Division of Botany

Department of Biological Sciences

School of basic sciences

Libyan academy of Graduate studies

Tripoli, Libya

Modification in Wheat Plant Gene Expression by Using *Bacillus Subtilis*

A Thesis submitted to the school of science at the Libyan Academy of Graduate studies in partial fulfillment of the requirements for the degree of Master of Science in botany

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Fall (2013)

دراسة تغيرات فى التعبير الجينى لنبات القمح باستخدام السماد الحيوى (الباسيلوس سابتاليس)

رسالة مقدمة لمدرسة العلوم الأساسية بالأكاديمية الليبية للدراسات العليا كجزء من متطلبات الحصول على درجة الإجازة العالية فى علم النبات

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الأكاديمية الليبية للدراسات العليا
طرابلس, ليبيا

الخريف 2013

بسم الله الرحمن الرحیم

"و قل رب زدنی علما"

صدق الله العظیم

الإهداء

إلى أبى و أمى ..... رمز المحبة و العطاء الدائم

أهدى ثمرة جهدى هذا لهما عرفانا بجميل صنعهما

و أدعو لهما بمديد عمر و صحة و عافية

كما

أهديها إلى إخوتى الأعزاء

الشكر و التقدير

الحمد لله رب العالمين الذى هدانى و وفقنى لإنجاز هذا العمل على هذا الوجة و أصلى و أسلم على خاتم المرسلين و إمام الأنبياء و العالمين سيدنا محمد الهادى الأمين.

يسرنى أن أتقدم بجزيل الشكر و التقدير لأستاذى الفاضل الدكتور أحمد فهمى حسين عطية أبو دومة على مساعدتى لإظهار هذا العمل على هذة الصورة بدأ من وضع الفكرة ثم تخطيط التجربة مرورا بكل مراحل هذا العمل إلى أن تم.

و أتقدم للأخ الدكتور شريف إدريس أحمد الأستاذ المساعد بقسم الوراثة بكلية الزراعة جامعة عين شمس و جميع الزملاء بمعمل البيولوجيا الجزيئية بالقسم الذين عاونونى لأداء الدراسات الجزيئية فى هذة الدراسة.

كما أتقدم بالشكر للأخوة الزملاء أعضاء هيئة التدريس و الباحثين بوحدة التسميد الحيوى بكلية الزراعة جامعة عين شمس على إمدادهم لى بسلالة الباسيلوس التى تمت عليها الدراسة.

لا يسعنى فى هذا المضمار إلا أن أشكر الأخوة الزملاء فى مركز البحوث الزراعية بطرابلس الذين أمدونى بحبوب الآصناف القمح التى تمت عليها الدراسة.

كما لا يفوتنى أن أشكر زملائى و أصدقائى الذين وفروا لى الدعم النفسى و المعنوى للوصول بهذا العمل للصورة التى هو عليها الأن و كل من مد لى يد العون لى لإتمام هذة الدراسة.

الملخص

يعتبر نبات القمح من المحاصيل ذات الأهمية الكبيرة لغذاء الإنسان. التسميد بالمركبات الكيماوية و الأملاح غير أمنة على صحة الإنسان كما أنها مرتفعة التكاليف. فمن الضرورى إيجاد بدائل أخرى للتسميد أكثر أمان و أقل تكلفة من التسميد الكيميائى. تتميز بكتيريا *B. subtilis* بأنها تعمل على تحليل مصادر الفوسفور العضوى و تحويلة لحمض فوسفوريك بواسطة انزيم الفيتيز الذى تفرزة هذة البكتيريا مما يساعد على زيادة ذوبان العناصر مما يسهل للنبات إمتصاصها. تمت الدراسة على نباتات صنفين من أصناف نباتات القمح هما بنى سويف و سلامبو. فقد تم تنمية نباتاتهما لمدة 90 يوم بعد الإنبات بحيث تكون إما ملقحة ببكتيريا *B. subtilis* (معاملة) أو غير ملقحة بالبكتيريا (كنترول). تم قياس سبعة صفات مختلفة و استخدمت هذة الصفات كأدلة على نمو نباتات القمح و إنتاجيتها. هذه الصفات هى طول النبات و عدد أشطاء النبات الواحد و الوزن الغض للمجموع الخضرى و عدد الأوراق للنبات و الطول الكلى للمجموع الجذرى و وزن أحدث ورقة تم إستطالتها (التالية لورقة العلم) و أخيرا عدد السنابل فى النبات الواحد. كما تم جمع عينات ورقية من نباتات كلا الصنفين معامل و غير معامل و ذلك بغرض إجراء تحليلات جزيئية بإستخدام تقنية التقدير شبة الكمى للتعبير الجينى عن طريق النسخ العكسى لنواتج الجينات المتحكمة فى إنتاج بروتينات إنزيم CDPKs و إنزيم PEPCs و إنزيم P5CS. أظهرت النتائج للصفات السبعة سالفة الذكر زيادة بنسب متفاوتة فى النباتات الملقحة ببكتيريا *B. subtilis* مقارنتا بتلك النباتات غير الملقحة بالبكتيريا بكلا الصنفين من أصناف القمح تحت الدراسة. و على المستوى الجزيئى فإن تلقيح جذور النباتات لكلا الصنفين تحت الدراسة ببكتيريا *B. subtilis* أدى إلى زيادة التعبير الجينى للجين المسؤل عن إنتاج البروتين CDPKs و المسؤل عن نقل الإشارات الخلوية و بذلك فإن له دور هاما فى تنظيم فسيولوجيات الخلية. و من

