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EFFECT OF SINGLE AND DUAL INOCULATION WITH *Rhizobium leguminosarum* BV. *TRIFIOLII* AND *Anabaena* SP. ON SEEDLING RICE GROWTH UNDER LABORATORY CONDITIONS

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ABSTRACT

Plant growth promoting activities for the local isolated and identified rhizobial and cyanobacterial strains were performed under gnotobiotic conditions in laboratory. The experiment contained four Egyptian rice varieties (Sakha ١٠١, Sakha ١٠٤, Giza ١٧٧ and Giza ١٧٨) that were tested against the *Rhizobium leguminosarum* (*RH*) strain E١١ and the *Anabaena* sp. strain ERC١٠٢ and their consortia. The results indicate that inoculation with the RH E١١ alone stimulated root and shoot growth of the three rice varieties Sakha ١٠١, Sakha ١٠٤ and Giza ١٧٨. The plant growth promotion provided by inoculation with Rlt E١١ alone was better than for inoculation with the cyanobacterial strain alone or when the strains were mixed in a consortia inoculum.

INTRODUCTION

Numerous species of soil bacteria that flourish in plant rhizosphere may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms. These bacteria are collectively known as PGPR (Plant Growth Promoting Rhizobacteria). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers which must be tested for their efficiencies in establishment of effective plant microbe interrelationships that can benefit plant growth and crop performances while do not have pathogenic effects to plants, humans, animals or adverse impact on the environment. Inoculation with those microorganisms, when tested and found effective, can assist plant growth stimulation by providing fixed nitrogen or secretion of growth hormones that induce better plant growth, nutrient uptake and increased tolerance towards drought and/or salinity stress. The cost/benefit ratio of biofertilizers is always higher. Costs of fertilization with nitrogen and phosphorous chemical fertilizers is so commonly an economic limiting factor in crop production in most of the world, which can be mitigated by biofertilization (Anandaraj and Delapierre, ٢٠١٠). Cyanobacterial strains belonging to Nostoc and *Anabaena* comprised ٨٠٪ of the rhizosphere isolates of cyanobacteria, which were found efficient in enhancing germination and growth of rice seeds and exhibited significantly high protein accumulation, IAA production and nitrogen fixation (Prasanna *et al*., ٢٠٠٩).

Endophytic bacteria, as defined by (Hallmann *et al.*, ١٩٩٧), are bacteria that can be isolated from inside surface-disinfested plant tissue and do not visibly harm the plant. It is a practical definition based on experimental limitations and is inclusive of bacterial symbionts, as well as internal plant-

colonizing nonpathogenic bacteria with unknown beneficial or detrimental effects on colonized plants. Historically, endophytic bacteria have been considered weakly virulent plant pathogens. Recently, been discovered to have several beneficial effects on host plants, containing plant growth promotion and increased resistance against plant pathogens and parasites.
The strategy followed to achieve this goal consisted of two consecutive steps that can be summarized in:

١-Isolation, purification and identification of strain belonging to N-fixing cyanobacteria from rice fields and *Rhizobium leguminosarum* biovar *trifoli*i that already tested and found successful for rice biofertilization in the Nile delta (Yanni and Dazzo ٢٠١٠).

٢-Testing the performance of these bacteria, individually and when used as consortia, in enhancement of rice seedling growth under gnotobiotic lab culture conditions.

