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Sweet pepper internal tissues are repositories for endophytic multifunctional diazotrophs supporting plant nutrition, health and fruit yield

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ABSTRACT

This work provides original evidence for the endophytic diazotroph community structure of sweet pepper internal tissues. A medium modification based on basal salts with a mixture of five carbon sources (mannitol, sucrose, glucose, malic acid and sodium lactate) to fulfill the requirements for the majority of endophytic N_2-fixers to exhibit good growth and biomass production was evaluated. Total diazotroph counts of *ca*. 10^6 cells g^{-1} were recorded inside root and stem sweet pepper samples, lower populations (< 10^4 cells g^{-1}) were encountered in leaves. Identification of representative endophytic isolates was performed using the API microtube systems; 20 E, 20 NE and 50 CH. API profiles of the isolates were closely related to those of the genera *Bacillus*, *Enterobacter*, *Klebsiella*, *Paenibacillus*, *Pseudomonas* and *Rahnella*. The majority of isolates successfully fixed N_2 (up to 18.2 mg N fixed/ g C consumed), reduced C_2H_2 (7.5-62.7 nmoles C_2H_4 culture hr^{-1}), produced IAA and GA_3 (9.1-71.2 and 0.8-10.4 ppm), as well as siderophores (39.4-90.4 SU%). This is beside their ability to solubilize phosphate (99.9-303.1 %) and production of extracellular polymeric substances (0.99-2.84 g l^{-1}). Adopting the dual Petri-dish culture assay, the isolates showed high lethality against the pathogenic fungi *A. solani*, *F. oxysporum, F. solani, R. solani* and *S. sclerotiorum* with growth inhibition percentages of 10 -> 60. In a greenhouse experiment, introduction of endophytic diazotroph bioagents to fungi-infested pepper plants did significantly alleviate the severity of the pathogens, an effect that was endophyte isolate-dependent. Field-grown pepper responded to diazotroph inoculation in presence of rational N fertilizer level. The endophytes *P. polymyxa* and *E. agglomerans* were the superiors among the single inocula resulting in fruit yields of 1.94 and 1.95 kg row^{-1} respectively. The highest fruit yield of 2.41 kg row^{-1} was obtained by the composite inoculum-treated peppers.

Keywords: Sweet pepper, endophytes, API profiles, pathogens, antibiosis, fruit yield.

Introduction

The traditional agricultural practices particularly the application of agrochemicals (mineral fertilizers and pesticides) are seriously affecting the agro-ecosystem balance resulting in harmful effects on plant nutrition and health. Fortunately, a great number of soil micro-residents particularly N_2-fixers and biocontrol agents are documented as key players in plant development and productivity in various environments.

The last decades witnessed that such creatures are occupying the outer plant surfaces besides the internal tissues, the latter are referred as endophytes. Baldani *et al.* (1997) reported that the endophytic microorganisms include facultative and obligate ones. The endophytic interactions between the endophytes and host plants represent a unique example for supporting the plant growth and safety. More than 80 bacterial candidates were found by Lodewyckx *et al*. (2002) to successfully reside the internal tissues of a wide range of plants. Surette *et al*. (2003) mentioned that multitudes of endophytic organisms present in all plant organs including seeds, roots, stems, leaves and fruits are seemingly neutral in terms of plant health. Barka *et al*. (2002) and Bailey *et al*. (2006), on the contrary, reported that the majority of endophytes are considered not neutral but are very beneficial in most cases.

Generally speaking, the endophytic microbiota are positively contributing to plant welfare *via* stimulating the growth (Kang *et al*., 2007), alleviating the deleterious and lethal impacts of plant pathogens (Senthikumar *et al.,* 2007), producing anti-herbivory products (Sullivan *et al*., 2007) and

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assimilating the nitrogen gas to provide a part of plant N needs through nitrogen fixation ((Jha and Kumar, 2007), in addition to increase the nutrients uptaken by plants (Malinowski *et al*., 2000). All of all, such benefits that the endophytes can provide to the associated hosts, did encourage the scientists interesting in the research area of microbe-plant interweave to use this very special group of micro-inhabitants in the biofertilization and biological control programs for both annual and perennial crops.

As a part of the on-going research attempts to guarantee productive and safe exporting agricultural commodities, the present study was executed. The major targets encompassed investigating the ability of a number of endophytic N_2-fixing bacteria, isolated from internal sweet pepper organs, to promote the vegetable growth and fruit yield, besides restricting the hazards of pathogenic fungi that infect the plant.

Materials and Methods

Endophytic diazotroph occurrence in sweet pepper

Sweet pepper plants were taken from different field sites of Ismailia governorate and separated into roots, stems and leaves. Endophytic residence of diazotrophs was examined in a set of samples by incubating 4 cm water-washed plant portions overnight in tubes ½ filled with tetrazolium-phosphate buffer solution (PBMT). This solution consists of 0.05 M potassium phosphate buffer (pH 7.0) that contains 0.625 g l^{-1} malate and 1.5 g l^{-1} 2, 3, 5- triphenyl tetrazolium chloride. The buffer malate mixture was autoclaved and tetrazolium added after autoclaving. An additional batch of samples was surface sterilized adopting various procedures as present in Table (1). Thereafter, the plant materials were thoroughly washed several times with sterile distilled water. Sterility check was made by inserting plant samples on the surface of nutrient agar plates. Finally; roots, stems and leaves were triturated for 5 min. in Warring blender using sufficient amount of half strength basal salt of liquid N-deficient combined carbon sources medium (CCM) of Hegazi *et al*. (1998) as a diluent. Further serial dilutions were prepared, using the same diluent, for counting total diazotroph communities of inner plant tissues. From each suitable dilution, 1 ml aliquots were transferred to 5 tubes containing 5 ml of semi-solid CCM, tubes were then incubated at 30 °C for 5 days. The most probable numbers (MPN) were calculated according to Alexander (1982). The used CCM comprised the following (g l^{-1}): glucose, 2.0; malic acid, 2.0; mannitol, 2.0; sucrose, 1.0; K_2HPO_4, 0.4; KH_2PO_4, 0.6; $MgSO_4$, 0.2; NaCl, 0.1; $MnSO_4$, 0.01; yeast extract, 0.2; fermentol (a local product of corn-steep liquor), 0.2; KOH, 1.5; $CaCl_2$, 0.02; $FeCl_3$, 0.015; Na_2MoO_4, 0.002. In addition, $CuSO_4$, 0.08 mg; $ZnSO_4$, 0.25 mg; sodium lactate, 0.6 ml (50 v/v) were added per litter.

Isolation and identification of N_2-fixing endophytes

Isolation of endophytic diazotrophs was performed by streaking loopfuls from positive MPN tubes on agar plates of CCM medium. Colonies of 3-4 day old were purified with single colony isolation. Eighty eight pure isolates were examined for acetylene reducing activity (Hegazi *et al*., 1980), then screened down to 15 superior N_2-fixing ones producing more than 10 nmoles C_2H_4 culture^{-1} hr^{-1}. Pure N_2-fixing isolates were tested for colony morphology, cell characteristics and some physiological characteristics according to Berge's Mannual of Systematic Bacteriology (Goodfellow *et al*., 2012). This is in addition to API microtube systems (Logan and Berkeley, 1984); API 20E for Enterobacteriaceae, 20 NE for non-Enterobacteriaceae and 50 CHB for Bacilliaceae. Other conventional tests such as Gram stain and motility were considered as well.