جهة أخرى أدى تلقيح جذور النباتات لكلا الصنفين تحت الدراسة ببكتيريا *B. subtilis* إلى زيادة التعبير الجينى للجين المسئول عن إنتاج بروتين إنزيم PEPCs و الذى لة دورا هاما فى عملية البناء الضوئى. فزيادة تركيز هذا الإنزيم يسهم بصورة مباشرة و كبيرة فى زيادة معدلات البناء الضوئى مما ينعكس على زيادة معدلات نمو نباتات القمح. و أخيرا فإن تلقيح جذور النباتات لكلا الصنفين من أصناف القمح ببكتيريا *B. subtilis* أدى إلى زيادة معدلات التعبير الجينى للجين الذى يحكم إنتاج انزيم P5CS مقارنتا بالنباتات غير الملقحة. و هذا الإنزيم هو المتحكم الأساسى فى التخليق الحيوى للحمض الأمينى البرولين و الذى يعمل كمعادل للإسموزية مما يزيد من قدرة النباتات على تحمل الإجهاد البيئى و خاصة غير الحيوى. و عموما فإن تلقيح جذور نباتات القمح ببكتيريا *B. Subtilis* أدى إلى التغير فى معدلات التعبير الجينى للعديد من الجينات بالمقارنة بتلك النباتات غير الملق

Abstract

Wheat is one of the most important crops for food consumption. Fertilization using minerals and chemicals compound is not safe for human health, as well as it is expensive. It is important to find the alternatives. *Bacillus subtilis* (BS) contain phytase enzyme which change phosphorus from phytate into phosphoric acid, the most available form of phosphorus for plant uptake.

Two wheat cultivars (*Triticum asitivum* L.) (Beni swafe and Salambo) were grown up to ninety days after germination as *B. subtilis* treated and none treated ones (control). Seven different yield related traits were measured and used as plant growth detectors. The traits were plant height (P.H.), number of tillers (N.T.), shoot fresh weight (S.F.W.), number of Leaves /plant (N.L.P.), Total root length (T.R.L.), weight of the youngest elongate blade leaf (Y.E.B.) and number spikes of plant (N.S.P). In the end of the experiment, leaf samples were collected from *B. subtilis* treated and non treated plants of the two cultivars under investigation, and subjected to molecular analysis using semi quantitative RT-PCR for three different genes conferring calcium dependant protein kinase (CDPKs), phosphoenol pyruvate carboxilase (PEPCs) and proline-5

carboxylate synthetase (P5CS). The results showed that all the aforementioned yield related traits were increased with different proportions as response for root inoculation with *B. subtilis* if compared with the non-inoculated plants of the two cultivars. These results reflected the great effects of root inoculation with *B. subtilis* on wheat plant growth and productivity. On the molecular level, wheat root inoculation with *B. subtilis* resulted in increasing expression of the gene conferring calcium dependant protein kinase (CDPKs) rather than the presence of a new band of (CDPKs) in the inoculated plants, but it was absent in non inoculated ones. This membranous protein acts as a secondary messenger for signaling transduction and thus plays an important role in the organization of cells physiology. Moreover, the gene conferring phosphoenol pyruvate carboxilase (PEPCs) increased as response for barley root inoculation with *B. subtilis*. Phosphoenol pyruvate carboxilase (PEPCs) has an important role in photosynthesis and the increase of this gene expression increased photosynthesis and thus plant growth. Finally, the expression of the gene conferring proline-5 carboxylate synthetase (P5CS) increased as response for wheat root inoculation with *B. subtilis*. This protein confers the concentration of proline which acts as osmolite and improving the plant tolerance for

abiotic stress. Commonly, the root inoculation with *B. subtilis* changes the rate of expression of several genes.