MATERIALS AND METHODS

Isolation of clover-nodulating *Rhizobium* from roots of field-grown rice

Rhizosphere soil and roots of the Japonica rice cultivar Giza-١٧٢ were collected from fields at Sakha, Kafr El- Sheikh Governorate, where rice has been rotated with Egyptian berseem clover for several hundred years. The roots were washed with running tap water followed by distilled water, surface-sterilized with ٧٠٪ ethanol followed by ١٠٪ sodium hypochlorite, rolled over Yeast Extract Mannitol (YEM) gar plates to verify root surface-sterilization, and macerated in sterile ٥ m*M* Na-phosphate buffer of pH ٧,٠. Authentication followed by enumeration of *Rhizobium.* in the surface-sterilized / macerated rice roots was performed by the five-tube most probable number (MPN) - plant infection test (Somasegaran and Hoben, ١٩٨٥) using berseem clover (*Trifolium alexandrinum*) as the legume trap host. Tubes containing Vincent's nitrogen-free agar medium were planted with surface-sterilized clover seeds and left for germination for ٣ days before inoculation with the pre-prepared rice root macerate. The seedlings were incubated under ١٦ hr day light, ٧٠٪ relative humidity, and ٢٢ ^{o}C day / ٢٠ ^{o}C night cycle for ٣٢ days in lab green house and then scored for root nodulation. Root nodules that developed on clover plants inoculated with the highest dilutions of rice root macerates were excised, surface-sterilized, and the nodule occupants were isolated into pure culture by plating on YEM agar followed by restreaking of the isolated colonies on defined BIII agar medium (Dazzo, ١٩٨٢).

Analyses of the symbiotic properties of the selected isolates in pure culture were performed on Egyptian berseem clover seedlings (٨ replicates per treatment) grown on agar slopes of N-free Fahraeus medium under microbiologically controlled conditions (Dazzo, ١٩٨٢). Seedling roots were inoculated with ١٠^{٦} cells of a ٥-day-old inoculum and incubated in growth chamber under the same conditions described above. Nodulation kinetics was assessed by periodically inspecting plants using stereomicroscopy for emergence of root nodules. The plants were harvested at ٤١ days after

inoculation and evaluated for effectiveness in symbiotic N fixation by comparison of their dry weight and N-content (estimated by the micro-Kjeldahl steam distillation method, Black *et al*., ١٩٦٥) to that of the non-inoculated control plants.

Analysis of rhizobial strain diversity and identification of endophytic isolates by molecular methods

Plasmid profiles were analyzed by the method of (Eckhardt, ١٩٧٨) as modified by (Espuny *et al.*, ١٩٨٧). Genomic Restriction Fragment Length Polymorphism (RFLP) of X*ba*١ digests was analyzed by pulsed- field gel electrophoresis as described by (Corich *et al.*, ١٩٩١). BOX-PCR amplification fragment length polymorphism was analyzed as described by (Versalovic *et al*., ١٩٩٤). Cells were also boiled in SDS gel buffer (Laemmli, ١٩٧٠) and the profiles of their total cellular proteins were compared by SDS-PAGE in ١٢٪ running gels stained with Coomassie blue. The phylogenetic relationships of the *R. leguminosarum* bv. *trifolii* strains (given the names E١١ E=Egypt) were analyzed by sequencing their total ١٦S rDNA. The ١٦S ribosomal RNA-encoding genes were amplified from genomic DNA using conserved eubac-terial primers ٨F and ١٥٤٠R. The amplified product was sequenced using dye terminators on the Applied Biosystems' DNA Sequencing System (Foster City, CA). Sequences were aligned against those in the Ribosomal Database Project (Larsen *et al.*, ١٩٩٣) on the basis of conserved regions of sequence and secondary structure. Phylogenetic relationships were inferred using regions of unambiguous alignment and the distance method of (DeSoete, ١٩٨٤).

Microscopical examination of *Rhizobium* endophytes within rice roots

Rice seedlings were grown for ٣٢ days in non-sterile potted soil in a growth chamber as previously described (de Bruijn *et al.,* ١٩٩٥). Roots were sampled, cleaned with running water, freehand sectioned, and processed for examination by scanning electron microscopy (Umali-Garcia *et al.*, ١٩٨٠). Other seedlings were grown under microbiologically controlled conditions in enclosed tubes inoculated with pure cultures of the rice endophyte RH strain E١١. Freehand sections were stained with ٠,٠١٪ acridine orange, washed and mounted in ١٪ sodium pyrophosphate, and examined by laser scanning microscopy in the epifluorescence confocal mode using computer-enhanced reconstruction of serial section overlays and digital image processing (Subba-Rao *et al*., ١٩٩٥).