Biochemical potential of endophytic diazotrophs

a- Efficiency of N_2- fixation

Nitrogen fixation efficiency of the endophytes expressed as mg N fixed/ g C consumed was determined using the method of Kanimozbi and Panneerselvan (2010). Fifty ml combined carbon sources liquid medium lacking nitrogen in 150 ml Kjeldahl flasks were sterilized @ 121 °C for 20 min. and inoculated with 0.5 ml suspensions of actively growing bacteria (96-hr old). Three replicates

for each tested strain were prepared. Flasks were incubated @ 30 °C for 15 days. Prior to chemical measurements, the bacterial cultures were brought back to the original volumes with distilled water and carfully shaken. Two ml of suspensions were estimated for organic carbon and the reminders for total nitrogen estimation (Jackson 1973).

b- Acetylene reducing activity

Tubes of 3-4 day old cultures were fitted with rubber closures and the gas phase inside was evacuated, then 10 % air and 10 % C_2H_2 were injected. Tubes were incubated @ 30 °C for 4 hrs. followed by measuring C_2H_4 produced using gas chromatograph (Hegazi *et al.*, 1980).

c- Indol acetic acid production

Production of IAA was determined by the modified technique of Sachdev *et al.* (2009). Diazotrophs were grown for 14 days in liquid CCM @ 30 °C on a rotary shaker in 100 ml medium in 500 ml flasks. Cultures were centrifuged @ 3000 rpm, supernatants were acidified to pH 2.8-3.0 with dilute HCl and extracted 3 times with ethyl acetate. The mixed ethyl acetate extracts were allowed to evaporate at room temperature to dryness and the residues were dissolved in methanol (0.5 ml). To 1.5 ml distilled water in test tube, 0.5 ml of the methanol fraction was mixed and 4 ml of fresh Salper's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) were added dropsies. The color developed was allowed to proceed for 1 hr. in dark and read in Bechman DU spectrophotometer @ 535 nm against a reagent blank. A series of known IAA solutions were similarly determined for preparing a standard curve from which the unknowns were calculated.

d- Gibberellin production

Adopting the procedure of Bilkay *et al*. (2010), gibberellin produced by the endophytes was estimated. Freshly prepared cultures were filtered, acidified to pH 2.5 by HCl and extracted using liquid-liquid (ethylacetate/ $NaHCO_3$) extraction. Gibberellic acid in the ethylacetate phase was assessed by UV spectrophotometer @ 254 nm. The amount of gibberellic acid was estimated from the standard curve.

e- Siderophore production

The technique described by Sayyed *et al.* (2005) was used. Iron-free SM medium (Meyer and Abdallah, 1978) consisting of (gl^{-1}): K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0 and succinic acid, 4.0 was inoculated with 24 hr- old cultures @ the rate of 1 % (v/v). Incubation took place for 24-30 hr @ 29 °C with shaking at 120 rpm. Cultures were centrifuged (10000 rpm for 15 min.) and supernatants were estimated for siderophores using CAS-shuttle assay (Payen, 1994). Five ml of culture supernatant was combined with 0.5 ml of CAS reagent, and absorbance was measured @ 630 nm against a reference consisting of 0.5 ml uninoculated broth and 0.5 ml CAS reagent. Siderophore content was calculated using the following formula:

% siderophore units (SU) = Ar-As x100 / Ar

where: Ar = absorbance of reference @ 630 nm (CAS reagent) and As = absorbance of sample @ 630 nm.

f- Phosphate solubilization efficiency

Tested strains were screened for their phosphate solubilizing ability on Pikovskaya's agar medium (Pikovskaya, 1948) supplemented with tri-calcium phosphate at a concentration of 0.5 % (w/v). The solubilization region and colony diameters were measured after five days of incubation at 30 °C. Results were expressed as phosphate solubilizing efficiency (SE) according to Nguyen *et al*. (1992) as the following:

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

g- Extracellular polymeric substances (EPS) production

The formation of EPS by the endophytes was determined by using a modified procedure of Mondel *et al*. (2008) as mentioned by Dante *et al.* (2016). Two day-old broth cultures of the examined organisms were decanted into 50 ml centrifuge tubes, vortexed for 5 min. and centrifuged @ 10000 rpm for 30 min. @ 4 °C to remove cells. The supernatants were collected and 2.0 volumes of acetone 80 % were added and kept overnight @ 4 °C for precipitation. The pellets, collected by centrifugation @ 12000 rpm for 10 min., were dissolved in deionized distilled water and dialyzed overnight at 4 °C against deionized distilled water. Dialyzed materials were lyophilized and weighed. Experiments were performed three times to ensure reproducibility.

Isolation of pathogenic fungi

Several root and stem samples taken from infected sweet pepper plants showing wilt disease symptoms were washed in tap water, dried between two filter papers and cut into 5 mm pieces. Those were surface sterilized with 3 % sodium hypochlorite for 3 min., rinsed in sterile distilled water and put on potato dextrose agar (PDA) plates. Incubation took place at 30 °C with daily observation of fungal growth. The isolated fungi were purified using the single spore technique (Hidlbrand, 1948) and / or hyphal tip procedure of Schneider and Kelly (2000). Purified fungi were transferred to PDA slants and kept @ 5 °C. Fungal identification was done at the Mycology Research and Disease Survey Department, Plant Pathology Research Institute, ARC, Giza using the methods of Gilman (1957), Booth (1971), (1977) and Domsch *et al.* (1980).

Endophytic diazotroph antibiosis towards fungal pathogens

The capability of selected diazotrophs to antagonize pathogenic fungi was experimented using dual Petri dish culture test described by Landa *et al*. (1997). Sterilized Petri dishes (9 cm diameter) that contain PDA medium were streaked by 2-day old culture of either bacterial antagonist at the periphery of the plate and incubated @ 28 °C for 24 hr. Following bacterial development, mycelial plugs (5mm) from the advancing edges of 7-day old fungal PDA cultures were deposited into plates. Plates with no bacteria were used as control. Plates were reincubated at 28 °C and checked daily. Three plates were prepared for every treatment. When growth of the pathogen covered the full plate surface in the control, the reduction in mycelial expansion of the tested fungi was determined. Reduction percentage in the linear growth of the pathogen on the nearest sides to the bacterial bioagent was determined according to Fokemma (1973) as the following:

Reduction in linear growth = R1-R2 X 100 / R1

where: R1, the radius of normal (control) growth; R2, the radius of inhibited growth.

Diazotroph inocula preparation

Based upon chemical potentials and antibiosis towards tested pathogenic fungi, the superior 4 endophytic diazotrophs were selected for inoculation experiments. The N_2-fixing members were individually grown in liquid CCM for 4 days @ 30 °C, this is to guarantee an inoculum density of *ca*. 10^8 cells ml^{-1} for either. For the composite inoculum preparation, equal volumes of cell suspensions were carefully mixed just prior to application.

Pathogenic fungi cultures preparation

Either *Fusarium oxysporum* or *Rhizoctonia solani* culture was prepared using sorghum grain medium in glass bottles (500 CC) and incubated @ 27 °C for 18 days. For infestation in the pot experiment, the pathogen inoculum was added to soil at the rate of 2 % (w/w) prior to planting.

Greenhouse experiment

The effectiveness of selected endophytic diazotrophs to restrict the pathogenicity of *F. oxysporum* and *R. solani* towards pepper plants was evaluated in a pot experiment. A sandy soil taken from Ismailia (pH, 8.2; EC, 0.94 dSm^{-1}; OC, 0.28 %, TN, 0.09 %) was distributed in plastic pots (30 cm diameter, 40 cm depth) as 10 kg pot^{-1}. Before transplanting, the NPK fertilization regime recommended for pepper cultivation was added for uninoculated treatments. This represented by the incorporation into soil of superphosphate (P_2O_5, 15 %), potassium sulphate (K_2O, 50 %) and urea (N, 46.5 %) in quantities equivalent to 150, 50 and 50 kg $acre^{-1}$ respectively. All inoculated treatments received 100 % PK levels but 50 % of N. Thirty-day old seedlings of sweet pepper cv. California Wonder were transplanted as 3 pot^{-1}. After soil infestation with pathogenic fungi, the different diazotroph formulations were introduced into plant-soil systems in several methods. Those are root dipping, seedling spray and over-head soil addition. Along the experimental period, plants were irrigated with tap water up to 70 % WHC. Pots were arranged in the greenhouse in a complete randomization with 5 replicates. For one set of pots, 45- and 60-day old plants were examined for disease percentage based on number of dead plants/ total number of grown plants x 100. The efficiency of endophytes to control pathogenic fungi was determined using the formulation of Wang *et al*. (1994) as follows: Efficiency % = A-B X100/A

where: A, disease percentage of bioproduct-untreated plants; B, disease percentage of treated ones. Another set of pots was kept for 90 days where plants were uprooted and separated into roots and shoots and determined for dry weights (70 °C to constant weight).

