, antibiotics and cyanide) that harm or inhibit other microbes but not plants, by limiting the availability of iron to pathogens (production of siderophores) or by altering the metabolism of the host plant to increase its resistance to pathogen infection. Biocontrol PGPB can also possess traits similar to PGPB, *e.g.* they may fix nitrogen or produce phytohormones (*76*).

Trichoderma spp. are thought to promote plant growth by at least two different mechanisms: (*i*) by controlling the population of pathogenic microorganisms in the rhizosphere (*21*), and (*ii*) by influencing plant physiology through mineral solubilization (*22*) or hormone secretion (*77*). Furthermore, plant cell wall-degrading enzymes produced by *Trichoderma*, such as xylanases and cellulases, are able to directly induce ethylene biosynthesis in plants, which is a well-known response to the presence of pathogens (*26*). Previously, El-Katatny (*20*) studied the antagonistic actions and production of polysaccharide-degrading enzymes by *T. harzianum* T24 against some phytopathogenic fungi.

Symbiotic N_2 fixation is a well-known process exclusively driven by bacteria, the only organisms possessing nitrogenase, the key enzyme which specifically reduces atmospheric N_2 to ammonia in the symbiotic root nodules (*78,79*). N_2 fixation is the first step for cycling N

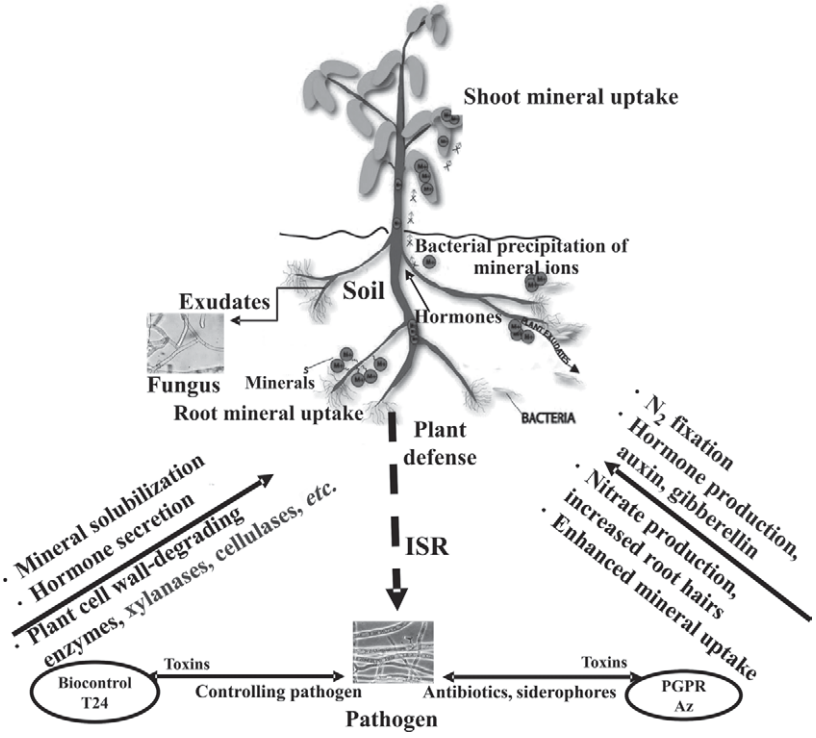

Fig. 5. Suggested modes of action of *Azospirillum* (PGPR Az) and *Trichoderma* (biocontrol T24) in promoting plant growth (ISR-induced systemic resistance)

to the biosphere from the atmosphere, a key input of N for plant productivity (*80*). In this study, a non-symbiotic *A. brasilense* had a weak capability to degrade pectin as compared to *T. harzianum* in free or immobilized cultures for PGase and N fixation. However, the mixed culture of *A. brasilense* and *T. harzianum* (free or coimmobilized) was able to degrade a large quantity of pectin and N fixation increased significantly. This may indicate that *A. brasilense* grows and fixes nitrogen in mixed culture at the expense of the fermentation products of pectin made available by the activity of *T. harzianum via* pectin degradation. These results are consistent with those obtained by El-Katatny *et al.* (*55*), who studied pectin decomposition by mixed cultures of *A. brasilense* and *Penicillium corylophilum*.