Isolation and identification of *Anabaena* spp.

Field grown rice plants were carefully uprooted and transferred to the "Blue-green Algae Research lab at Sakha Agricultural research Station, Kafr El-Sheikh". The roots were washed several times with running distilled water to remove mud and soil particles. The major roots were excised, surface sterilized with ٧٠ % ethanol followed by ١ % sodium hypochlorite solution, cut into pieces under sterile conditions, and then macerated in sterile ٥ m M Na- phosphate buffer (pH ٧). Sterile dilutions from $١٠^{-١}$ to $١٠^{-٨}$ of the sterile rice root macerates. The dilutions containing the root macerates were used for inoculation of N-free mineral plant nutrient medium (The Modified Watanabe medium, El – Nawawy *et al.* ١٩٥٨), with incubation under photosynthetic illumination of ١٤ light and ١٠ dark for ١٠ days under temperatures of ٢٤ to ٣٠ ^{o}C for isolation of the

cyanobacterium. Standard microbiological methods were used to establish pure cultures of the isolated organisms and maintain their viability in culture collections. Grouping of the isolates into genomically distinct strains was done by plasmid profiling and BOX-PCR genomic fingerprinting techniques. Confirmation of their taxonomic status is made by the aforementioned bioinformatic comparison of their ١٦S rRNA sequences to appropriate databases.

Growth of rice under gnotobiotic conditions

Laboratory studies of the interaction between rice and clover-nodulating rhizobial endophytes were performed using an enclosed sterile tube culture system under climate-controlled growth chamber conditions. The rice varieties Sakha ١٠١, Sakha ١٠٤, Giza ١٧٧ and Giza ١٧٨ were used for this study. The *Rhizobium leguminosarum* bv. trifolii strain E١١ was used among Separate tests established that seeds of the four rice cultivars harbored no endophytic clover-nodulating rhizobia that would survive surface-sterilization. Seeds weighing approximately ٣٠ mg each were surface-sterilized by treatment with ٧٠٪ ethanol for ١ min followed by ١٠٪ sodium hypochlorite solution for ٤ min, and then washed (four times one min each) with sterile water, then transferred to twenty-five ٢٠٠ mm tubes, each enclosed with foam plugs and containing ٢٠ ml of Hoagland's plant growth medium (Sigma Chem. Co., St. Louis, MO) solidified with ١٪ purified agar (United States Biochemical, Cleve- land, OH), above which was layered ٥ g of sterile acid-washed quartz sand and ٤ mL sterile Hoagland's #٢ liquid medium. The tubes were incubated for ٢ days in the dark at ٣٠ ^{o}C for seed germination. Rhizobial inoculum was grown separately on BIII agar for ٥ days at ٣٠ oC, suspended in sterile Hoagland's medium and adjusted to a density of ١٠٧ cells mL-١. The cyanobacterial inoculate was grown on N-free medium for ٥ days under illumination program of ١٤ hrs light / ١٠ hrs dark in the lab growth chamber maintained at ٣١+ ٠,٥ ^{o}C. Each seedling root was inoculated with ١٠٦ CFU of each of the two organisms or them both as a consortium, and tested against a non-inoculated control treatment. Six plant replicates were used for each treatment). When grown to sufficient length, the stem was repositioned through a slit on the edge of the foam plug to allow continuous growth while preventing microbial contamination of the root system. Tubes were irrigated with sterile water alternating with equal quantities of sterile Hoagland's solution as needed. Rice plants in tube culture were gently uprooted ٣٢ days after inoculation and excised at the stem base. Root biovolume and shoot height, are tabulated later in the results section.

Screening of plant growth-promotion activities

Bioassays were performed using different varieties of rice seedlings maintained under microbiologically controlled, gnotobiotic growth conditions in plant growth chambers. A variety of microscopy methods are employed to study their colonization of rice roots, including immunofluorescence microscopy, confocal laser-scanning microscopy and scanning electron microscopy. Quantization of their colonization patterns is done by computer-assisted microscopy and digital image analysis using the

software CMEIAS [Center of Microbial Ecology Image Analysis System (http://cme.msu.edu/cmeias/)].