Field trial

A field experiment was carried on 18 April, 2016 in a private farm at Ismailia. The recommended agronomic practices for pepper cultivation were applied. The experimental area was divided into plots with 3 rows of 38 m long in each. Before planting, the P, K and S fertilization regimes of 6.0, 0.5 and 0.5 kg row^{-1} of superphosphate (P_2O_5, 15 %), potassium sulphate (K_2O, 50 %) and sulpher respectively were used. Rational N fertilizer dose of urea (N, 46.5 %) was incorporated into soil at the rate of 0.25 kg N row^{-1}. Besides, a slow release organic fertilizer in the form of chicken manure (OC, 24.3 %; TN, 3.1 %) was applied as 15 m^3 $acre^{-1}$. The endophytic diazotrophs used in the pot experiment either separately or all in combination were applied in this experiment. *In situ*, the root system of one set of 30-day old seedlings cv. California Wonder was dipped for 20 min. in either diazotroph liquid culture of cell density of *ca.* 10^8 ml^{-1}. Another group of seedlings was over-head soil inoculated after cultivation. Along the growth period, plants were fertigated with water containing potassium sulphate and phosphoric acid in quantities equivalent to 10.0 and 1.0 kg $acre^{-1}$ respectively. The experimental design was a split-plot where diazotroph members allocated the main plot and sub-plot for inoculation method. Six months after cultivation, pepper plants were taken and determined for leaves and fruits traits. Those included chlorophyll a, b and carotenoids (Inskeep and Bloom, 1985) as well as nitrogen (Horneck and Miller, 1998), phosphorus (Horneck and Hanson, 1998) and potassium (Sandell, 1950) for leaves. While fruits were tested for length, diameter, total yield and NPK contents.

Statistical analysis

Data were statistically analyzed for least significant differences ($p < 0.05$) as mentioned by Huang and Chen (2008).

Results

Endophytic occurrence of diazotrophs in pepper tissues

After overnight incubation with 2,3,5-triphenyl tetrazolium chloride; pepper roots, stems and leaves appeared red-colored as a result of 2,3,5-triphenyl formazan production. The color intensity was naked eye-distinguishable particularly for surface sterilized samples; indicating the endophytic

occurrence of dense populations of diazotrophs. It was verified by light microscopic observations where bacteria were present inter-and intra-cellular.

Almost all surface sterilization procedures used successfully eliminated great parts of roots, stems and leaves surface-colonizing microorganisms in addition to those had the ability to inter plant organs and create endophytic association. Among the applied methods, treatment with 95 % ethanol for 5-10 min. followed by 3 % sodium hypochlorite for 30 min. appeared appropriate and facilitates the isolation of the endophytes. Based on MPN estimations using N-deficient combined carbon sources medium (CCM) and avoiding the tap water -treatment, roots supported the greatest existence of endophytic diazotrophs (0.8-71.9 x10^4 cells g^{-1}) followed by stem that recorded 0.3-34.4 x 10^4 cells g^{-1} (Table 1). This proves that both plant roots and stems deem suitable repositories for diazotroph endophytic establishment. N_2-fixing microbiota rarely existed in leaves being < 10^4 g^{-1} or completely absent.

Table 1: Most probable numbers (MPN) of endophytic diazotrophs in sweet pepper roots, stems and leaves surfacely sterilized with various procedures

Procedures	References	MPN (10^4 cells g^{-1})		
		Roots	Stems	Leaves
1. Tap water	Cavalcante and Dobereiner (1988)	118.6	15.3	0.9
2. Chloramine T (1 %, 5 min.)	Paula *et al*. (1991)	26.4	9.1	0.2
3. Ethanol (95 %, 5-10 sec.), sodium hypochlorite (3 %, 30 min.)	Somasegaran and Hoben (1985)	71.9	34.4	0.2
4. Hydrogen peroxide (3 %, 5-10 min.)	Somasegaran and Hoben (1985)	16.7	8.0	ND
5. Mercuric chloride (0.1 %, 5-10 min.)	Somasegaran and Hoben (1985)	2.2	0.3	ND
6. Mercuric chloride (0.2 % in ethanol 50 %, 4 min.)	Gagen *et al*. (1987)	0.8	1.1	ND
7. Sodium hypochlorite (3 %, 3-5 min.)	Somasegaran and Hoben (1985)	29.4	8.3	0.8
8. Sodium hypochlorite (5 %, 30 min.)	Bilal *et al*. (1990)	43.3	1.6	0.8
9. Sodium hypochlorite (6 %, 2 min.), ethanol (95 %, 15 sec.), flame	Gagen *et al*. (1987)	3.6	3.8	0.1

ND, not detected.

A major advantage of CCM is that colonies of various diazotrophs displayed distinguished characteristics enabling easy discrimination. The typical colonies developed on CCM agar plates after inoculation from the positive MPN tubes were examined. *Bacillus* spp. grew well producing tear-like, slimy and transparent colonies. *Klebsiella* spp. colonies were slimy, not watery and overspread other colonies. *Enterobacter* spp. produced smooth, irregular and translucent colonies. Non-pigmented, confined and opaque colonies characterized *Rahnella* spp. While fluorescency of *Pseudomonas* spp. was easily distinguished against bright daylight and UV.

Prevailing endophytic diazotrophs

Single-colony isolation technique was adopted for isolation followed by purification of a number of diazotroph representatives. A total number of 88 pure isolates was tested for acetylene reducing activity. Those producing > 10 nmoles C_2H_4 $culture^{-1}$ hr^{-1} (15 isolates) were selected for identification. Based on Gram reaction and motility, in addition to API 20 E, 20 N E and 50 CH profiles (Table 2), the N_2-fixing spore-forming bacilli were predominant (40 %) represented by the species *Bacillus circulans* (1), *Bacillus macerans* (1), *Bacillus megaterium* (1) *Bacillus pumilus* (1) beside *Paenibacillus polymyxa* (2). The non-sporing members were represented by 9 isolates. They belonged to the genera *Pseudomonas* (*Ps. Luteola*, 4 isolates); *Enterobacter* (*E. cloacae*, 2 isolates; *E. agglomerans*, 1 isolate); *Klebsiella* (*K. oxytoca*, 1 isolate) and *Rahnella* (*R. aguatits*, 1 isolate).

Biochemical characteristics of the endophytes

Special attention was given to the most biochemically active isolates to be selected for inoculation experiments. Table (3) shows the biochemical potentials of the 15 N_2-fixers.