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INTRODUCTION

Wheat is one of the most important crops for food consumption. Fertilization using minerals and chemicals compound is not safe for human health, as well as it is expensive. Its important to find the alternatives. *Bacillus subtilis* is considered as one of the biofertilizers, it could be used as an alternative for chemical fertilization.

Bacillus subtilis, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium. A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct.

The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination. Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal.

Bacillus subtilis conduct a kind of symbiotic relation with the plant root cells of wide range of the plants. Bacillus subtilis by a mechanism or

another increases the availability of elements for plant uptake. The increase of the availability of elements specially, calcium which plays the main role in cell signaling which could be lead to switch on several genes such as, protein kinases and other genes related with photosynthesis and that finally, affect the plant growth. This investigation aimed to study the changes in wheat genes expression as response for root inoculation with *Bacillus subtilis*.

OBJECTIVES

This investigation aimed to study**:**

1- The effect of wheat root inoculation with *Bacillus subtilis* on the plant growth as detected using different yield related traits.

2- The changes in gene expression of some genes in wheat plants as response for their inoculation with *Bacillus subtilis* on the molecular level either using semi quantitative RT-PCR or real time RT-PCR.

   a- The genes planed to be under investigation conferring proline 5-carboxylate synthetase (P_5CS).

   b- The genes conferring calcium dependent protein kinase (CDPKs).

   c- The genes conferring phosphoenol pyruvate carboxilase (PEPCs).

REVIEW OF LITERATURE

Merriman *et al.* (1974) studied the effect of *Bacillus subtilis* A13 and Streptomyces griseus 2-A24 on the yield of wheat and carrots and with a known incidence of *R. solani*. They found that root disease was not effectively controlled; the seed treatments increased grain yield and dry matter production at one site, advanced time of heading at another site and increased tiller number at two sites. Evidence was obtained that the organisms persisted on pericarps in soil for 5 weeks after sowing. In subsequent trials application of *B. subtilis* and *S. griseus* in combination with a pellet treatment increased marketable yields of carrots by 48% and 15%, respectively over control. Addition of the organisms to seed without pelleting increased yields by 17% over controls. These results indicated a non-specific effect of *B. subtilis* and *S. griseus*, since they increased growth of plants belonging to two different families, the Gramineae and Umbelliferae, in different soils.

Plasmids constructed can also transform PEG-treated protoplasts, and increased the protoplast tolerance for drought. **Van Elsas *et al.* (1986)** characterized Antibiotic-resistant strains of *Pseudomonas fluorescens* and

Bacillus subtilis, produced by transposon Tn5 mutagenesis and transformation with plasmid pFT30, respectively. Both strains grew at a rate comparable to that of the wild-type strains, and the antibiotic resistance remained stable for over 50 generations without selective pressure. During the growing season, the survival of these strains was studied in two soils of different texture cropped with wheat. The *B. subtilis* populations declined rapidly in both soils and then stabilized at the levels of added spores. *P. fluorescens* showed a slow, steady decline in both soils. No significant rhizosphere effects were detected in either of the two soils.

Khanna and Stotzky (1992) worked on the equilibrium adsorption and its relation to the soil inoculation with *Bacillus subtilis* on the clay mineral. Maximum adsorption of elements from the clay occurred after 90 min of contact and was followed by a plateau. Adsorption was pH dependent and was greatest at pH 1.0

Kong and Dubnau (1994) stated that the expression of competence genes in *Bacillus subtilis* is controlled by a signal transduction cascade which increases the expression of a competence transcription factor (CTF, encoded by comK) during the transition from exponential growth to stationary phase. The transcription of CTF (ComK) is decreased by the product of the mecA gene, and this inhibition is relieved in response to an unknown signal

received from upstream in the regulatory pathway. Inactivation of either mecA or another gene, mecB, results in overproduction of ComK. They showed that the concentration of MecA protein does not vary markedly with culture medium, as a function of growth stage, or in competent and non competent cells. They also reported that MecA can interact directly with ComK. Finally, evidence is presented suggesting that MecB functions prior to MecA in the signaling pathway. A model is discussed which involves the sequestration of ComK by MecA binding and the release of the transcription factor when an appropriate signal is relayed to MecA by MecB.