RESULTS AND DISCUSSION

Authenticity of the tested bacterial strains

Isolates of cyanobacteria and rhizobia isolated from surface-sterilized rice roots grown in the Nile delta were examined for authenticity using répétitive extragenomic palindromic-PCR fingerprinting and/or PCR-restriction fragment length polymorphism (RFLP) of ١٦S-ribosomal (cultivars Giza ١٧٧ and Sakha ١٠٢). Fig. (١A and B) showing electron micrographes of the epidermal root surface of rice (Sakha ١٠٢) colonized by the rice endophyte *Rhizobium leguminosarum* bv. trifolii (Strain E١١).

DNA as intergenic spacers and PCR-RFLP of a nodulation gene region, *nodD,* as a marker of the symbiotic component of the genome. A ١٦S rDNA sequence analysis for cyanobacterial isolate revealed that its nucleotide sequences are most closely matching the order Nostocales, genus *Anabaena*. The strain was tested for atmospheric N-fixation and found positive regarding its ability to grow on N-free medium. Phase-contrast and fluorescence photomicrographs are shown below in Fig. ٢ A, B, C, D and E and Fig. ٣. Red and blue arrows indicate examples of differentiated $N_٢$-fixing heterocysts in the filaments.

Promotion of rice growth

Results of the plant growth promotion test for the rhizobial and cyanobacterial strains (appearing in Figs ١, ٢ and ٣) performed under gnotobiotic controlled condition in a growth chambers (Fig ٤) are reported in Table (١). The experiment contained four Egyptian rice varieties tested against the *Rhizobium leguminosarum* bv. trifiolii (*Rl*t) strain E١١ and the *Anabaena* sp. strain ERC١٠٢ and their consortia. Root biovolume and shoot length were taken as index for the plant growth promotion activity of the tested strains.

Fig. ١ A and B: Scanning electron micrographs of the rice Sakha ١٠٢ epidermal root surface colonized by an endophyte strain of rice-adapted *Rhizobium leguminosarum* bv. trifolii (Strain E١١).

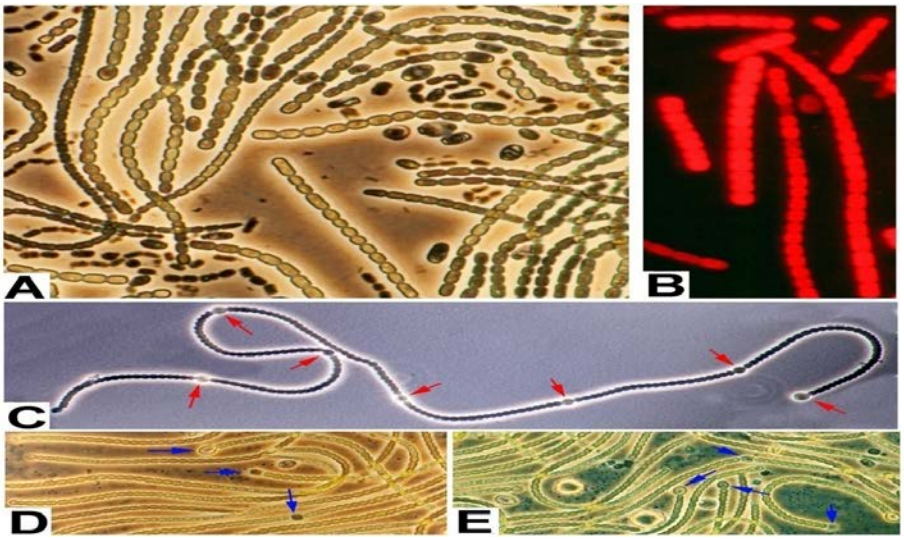

Fig. ٢. A and B: Photomicrographs of the cyanobacterial isolates for this study. Arrows in C, D and E point to cells of differentiat heterocysts.