While *P. polymyxa* (BR2) exhibited the highest N_2-fixation efficiency of > 18 mg N fixed/ g C consumed, the leaves endophyte *Ps. luteola* (BL2) scored the lowest efficiency of 1.1. *B. megaterium* seemed an efficient N_2-fixer and assimilated *ca.* 17 mg N. Other endophytic diazotrophs variably fixed atmospheric dinitrogen with efficiencies of 1.9-13.9 mg N fixed/ g C oxidized. *P. polymyxa* (BR2) exhibited up to > 62 nmoles C_2H_4 culture hr^{-1} followed by *Ps. luteola*, CR2 (45.8) while the lowest acetylene reducing activity (7.5 nmoles C_2H_4 culture hr^{-1}) was scored by *K. oxytoca*. The microorganism *E. agglomerans* successfully expressed itself as a true N_2-fixer recording activity of 43.8 nmoles C_2H_4 culture hr^{-1}. Almost all the effective ethylene producers did possess great capacity to produce a number of biologically active substances. Appreciable quantities of IAA (up to 71.2 ppm) were produced by the tested diazotrophs, *P. polymyxa* (BR2) was the pioneeric. This diazotroph appeared a good producer for gibberellins (8.3 ppm) and siderophores (61.9 ppm). *B. megaterium*, *E. agglomerans* and *Ps. luteola* are considered as well among the plant growth promoting diazotrophs. All the examined endophytes had high phosphate solubilizing efficiency (PSE) where the estimated percentages ranged from 99.9 to 303.1. Spore-forming bacilli ranked the first among the tested endophytes in respect to their capability to generate extracellular polymers, amounts were falling in the range 0.85-2.84 g l^{-1} being the highest by *B. macerans*. Except *E. cloacae* (BL1), other non-spore formers produced amounts not exceeding 2.0 g l^{-1}. As low as 0.85 g l^{-1} extracellular polymeric substances was detected in *Ps. luteola* (BL2) culture supernatant.

Table 2: Cell characteristics and taxonomic position of the selected endophytes based on the API microtube systems

Isolate code*	Plant sphere	Gram reaction	Motility	Proposed position			Identity %
				API 20 E	API 20 NE	API 50 CH	
AR1	Roots	+	Motile			*Bacillus megaterium*	98
AR2	Roots	-	Non-motile	*Enterobacter cloacae*			98
AS1	Stems	-	Non-motile	*Klebsiella oxytoca*			98
AS2	Stems	+	Motile			*Bacillus circulans*	89
AL1	Leaves	-	Motile		*Pseudomonas luteola*		99
BR1	Roots	+	Motile			*Bacillus macerans*	98
BR2	Roots	+	Motile			*Paenibacillus polymyxa*	98
BS1	Stems	-	Motile	*Enterobacter agglomerans*			97
BL1	Leaves	-	Non-motile	*Enterobacter cloacae*			88
BL2	Leaves	-	Motile		*Pseudomonas luteola*		89
CR1	Roots	+	Motile			*Paenibacillus pumilus*	98
CR2	Roots	-	Motile		*Pseudomonas luteola*		98
CS1	Stems	+	Motile			*Paenibacillus polymyxa*	99
CS2	Stems	-	Non-motile	*Rahnella aguatits*			97
CL1	Leaves	-	Motile		*Pseudomonas luteola*		98

*; A,B,C represent sampling sites.

Table 3: Biochemical activities* of selected endophytic diazotrophs

Endophytes	NFE	ARA	IAA	GA_3	Sider	PSE	EPS
B. circulans	11.4	36.6	44.4	2.4	80.1	250.6	2.24
B. macerans	10.3	18.1	28.9	0.9	66.5	150.4	2.84
B. megaterium	16.6	44.1	59.4	10.4	80.4	211.1	2.09
***P. polymyxa* (BR2)**	18.2	62.7	71.2	8.3	61.9	240.0	1.88
***P. polymyxa* (CS1)**	8.8	24.6	45.5	9.1	74.6	210.2	2.66
P. pumilus	4.7	10.8	66.6	3.4	39.4	181.1	1.91
E. agglomerans	13.9	43.8	59.7	8.3	90.4	303.1	1.97
***E. cloacae* (AR2)**	9.4	24.5	59.2	2.9	52.3	198.7	1.69
***E. cloacae* (BL1)**	6.8	11.6	43.3	10.3	61.8	201.4	2.14
K. oxytoca	3.3	7.5	38.1	0.8	51.8	ND	1.33
R. aguatits	1.9	10.9	9.7	3.7	42.6	150.6	0.99
***Ps. luteola* (AL1)**	5.6	15.6	28.8	4.1	81.2	99.9	1.73
***Ps. luteola* (BL2)**	1.1	11.7	11.6	8.5	47.4	108.3	0.85
***Ps. luteola* (CR2)**	9.9	45.8	54.7	6.4	76.5	152.4	1.46
***Ps. luteola* (CL1)**	4.8	20.7	9.1	2.9	46.4	ND	1.06

*; NFE (nitrogen fixation efficiency, mg N fixed/ g C-oxidized), ARA (acetylene reducing activity, nmoles C_2H_4 $culture^{-1}$ h^{-1}), IAA (indole acetic acid, ppm), GA_3 (gibberellins, ppm), PSE (phosphate solubilizing efficiency, %), Sider. (siderophores, SU %), EPS (extracellular polymeric substances, g l^{-1}).

Antibiosis of endophytes against pathogenic fungi

Using the dual Petri-dish culture assay, the 15 Gram-positive and-negative endophytic diazotrophs were experimented for antibiosis towards 5 pathogenic fungal strains representing 4 genera. All the tested diazotrophic bioagents did conspicuously inhibit the majority of neighboured fungal candidates, an effect that differed from a fungus to the other (Table 4). Apart from pathogen, *P. polymyxa* isolated from pepper roots was the most suppressive recording fungal growth inhibition percentages of 34-55 with an average of 46.8. This was followed by *B. megaterium* that scored average growth inhibition of 40.4 % although it failed to antagonize the pathogen *R. solani*. On the contrary, *K. oxytoca* deemed the inferior as a bioagent, an average growth inhibition percentage of 13.0 was estimated. Compared to other pathogens, the fungus *R. solani* exhibited the highest resistance patterns showing average of 17.1 % growth inhibition. On the other hand, both *A. solani* and *S. sclerotiorum* showed a unique susceptibility to the tested endophytic bioagents with average growth inhibition of approximately 31 %. Gram positive bioagents, in general, showed higher lethality against pathogenic fungi than Gram negatives, the average inhibition percentage was 34.1 for the former and 27.9 for the latter.

Table 4: Endophytic diazotrophs antibiosis towards pathogenic fungi (expressed as percentage of growth inhibition)

Endophytes	*Alternaria solani*	*Fusarium oxysporum*	*Fusarium solani*	*Rhizoctonia solani*	*Sclerotinia sclorotiorum*
B. circulans	28	42	23	39	48
B. macerans	36	22	36	10	11
B. megaterium	62	45	41	0	54
***P. polymyxa* (BR2)**	41	52	55	34	42
***P. polymyxa* (CS1)**	32	27	30	35	33
P. pumilus	40	18	26	0	22
E. agglomerans	44	28	46	30	48
***E. cloacae* (AR2)**	51	35	28	0	16
***E. cloacae* (BL1)**	27	26	0	0	33
K. oxytoca	0	16	26	23	0
R. aguatits	15	20	18	40	0
***Ps. luteola* (AL1)**	0	21	30	0	40
***Ps. luteola* (BL2)**	18	0	15	0	32
***Ps. luteola* (CR2)**	52	48	30	18	38
***Ps. luteola* (CL1)**	18	22	38	27	44

Endophytic bioagent-pathogen interaction in greenhouse

Soil infestation with the pathogens *F. oxysporum* or *R. solani*, in absence of endophytic bioagents, increased disease percentages of sweet pepper plants, a finding that was more obvious with the latter fungus (Fig. 1). Apart from inoculation method and plan age, respective estimates of 26.3 and 35.2 % were scored. Among the used endophytic diazotrophs, *P. polymyxa* was the pioneeric and succeeded to reduce disease percentages to the lowest limits of 15.7 for *F. oxysporum*-treated pepper and 18.3 for those infected by *R. solani*. It is an interesting observation that although *R. solani* seemed more aggressive than *F. oxysporum*, as recorded in Petri dish- culture assay, the former more severely injured by diazotrophic bioagent treatment than the other one where average reductions in disease percentages as a result of inoculation were 33.9 and 29.0 respectively. The composite inoculum of all diazotrophs was not that successful to restrict the pathogenicity of fungi towards plant vigor.