The *Bacillus subtilis* strain VTT E-68013 was chosen for purification and characterization of its excreted phytase by **Kerovuo *et al.* (1998)**. They reported that purified enzyme had maximal phytase activity at pH 7 and 55◦C. Isolated enzyme required calcium for its activity.

Wieser *et al.* (1998) illustrated that Pyrrole-2- carboxylate decarboxylase from *Bacillus megaterium* PYR2910 attains a balanced reaction equilibrium with an equilibrium constant of 0.320.4 M. Therefore, the enzyme catalyzes the reverse carboxylation of pyrrole after addition of bicarbonate. For the synthesis of pyrrole-2-carboxylate, the reverse reaction was optimized and the equilibrium was shifted towards the carboxylate. The product yield was 230 mM (25.5 g/l) pyrrole-2-carboxylate from 300 mM pyrrole in a batch

reaction and 325 mM (36.1 g/l) from 400 mM pyrrole in a fed-batch reaction, using both whole cells and the purified enzyme in a pH 8.0 reaction mixture with bicarbonate saturation of 1.9 M. Kinetic studies indicated, that bicarbonate is the reactive species used by this carbon dioxide-fixation enzyme.

Idriss *et al*. (2002) showed that several *Bacillus* strains belonging to the *B. subtilis*/*amyloliquefaciens* group isolated from plant-pathogen-infested soil possess plant-growth-promoting activity. Three out of the four strains investigated were identified as *B. amyloliquefaciens* and were able to degrade extracellular phytate (*myo*-inositol hexakisphosphate). The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. The amino acid sequence deduced from the phytase *phyA* gene cloned from FZB45 displayed a high degree of similarity to known *Bacillus* phytases. Weak similarity between FZB45 phytase and *B. subtilis* alkaline phosphatase IV pointed to a possible common origin of these two enzymes. The recombinant protein expressed by *B. subtilis* MU331 displayed 3(1)-phytase activity yielding D/L-Ins(1,2,4,5,6)P5 as the first product of phytate hydrolysis. A phytase-negative mutant strain, FZB45/M2, whose *phyA* gene is disrupted, was

generated by replacing the entire wild-type gene on the chromosome of FZB45 with a *km*::*phyA* fragment, and culture filtrates obtained from FZB45/M2 did not stimulate plant growth. In addition, the growth of maize seedlings was promoted in the presence of purified phytase and the absence of culture filtrate. These genetic and biochemical experiments provide strong evidence that phytase activity of *B. amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation.

Yip *et al*. (2003) reported that phytate is the main form of phosphorus storage in plant seeds. To improve phosphorus bio-availability, they introduced a recently characterized phytase from *Bacillus subtilis* into the cytoplasm of tobacco cells. They also reported that tobacco line transformed with a neutral phytase exhibited phenotypic changes in flowering, seed development, and response to phosphate deficiency. In addition they declared that the transgenic line showed an increase in flower and fruit numbers and enhanced growth under phosphate-starvation conditions compared with the wild type. Moreover, the results suggested that the over-expression of Bacillus phytase in the cytoplasm of tobacco cells shifts the equilibrium of the inositol phosphate biosynthesis pathway, thereby making more phosphate available for primary metabolism. The approach presented

here can be applied as a strategy for boosting productivity in agriculture and horticulture.

Kong *et al*. (2005) reported that phytate is the major form of organic phosphorus in soil. Elevating the phytase activity in transgenic plants may be an effective approach to promote their phytate-phosphorus utilization. In this study, several transgenic tobacco lines carrying *Bacillus subtilis* phytase gene were compared with the wild-type tobacco, in terms of their ability in acquiring phosphorus from phytate in sterilized agar, sand and soil. In sterilized agar, transgenic tobacco plants were more efficient in phytate-phosphorus uptake and utilization, and their biomass and total phosphorus content were 3.6-10.7 and 2.2-4.6 folds of the wild-type's, respectively. **George *et al*. (2005)** stated that transgenic *Nicotiana tabacum* plants grown in sterile agar supplied with phosphorus (P) as phytate accumulated 3.7-fold more P than vector control plants. Despite of this, the expression of phy-A in plants did not lead to an improved accumulation of phosphorus. However, when soils were amended with either phytate or phosphate and lime, transgenic plants accumulated up to 52% more P than control. Positive responses by transgenic plants were, in some instances, coincident with a putative increase in soil phytate. They concluded that if plants that express phy-A are combined with soil amendments that promote the availability of

phytate, there is the potential to enhance the P nutrition of crop plants and to improve the efficiency of P fertilizer use in agricultural systems. Moreover, it will improve the plant growth as it could be change some of the plants transcriptomics.