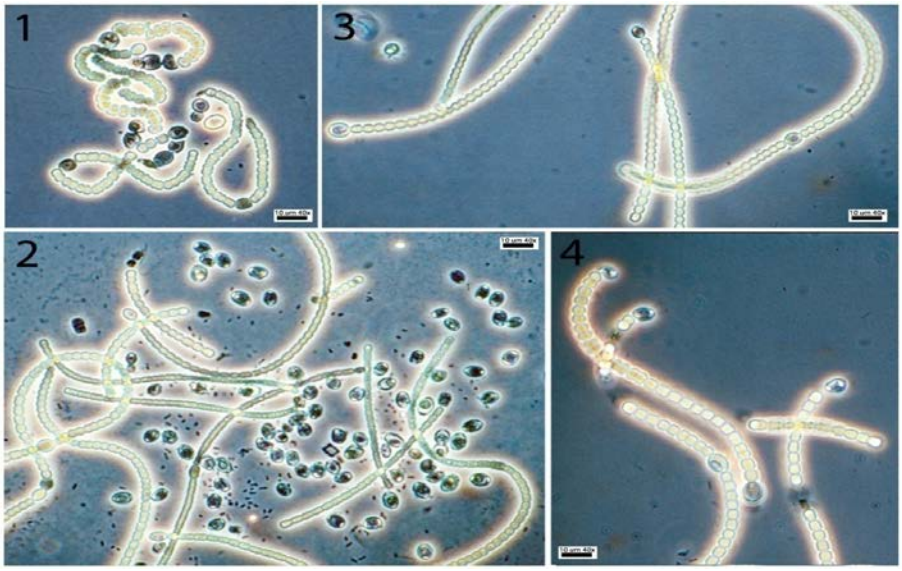

Fig. (٣): Filamentous heterocyst-forming cyanobacteria isolated for this study.

The results indicate that inoculation with the RH E١١ alone stimulated root and shoot growth of the three rice varieties Sakha ١٠١, Sakha ١٠٤ and Giza ١٧٨. The plant growth promotion provided by inoculation with RH E١١ alone was better than for inoculation with the cyanobacterial strain alone or when the strains were mixed in a consortia inoculum.

Table١: Effect of inoculation with *RH* strain E١١ and *Anabaena* sp. strain ERC١٠٢ on root biovolume and shoot height of four rice varieties.

Rice variety	Bacterial inoculum	Root biovolume (mm^3)	Shoot height(mm)
Sakha ١٠١	None	٦٠٠ ± ٢٣١	٢٨٠ ± ١٥
	E١١	١١٠٠ ± ١٤١	٢٨٤ ± ٠
	ERC١٠٢	٥٧٥ ± ٥٠	٢٥٩ ± ١٢
	E١١ + ERC١٠٢	٦٤٠ ± ٥٥	٢٧٥ ± ١٣
Sakha ١٠٤	None	٦٥٠ ± ١٢٩	٣١١ ± ١٢
	E١١	١٠٢٠ ± ١١٠	٣٠٠ ± ٨
	ERC١٠٢	٨٢٠ ± ٤٥	٢٨٤ ± ٢
	E١١ + ERC١٠٢	٨٢٠ ± ٨٤	٢٨٦ ± ٨
Giza ١٧٧	None	١٠٦٧ ± ١١٥	٢٩٥ ± ١٤
	E١١	١٠٠٠ ± ٨٢	٢٨٣ ± ١٤
	ERC١٠٢	٤٥٠ ± ١٢٩	٢٧١ ± ١٩
	E١١ + ERC١٠٢	٨٧٥ ± ١٥٠	٣٠٠ ± ١٥
Giza ١٧٨	None	٣٢٥ ± ٥٠	٢٥٠ ± ٢٣
	E١١	٥٥٠ ± ١٠٠	٥٥٠ ± ٢٧٤
	ERC١٠٢	٣٠٠ ± ٠	٣٠٠ ± ٢١٩
	E١١ + ERC١٠٢	٤٠٠ ± ٧١	٤٠٠ ± ٢٢٢