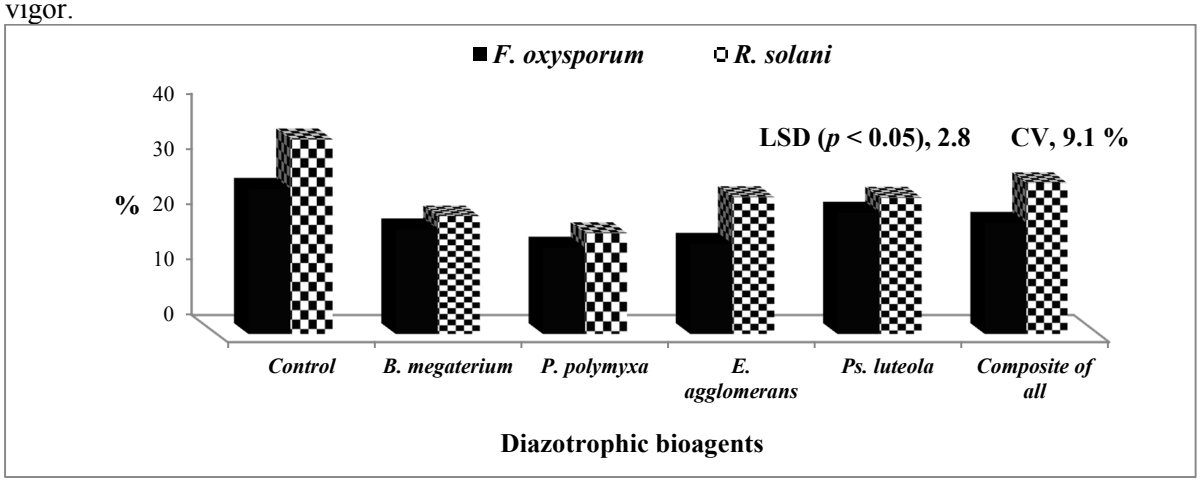


Fig. 1: Disease percentages of sweet pepper plants due to *F. oxysporum* and *R. solani* infestation in presence of diazotrophic bioagents (averages of 45-and 60- day old plants).

The ability of tested bioagents to control the lethal impact of fungal pathogens is expressed as efficiency percentages (Fig. 2). The endophytic *P. polymyxa* proved its antibiosis capability towards *F. oxysporum* and *R. solani* with the highest average efficiencies of 38.2 and 47.8 % respectively. *B. megaterium* seemed efficient to a great extent recording respective values of 25.6 and 39.1 %. The diazotrophic bioagents *Ps. luteola* and the composite inoculum were the inferiors among the tested bioformulations.

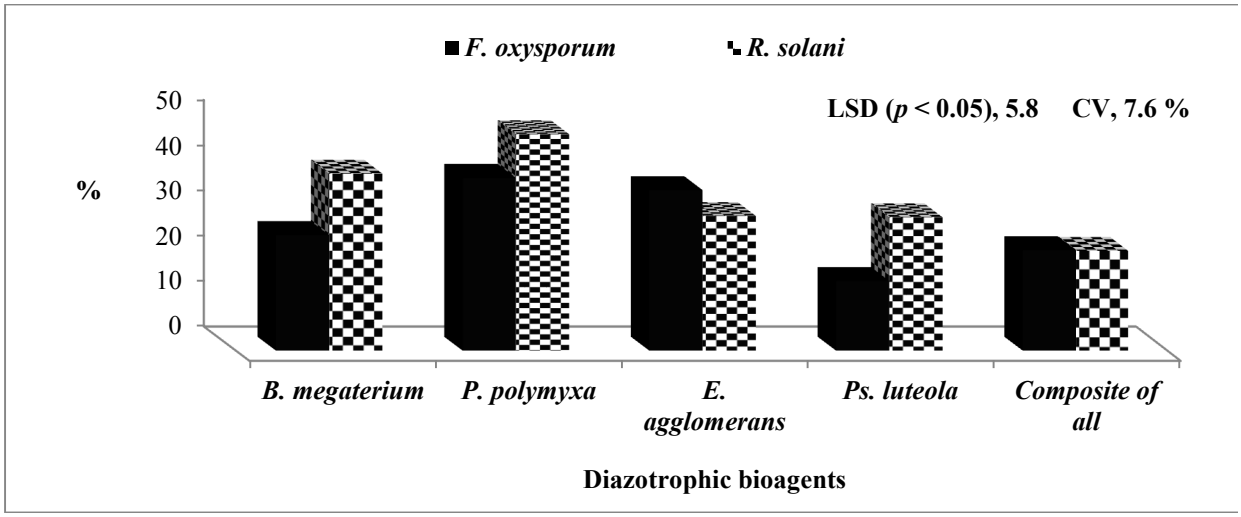


Fig. 2: Efficiency percentages of diazotrophic bioagents in alleviating the pathogenicity of *F. oxysporum* and *R. solani* (averages of inoculation methods and plant age).

The 90-day old plants variably responded either to fungal or diazotrophic inocula. Both roots and shoots severely harmed due to pathogen treatments (Table 5). Inoculation with endophytes, whatever the bioproduct, significantly overcame the deleterious influence of fungi, an effect that was inoculum-and inoculation method-dependent. *P. polymyxa*, in general, was the superior in supporting better root and shoot development followed by *B. megaterium* and *E. agglomerans*, *Ps. luteola* was the inferior. Root dipping in diazotrophic bioagents seemed more appropriate than over-head soil treatment. Slight promotion for shoot biomass of fungal-infected plants was attributed to seedling spray with the antagonistic endophytes.

The harmful and beneficial effects of the experimented microbiota on root and shoot development are expressed in change percentages in dry matter production (Fig. 3). Related to untreated plants, reductions in root dry weights of 60.6-70.0 % and 55.0-66.7 % were attributed to *F. oxysporum* and *R. solani* infestation, respectively. The corresponding estimates for shoots were 47.4-77.6 and 59.9-74.5 %. Inoculation with endophytic diazotrophs overcame the lethality of pathogens beside additional increases in both root and shoot dry matter yields.

Table 5: Root (R) and shoot (Sh) biomass (mg plant^{-1}) of 90-day old pepper plants infested by *Fusarium oxysporum* or *Rhizoctonia solani* simultaneously inoculated with endophytic diazotrophs

Treatments*	**Root dipping**			**Over-head soil**			**Seedling spray**		
	Root	**Shoot**	**R/Sh**	**Root**	**Shoot**	**R/Sh**	**Root**	**Shoot**	**R/Sh**
Control	180	192	0.94	180	192	0.94	180	192	0.94
***Fusarium oxysporum* (F.o)**	71	88	0.81	66	101	0.65	54	43	1.26
F.o* + *B. megaterium	286	249	1.15	199	201	0.99	187	222	0.84
F.o* + *P. polymyxa	322	296	1.09	216	238	0.91	201	242	0.83
F.o* + *E. agglomerans	204	244	0.84	223	202	1.10	172	199	0.86
F.o* + *Ps. luteola	172	223	0.77	207	201	1.03	148	187	0.79
F.o* + *composite inoculum	290	241	1.20	202	199	1.02	182	215	0.85
***Rhizoctonia solani* (R.s)**	60	77	0.78	81	69	1.17	63	49	1.29
R.s* + *B. megaterium	255	211	1.21	200	211	0.95	149	166	0.90
R.s* + *P. polymyxa	243	254	0.96	197	179	1.10	138	182	0.76
R.s* + *E. agglomerans	199	201	0.99	226	282	0.80	155	201	0.77
R.s* + *Ps. luteola	187	166	1.13	188	207	0.91	177	209	0.85
***R.s* + composite inoculum**	201	212	0.95	191	190	1.01	183	206	0.89

***;** Control plants are those supplemented with the recommended NPK fertilization regimes but biologically-untreated. Other treatments received 100 % PK but 50 % N.
LSDs ($p < 0.05$) for root and shoot dry weights are 26 and 33, CV (%) are 11.4 and 6.8 respectively.