A tendency to increase nitrogen fixation was also observed in sterile soil with different C sources added upon the inoculation with *A. brasilense* alone or in combination with *T. harzianum.* The highest values of nitrogen fixation were recorded when monosaccharides (glucose) were added to the soil. The extracellular microbial hydrolysis of polymers in soil resulted in the increase of the concentration of easily available mono- and oligomeric carbohydrates. In turn, this resulted in the activation of nitrogen fixation by the free-living heterotrophic bacteria (*27*). It had been demonstrated earlier that the nitrogenase activity of bacteria, including bacilli, in nutrient media increase when grown together with *T. hamatum, T. harzianum, Penicillium claviforme, Mortierella ramanniana* or *Cladosporium herbarum* (*30,81*). This would answer why growth parameters of seeds treated with *A. brasilense* Az in combination with *T. harzianum* T24 were improved significantly when soil was enriched with different C sources.

The results provide evidence that seedlings of tomato plant are potentially able to create a symbiosis with a diazotrophic bacterium *A. brasilense*, which colonizes seedling root, promoting a higher level of N_2 fixation and may play a role in better growth and development of the plant. *Azospirillum* species are also considered to be PGPR (*82,83*). A significant activity of *Azospirillum* could be related to the production of auxin-type phytohormones that affect root morphology and thereby improve nutrient uptake from the soil (Fig. 5). This may be more important for plant growth than the N_2-fixing activity (*84*). Inoculation with *Azospirillum* sp. mainly changes the growth or morphology of roots by increasing the number of lateral roots and root hairs (*85*). The enlargement of the root surface results in better nutrient uptake and an improved water status, which may be the main factors enhancing plant growth (*86*).

Increased growth response (IGR) has been demonstrated numerous times in mineral-limited soil environments, and a direct role for *T. harzianum* in the uptake of minerals and growth promotion was suggested (*87*). Results presented by Yedidia *et al.* (*88*) confirmed that the concentrations of important minerals including P, Fe, Mn and Zn are increased in plants grown exclusively with *T. harzianum*. In addition, the pathogen-free environment (sterile soil) enabled this research plan to study the direct influence of *T. harzianum* as a double inoculant with *A. brasilense* on the growth response of tomato seedlings.

Dual inoculation with *Azospirillum* and *Trichoderma* has some advantages over single inoculation. Possible explanation of this phenomenon includes the control of minor pathogens that lead to stronger growth and nutrient uptake of the plant (Fig. 5) (*89*). Janzen *et al.* (*90*)

found that co-culture of *A. brasilense* and the barley straw-degrading fungus *T. harzianum* produced a substantial increase of gibberellin (GA), a growth hormone. The amounts of gibberellins and cytokinins produced by the mixed culture of *A. brasilense* and *Arthrobacter giacomelloi* were higher than those of the single cultures (*91*), suggesting that the interactions between the rhizosphere inhabitants may affect their secondary metabolism and indirectly the plants (*91*).

Previously, mixed inoculation of *Azospirillum* and the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus intraradices* in sorghum created a synergistic interaction resulting in a significant increase in many plant growth parameters including a concomitant increase in the levels of root phosphatases (both alkaline and acidic), an increase in the phosphorus content in plants, and enhanced uptake of nitrogen, zinc, copper, and iron. This double inoculation could replace the application of N and P fertilizers (*92*). Inoculation of wheat seeds of various genotypes with *Glomus fasciculatum* increased the grain and straw yields significantly. However, further increase in grain yield was recorded by co-inoculation with *Azospirillum brasilense* (*93*). The plant growth promotion of tomato seedlings could be related mainly to the hormonal effect or plant growth regulators that regulate the expression of growth-related genes. The production of plant growth regulators by microorganisms is an important mechanism often associated with growth stimulation (*94*).

Conclusion

In the present study, the ability of a rhizobacterium (*Azospirillum brasilense*) and a biocontrol fungus (*Trichoderma harzianum*) was evaluated for their individual and combined effects on the production of hydrolytic enzymes and growth promotion of tomato seedlings. Co-inoculation of *T. harzianum* and *A. brasilense* (free or coimmobilized) could positively affect the production of lytic enzymes, nitrogen fixation, and significantly improve plant growth promotion when tested on tomato seedling growth. The enhancement of tomato seedling growth following co-inoculation could be due to the synergistic effect of both *Trichoderma* and *Azospirillum.*

Applying plant growth promotion by microbial inoculants to barren (infertile) ground areas, the roles of microorganisms and the method of formulating the microbial inoculant need further investigation. The synergistic effect of inoculants would be improved by the tools of immobilization and could reveal benefits for different applications in the fields of biotechnology.

It can be concluded from this study that coimmobilization of two or more cultures can derive benefits from all of them better than from their individual immobilizations. Co-inoculation with *Azospirillum* and other microorganisms is one of the major frontiers of *Azospirillum* technology and perhaps the main area for future applications.

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