The Table reports the means +/- standard deviation for the root biovolume (mm^3) and shoot length (mm) for the four rice varieties vs. three inoculation treatments

Fig.٤: Gnotobiotic tube cultures showing the growth response of Sakha ١٠١ rice to inoculation with *R. leguminosarum* bv. trifolii E١١, with a cyanobacterial isolate, and with a consortium containing rhizobial strain E١١ plus the same cyanobacterial isolate. (Note stunted vegetative growth of plants inoculated with the cyanobacterial isolate).

Results of the gnotobiotic experiment performed in glass tube culture using ٥٠٪ Hoagland #٢ (the composite Fig ٤, supported by Table ١) showed that that in the closed habitat of the culture glass tube, the inoculated cyanobacteria competed with the plant for available macro- and micronutrients necessary for growth of both of them. The endophytic rhizobia, in turn, assisted the plant to absorb more nutrients due to mechanisms involving enhanced root architecture (increased biovolume, cumulative length and surface area as described in: Yanni *et al.*, ١٩٩٧, Biswas *et al.*

,٢٠٠٠a,b; Chaintreuil *et al.*, ٢٠٠٠, Yanni *et al.*, ٢٠٠١, Ladha and Reddy, ٢٠٠٣ and Chi *et al.*, ٢٠٠٥). The plant and the cyanobacterium then competed in the gnotobiotic habitat against each other for the nutrients provided by the growth medium. Although the cyanobacteria is an active N-fixer, the inoculated plants seems faint, weak and suffering a sort of growth retardation than those which were inoculated by t *Rhizobium*. However, rice plant growth in open fields do not suffer consequences of such competition as supply of micronutrients from different sources, importantly soil, flood and irrigation water, decay of plant residues, small animals, macro and microorganisms etc., normally secure continues supply of micronutrients all over the plant growth period. Supply with phosphorus may be an exception as the element accumulates in the algal and cyanobacterial cells during the early stages of plant growth, during which sunlight incidence is high and proliferation of algal and cyanobacterial blooms is at its maximum. Later on, phosphorus, nitrogen and other macro and micronutrients liberate during the later rice growth stages when the algal blooms start to decay because of the increased rice plant canopy that prevent sunlight needed for continuity of algal photosynthesis.. This mechanism is very beneficial to the crop, as this phosphorus liberation is highly needed during the panicle initiation stage. Data presented by (Subrahmanyan *et al.*, ١٩٦٥), (Arora, ١٩٦٩) and (Roger and Kulasooriya, ١٩٨٠), and references herewith explained and discussed this mechanism in rice fields. However, seems that the short duration of the gnotobiotic bioassay experiment was only enough for nutrient absorption by the cyanobacterial cells that competed with the plant on available nutrients. However, the results indicate clear difference between nutrient competition between the plant and cyanobacteria on one hand versus the enhancement relationship between the rhizobia and the plant with enhanced nutrients uptake is evident!

When N-fixing cyanobacteria are to be used in rice biofertilization, it is necessary to apply a starter chemical N-fertilizer dose of around ١/٤ to ١/٣ of its N-demand to help the plant to grow well during the early period in which the cyanobacterial population establishes well in the field and starts to fix adequate amounts of atmospheric-$N_{٢}$, followed by decomposition/mineralization of the cyanobacteria after the rice canopy develops, liberating both the biologically fixed-N and the other macro- and micro-nutrients consumed by the cyanobacteria during their early stages of growth. Only then will those fixed-N and nutrients become available to the rice plant.