Comparatively higher total biomass production was attributed to root dipping in diazotrophic preparations in presence of pathogenic fungi, average increase of *ca.* 25 % over uninoculated plants was estimated. Over-head soil-inoculated plants ranked the second (*ca.* 12 % increase) while seedling spray inoculation method was not that successful.

Field-grown pepper inoculated with endophytic diazotrophs

The photosynthetic pigments and NPK profiles of field-grown sweet pepper leaves are presented in Figure (4). In *ca.* 50 % of cases, diazotroph inoculation significantly increased chlorophylls a and b production and the effect varied from inoculum to another.

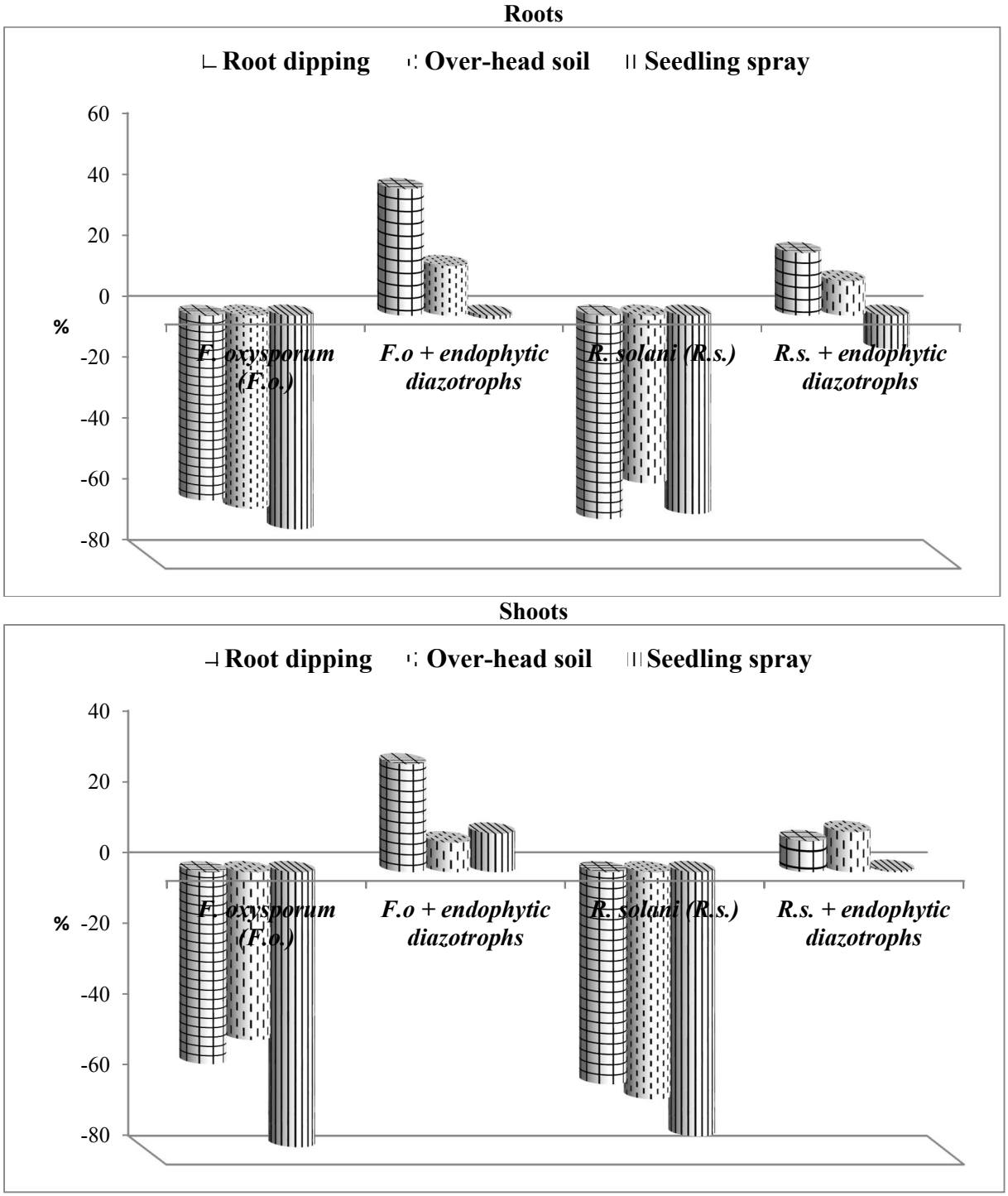
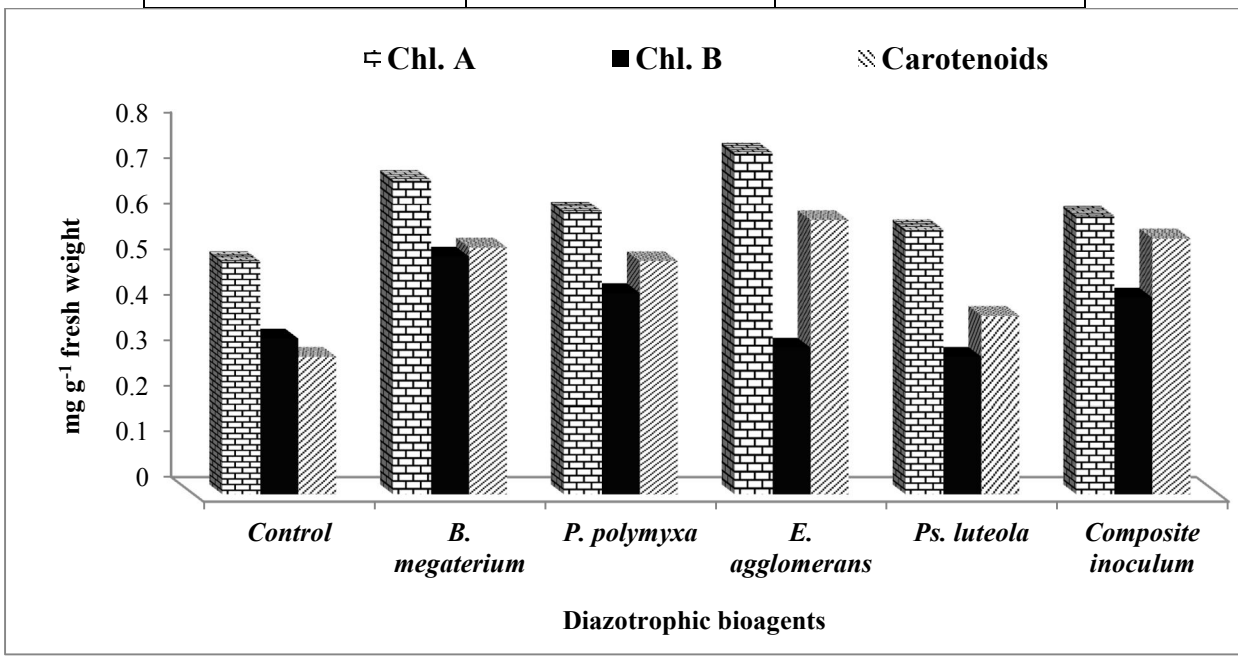


Fig. 3: Change percentages in pepper root and shoot biomass as affected by pathogen infestation and endophytic diazotroph inoculation (related to untreated plants) *F.o., F. oxysporum*; *R.o., R. solani*.

Chlorophyll a (LSD, 0.05; 0.10) (CV, 5.6 %)	Chlorophyll b (LSD, 0.05; 0.10) (CV, 12.4 %)	Carotenoids (LSD, 0.05; 0.12) (CV, 3.6 %)



Nitrogen (LSD, 0.05; 0.23) (CV, 8.4 %)	Phosphorus (LSD, 0.05; 0.06) (CV, 6.7 %)	Potassium (LSD, 0.05; 0.24) (CV, 11.5 %)

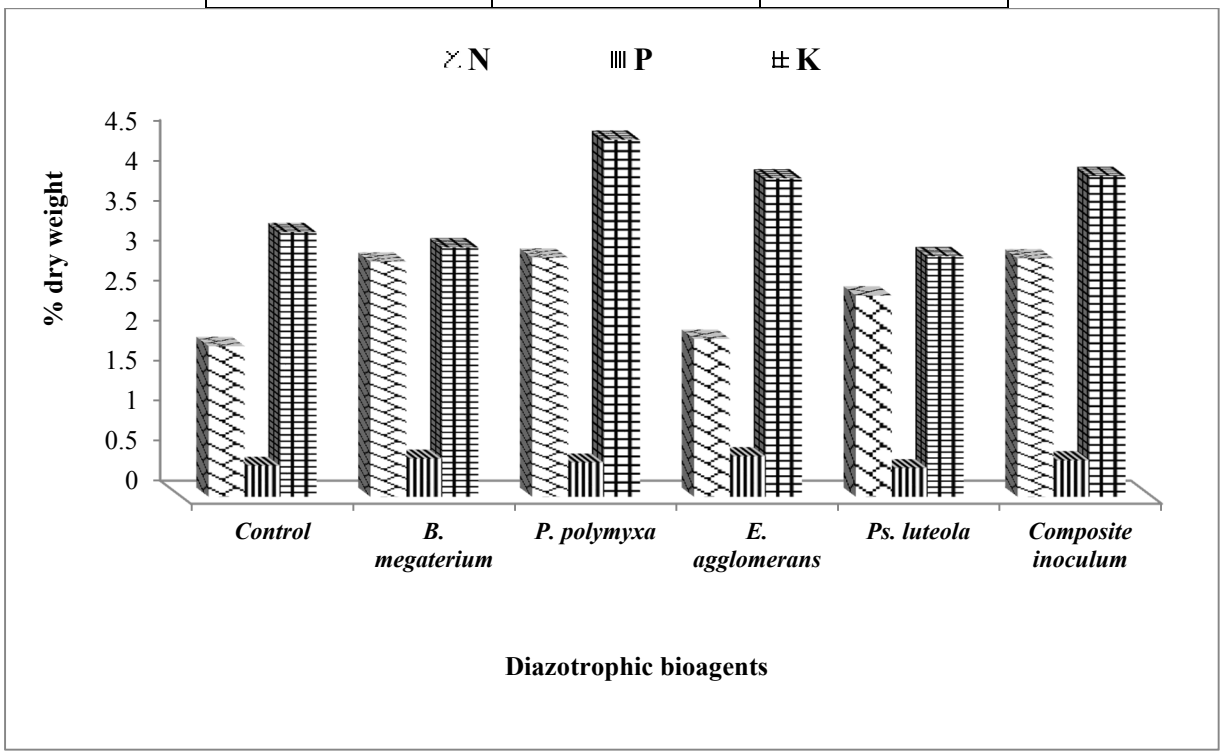


Fig. 4: Photosynthetic pigments and nutrient profile of sweet pepper leaves as affected by diazotroph inoculation methods.

Regardless the introduced endophyte, increases in chlorophylls a and b of root dipped plants were 27.5 and 17.7 % over control. Increases for the overhead soil-inoculated ones were 23.5 and 14.7 % respectively. Effect of inoculation on carotenoid pool was more conspicuous where respective increases were 73.3 and 63.3 %.

As expected, extraordinary quantities of N were accumulated in plant leaves as a result of diazotroph inoculation. Increases of > 58 % were attributed to *P. polymyxa*. *B. megaterium* nicely acted as well recording up to 56 % increase in N content. Root dipping in diazotroph inocula deemed more appropriate for nitrogen accumulation compared to overhead soil application, the average increases were 41.8 % for the former and 30.2 % for the latter. The influence of inoculation method on phosphorus levels in pepper levels was not that obvious where 15.4 % increase was attributed to root dipping against 12.8 % in case of overhead soil treatment. *E. agglomerans* in both application methods supported the highest P yield in pepper leaves compared to other bioformulations. Uninoculated plants contained 3.31 % potassium in their leaves, an amount that increased due to endophytic diazotroph inoculation. *P. polymyxa* overcame the other inocula resulting in 34.7 and 40.8 % increases for root dipping and overhead soil treatments respectively.

Endophytic diazotroph inoculation strikingly improved pepper fruit traits and total yield (Table 6). While uninoculated plants bore the smallest fruits of 6.8 cm length and 5.6 cm diameter, inoculated ones produced remarkably bigger fruits. Mono-bacterial culture of *B. megaterium* and the mixture of all were the superior while *Ps. luteola* was the inferior. No apparent differences were attributed to inoculation method.

As reported with the vegetable leaves, significant increases in fruit NPK contents were estimated for diazotroph-treated plants. Increases of 4.9-45.9 %, 9.1-100.0 % and 4.4-38.9 % in N, P and K contents respectively were scored as a result of inoculation. The NPK pool of root-dipped plants was slightly higher than that of overhead inoculated correspondings.

As low as 0.86 kg row^{-1} total fruit yield was produced by uninoculated pepper plants, those received the inocula produced > 2.6 kg row^{-1}. For both inoculation methods, the composite inoculum seemed the most promising. Generally speaking, the total fruit yields of root-dipped plants were > 7 % higher than those overhead soil treated.

Table 6: Pepper fruit characteristics and total yield of the different inoculation treatments

Endophytes	Length (cm)	Diameter (cm)	**Nutrient (% DW)**			Total yield (kg row^{-1})
			N	**P**	**K**	
Uninoculated	6.8	5.6	1.22	0.22	1.8	0.86
Root dipping						
B. megaterium	10.0	6.9	1.44	0.24	2.50	1.78
P. polymyxa	9.9	7.2	1.62	0.40	2.13	2.11
E. agglomerans	8.9	6.9	1.29	0.38	1.99	2.02
Ps. luteola	7.9	7.0	1.04	0.39	2.01	1.84
Composite of all	11.1	7.8	1.78	0.44	2.34	2.66
Over-head soil						
B. megaterium	10.9	5.9	1.54	0.26	2.34	1.88
P. polymyxa	8.3	6.8	1.33	0.33	1.91	1.76
E. agglomerans	8.8	6.3	1.00	0.31	2.00	1.88
Ps. luteola	6.9	7.1	1.28	0.40	1.88	2.02
Composite of all	9.8	6.6	1.47	0.39	2.01	2.16
LSD ($p < 0.05$)	1.2	0.3	0.07	0.01	0.42	0.49
CV (%)	9.3	2.7	12.4	7.6	6.3	2.9

Discussion

Plants are recognized as having a diverse bacterial community associated with them (Manter *et al*., 2010; Pini *et al*., 2012). Microbial communities accommodating both the belowground and aboveground parts of plants can be found inside plant organs as endophytes. These particular groups of microbiota that reside both vascular tissues and intercellular areas are diverse and likely originate from soil in close contact with root theater or from the leaf surface (Rosenblueth and Martinez-Romero, 2006). Nearly all plants investigated harboured isolates of endophytic microorganisms, some

may be plant pathogens, others may act as commensals or symbionts, significantly contributing to plant growth and/or disease resistance (Hardoim *et al*., 2008).

In the recent years, endogenous microbes from the internal plant tissues have attracted the attention of researchers as plant growth promoters and/or biological control agents due to their plant colonizing ability. In the present study; samples of sweet pepper roots, stems and leaves were analyzed by culture-dependent approach for endophytic multifunctional diazotrophs. Various methods are recommended for isolation of bacteria present in the internal tissues of their host plants, those include vacuum technique (Hallmann *et al*., 1997), centrifugation (Dong *et al*., 1994) and surface sterilization. The latter is the most frequent procedure and based on triturating of surface sterilized plant organs using different disinfectants such as sodium hypochlorite (Hinton and Bacon, 1995), chloramine T (Barrquio *et al*., 1997), ethanol (Dong *et al*., 1994) and others; this followed by several washes in sterile water or buffer solutions. In the present work, treatment with ethanol 95 % for 5-10 min followed by sodium hypochlorite 3 % for 30 min was sufficient enough to get rid of tissue surface-colonizing microorganisms beside facilitating the isolation of those endophytically exist.