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تـأثيرالتلقيح الفـردى والمـزدوج بكل مـن *Rhizobium leguminosarum* bv. *trifiolii* and *Anabaena* spعلـى نمـو بـادرات الأرز تحـت الظـروف المعملية

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أجريت هذه الدراسة للحصول على عزلة الريزوبيم من داخل الجذور المعقمة لنبات الأرز النامي في مناطق بشمال ووسط الدلتا باستخدام طرق ميكروبيولوجية قياسية ، وجمع عددا من عزلات***Anabaena*** من مزارع الأرز ومصادر المياه المختلفة في مناطق وسط الدلتا واختبار قدرتها على تثبيت الأزوت الجوى معمليا في تمعامل قسم بحوث الطحالب بمحطة البحوث الزراعية بسخا – كفر الشيخ كما تم دراسة مدى قرب أو بعد هذه العزلات من بعضها من ناحية التركيب الوراثي وعلاقاتها مع جذور النبات. في معامل قسم علوم الأحياء الدقيقة والوراثة الجزيئية ومركز دراسات البيئة الميكروبية بجامعة ولاية ميتشجان بالولايات المتحدة الأمريكية (Michigan State University – Center for Microbial Ecology, USA

كما تم دراسة نشاطات الكائنات المصنفة عند استخدامها في لقاحات منفردة أو عند استخدامها مجتمعة في التأثير على نمو أربعة أصناف من الأرز هي: جيزة ١٧٧ ، جيزة ١٧٨ ، سخا ١٠١ و سخا ١٠٤، اختبرت مع التلقيح بالريزوبيم اوالسيانوبكتيريم أو كلاهما

أظهرت نتائج تصنيف العزلات كونها تنتمي إلى الأجناس والأنواع الآتية: (وأعطى اسما لكل منها كما هو موضح): *Rhizobium leguminosarum* bv. *trifolii* (strain E١١) and *Anabaena* sp. (strain ERC١٠٢).

أدى التلقيح بسلالة الريزوبيا إلى زيادة أحجام المجموع الجذري لأصناف الأرز سخا ١٠١، سخا ١٠٤ و جيزة ١٧٨ ، بينما سجل حجم المجموع الجذري للصنف جيزة ١٧٧ ثباتا نسبيا (يميل إلى التناقص) مقارنة بالمعاملات غير الملقحة. أدت المعاملة أيضا إلى زيادة واضحة لأطوال البادرات للصنف جيزة ١٧٨، مع تغيرات طفيفة بالزيادة أو النقصان في باقي الأصناف؛

أدى التلقيح بسلالة السيانوبكتيريا إلى نقص في حجم المجموع الجذري للأصناف سخا ١٠١، جيزة ١٧٧ و جيزة ١٧٨، وزيادة في الحجم للصنف جيزة ١٧٨، بينما أدى التلقيح بها إلى نقصا في طول البادرات لجميع الأصناف ما عدا الصنف جيزة ١٧٨؛

أدى التلقيح بالسلالتين معا إلى زيادة حجم المجموع الجذري للأصناف ما عدا الصنف جيزة ١٧٧ الذي سجل متوسطه ٨٢% من الحجم الخاص بالمعاملة غير الملقحة. كما أدى التلقيح إلى ثبات طول البادرات عموما فيما عدا بادرات الصنف جيزة ١٧٨ الذي سجل زيادة بلغت ٤٠% من طولها عند عدم التلقيح.

أوضحت هذه النتائج تفاوتا لاستجابة حجم المجموع الجذري وطول البادرات للأصناف المختبرة للتلقيح بالسلالات تحت الدراسة. أدى التلقيح بالسيانوبكتيريا إلى نوع من التنافس بين النبات والميكروب على المغذيات المتاحة لكليهما في الوسط المغلق الذي تمت فيه التجربة. ما كان له أثرا عكسيا على الصفات المورفولوجية المختبرة. من المهم هنا ملاحظة أن التلقيح بكلا السلالتين معا قد أدى إلى تقليل ذاك الأثر السلبي الناتج عن التلقيح بالسيانوبكتيريا بمفردها. ما يرجح التأثير المشجع للتلقيح بالريزوبيا على زيادة قدرة بادرات الأرز على امتصاص المغذيات من وسط النمو.

قام بتحكيم البحث

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