The MPN estimates indicated that pepper roots harboured populations of N_2-fixers more than those occupying the endospheres of stems and leaves. This proves that the roots are the primary sites where bacteria invade and penetrate into the plant (Lodewyckx *et al*., 2002). Single-colony isolation was applied to isolate 88 colonies appeared on the N-deficient combined carbon sources (CCM) agar medium. Based on nitrogenase activity measurements, the superior 15 candidates were chosen for identification. According to their morphological, cultural and physiological characteristics, the pure isolates were found to belong to the genera *Bacillus* spp., *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas* spp., *Paenibacillus* spp. and *Rahnella* spp. Those isolates possessed great abilities to reduce acetylene actively in CCM. This indicates that the proposed medium is suitable for supporting growth and activity of true not putative diazotrophs tested, bacilli represented > 33 % followed by *Pseudomonas* spp. (26.7 %) and *Enterobacter* spp. (20 %). *Klebsiella* spp. and *Rahnella* spp. were represented by one isolate for either. These findings confirm those of Lodewyckx *et al*. (2002) who found that a great part of endophytic microorganisms secured from black pepper roots and stems were Gram positive (80 %), but Gram negative ones represented only 20 %. Of the Gram positives, *Bacillus* spp. followed by *Arthrobacter* spp., *Micrococcus* spp. and *Curtobacterium* sp. were the most residential. In respect to Gram negatives, *Pseudomonas* spp. was the most common followed by *Serratia* sp. Other bacterial genera reported as endophytes are *Agrobacterium*, *Bacillus*, *Bradyrhizobium*, *Cellulomonas*, *Calvibacter*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Microbacterium*, *Micrococcus*, *Pseudomonas*, *Rothia* and *Xanthomonas* (Kobayashi and Palumbo, 2000; Zinniel *et al*., 2002).

It could be mentioned that endophytic bacteria have typically been enumerated and identified, in the present study and some others, using traditional culture-based approaches, although such methods are medium- and cultivation condition-dependent. Here, it has to be realized that, culture-independent 16S rRNA-based methods can discover some unculturable bacterial members or those bacteria that are in low abundance or slowly grow and missed by traditional culture-based protocols.

The selected isolates in the present investigation exhibited N_2-fixation efficiencies of up to 18.2 mg N fixed /g C oxidized, in addition to their ability to produce the plant hormones; IAA (9.1-71.2 ppm) and GA_3 (0.8-10.4 ppm). Also, they produced siderophores (39.4-90.4 SU %) and extracellular polymeric substances (0.85-2.84 g l^{-1}) with phosphate solubilizing efficiencies of 99.9-303.1 %. In this respect, a vast array of literature is available on the biochemical potentials of endophytic diazotrophs. Besides fixation of atmospheric dinitrogen by endophytic diazotrophs, the secondary metabolites they produce are biologically active. The production of IAA and GA_3 is suggested to support colonization capability (Shi *et al*., 2009; Merzaeva and Shirokikh, 2010) possibly *via* interference with the host defense system. Endophytes are producers of vivid siderophores (Araujo *et al*., 2008; Rosconi *et al*., 2013).

The biologically active compounds the endophytes produced are very important sources for anticancer, antioxidant, antidiabetic, immunosuppressive, antifungal, antioomycete, antibacterial, insecticidal, nematicidal and antiviral agents (Aly *et al*., 2011; Brader *et al*., 2014). In addition, endophyte metabolites are involved in mechanisms of signaling, defense and genetic regulation of the symbiosis establishment (Schulz and Boyle, 2005).

Nearly all of tested endophytes did strongly *in vivo* inhibit all the five examined pathogenic fungi, in some cases, inhibition exceeded 60 %. Dijksterhuis *et al*. (1999) reported that less than half of all bacterial strains from Geumsan-gum and Jinan-gum and one fourth of all strains from Ganghwa-gum exuded growth-inhibitory substances against fungal pathogens when tested *in vitro*. As stated by Mavingui and Heulin (1994), the lethal impact of bioagents including *Bacillus* sp. on phytopathogenic fungi might be associated with enzymes produced that can act against the fungal cell wall. Chitinases, produced by *Bacillus* spp., are having a synergistic action with Cry proteins which results in restricting the growth of pathogens (Barboza *et al*., 1999). Several endophytes are able to antagonize certain plant pathogens *via* production of a number of growth-inhibitory substances. These substances comprise alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols and chlorinated chemicals (Tejesvi *et al*., 2013). Alkaloids produced by endophytes are among the most-described compounds. For example, the neurotoxic indole-diterpenoid alkaloids, so-called lolitrems, are responsible for intoxication of cattle grazing on the endophyte-infected grass (Gallagher *et al*., 1984). Other alkaloids are important for protection of plants against insect herbivores (Siegal *et al*., 1990). The endophyte *Enterobacter* sp. strain 638 produced antibiotic substances encompassing 2-phenylethanol and 4-hydroxybenzoate (Taghavi *et al*., 2010).

Some of the examined endophytic diazotrophs, particularly *B. megaterium*, did reduce the pathogencity of *F. oxysporum* and *R. solani*. This is indicated by compensating a great portion of pepper biomass yield losses. This is besides modifying the root/shoot ratios for better nutrient and water uptake. In fact, the use of endophytic N_2-fxers to protect crops from fungal or bacterial attack has been experimented with few plant species, but is of special interest because the same bacterium may both promote the host growth and provide antibiosis against pathogens. Actually, some endophytic diazotrophs such as *Bacillus* spp., *Enterobacter* spp. and *Pseudomonas* spp. can have protective antifungal effects on cotton, potato, tomato and ballon flower roots (Berg *et al*., 2005). *Bacillus* spp. and *Paenibacillus* spp. are forming spores and this is advantageous for ease of handling and production of antifungal spore-specific lipopeptides. Furthermore, *Paenibacillus* spp. is able to secrete antimicrobial and biosurfactant materials such as surfactin, iturin and fengysin (Yao *et al*., 2003). Of rather interest in the endophyte-pathogen interaction is the role of the ACC deaminase enzyme of the endophyte that is often associated with the alleviation of pant stress. This enzyme is responsible for lowering the levels of ethylene in the plant by cleaving the plant-produced ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) to ammonia and 2-oxobutanoate, preventing ethylene signaling. The ethylene contributes in seed germination in response to various stresses and it is the key regulator of colonization of plant tissues by the endophytes (Iniguez *et al*., 2005). When the ACC deaminase gene of *B. phytofirmans* PsJN was inactivated, the endophyte lost the potential to promote root elongation in canola seedlings (Sun *et al*., 2009). An additional study performed on cut flowers indicated that microbial endophytes are able to colonize the shoot and that ACC deaminase delays flower senescence (Ali *et al*., 2012).

Conclusion

The documented ability of endophytic diazotrophs to interact obviously with pepper or even other vegetable and cereal plants flashes light on the use of endophytic/rhizospheric bacteria for improving plant growth, health and yield. However, to magnify benefits from these microbiota, especially those of endophytic nature, either through BNF or plant growth promotion, a better understanding of endophytic ecology and mechanism of interaction at molecular level is required. With complete genome sequencing of various endophytes, genes that are induced or repressed during colonization could be identified. Also, it would be very useful to have knowledge how different endophytic strains work together, in a consortium, for the synergistic promotion of plant growth. This insight in the mechanism will be promising in developing a more successful plant-diazotroph interweave to support sustainable production of biomass in the field.

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