Lim *et al*. (2007) reported that phytate, the most abundant organic phosphorus compound in soil, dominates the biotic phosphorus input from terrestrial runoffs into aquatic systems. Microbial mineralization of phytate by phytases is a key process for recycling phosphorus in the biosphere. Bioinformatic studies were carried out on microbial genomes and environmental metagenomes in the NCBI and the CAMERA databases to determine the distribution of the four known classes of phytase in the microbial world. Analysis of the genetic contexts of these subgroups showed that beta-propeller phytase genes exist either as an independent gene or are closely associated with a Ton B-dependent receptor-like gene in operons, suggesting that these two genes are functionally linked and thus may play an important role in the cycles of phosphorus and iron. **Lung *et al*. (2008)** reported that phytases are enzymes that catalyze liberation of inorganic phosphates from phytate, the major organic phosphorus in soil. Tobacco (*Nicotiana tabacum*) responds to phosphorus starvation with an increase in extra cellular phytase activity. By three-step purification scheme, a

phosphatase with phytase activity was purified 486-fold from tobacco root exudates to a specific activity and an overall yield of 3%. SDS-PAGE revealed a single polypeptide of 64 kDa, thus indicating apparent homogeneity of the final enzyme preparation. Gel filtration chromatography suggested that the enzyme was a ca. 56 kDa monomeric protein. De novo sequencing by tandem mass spectrometry resulted in a peptide sequence that shares high homology with several plant acid phosphatases. The identity of the enzyme was further confirmed by molybdate-inhibition assay and cDNA cloning. Although a broad specificity of the enzyme was observed for a range of physiological substrates in soil, maximum activity was achieved using mononucleotides as substrates. They concluded that the phytase activity in tobacco root exudates is exhibited by acid phosphatase and its catalytic properties are pertinent to its role in mobilizing organic P in soil.

Liu *et al* (2009) studied the bacterial strain E1R-j, isolated as an endophyte from wheat roots, exhibited high antifungal activity to *Gaeumannomyces graminis* var. *tritici* (*Ggt*). Strain E1R-j was identified as *Bacillus subtilis* based on morphological, physiological and biochemical methods, as well as, on 16S rDNA analysis. This strain inhibited mycelium growth *in vitro* of numerous plant pathogenic fungi, especially of *Ggt*, *Coniothyrium diplodiella*, *Phomopsis* sp. and *Sclerotinia sclerotiorum*. In greenhouse

experiments, soil drenches with different cell densities of E1R-j strain reduced significantly take-all disease, caused by *Ggt*, in wheat seedling by 62.6%, 68.6% and 70.7%, respectively, compared to the inoculated control, 4 weeks after sowing. Growth parameters such as lengths and fresh weights of roots and shoots of Ggt-inoculated control plants were significantly lower compared to Ggt-inoculated and E1R-j treated plants. The results indicated that the endophytic strain E1R-j of *B. subtilis* meets demands required for biocontrol of take-all.

Ma'ayan and JC (2010) concluded that Genome-wide messenger RNA profiling provides a snapshot of the global state of the cell under different experimental conditions such as diseased versus normal cellular states. However, because measurements are in the form of quantitative changes in messenger RNA levels, such experimental data does not provide direct understanding of the regulatory molecular mechanisms responsible for the observed changes. Identifying potential cell signaling regulatory mechanisms responsible for changes in gene expression under different experimental conditions or in different tissues has been the focus of many computational systems biology studies. Most popular approaches include promoter analysis, gene ontology, or pathway enrichment analysis, as well as reverse engineering of networks from messenger RNA expression data.

By combining promoter analysis; data from various chromatin immune-precipitation studies such as chromatin immune-precipitation sequencing, chromatin immune-precipitation coupled with paired-end ditag, and chromatin immune-precipitation-on-chip; protein-protein interactions; and kinase-protein phosphorylation reactions collected from the literature, they can identify and rank candidate protein kinases for knock-down, or other types of functional validations, based on genome-wide changes in gene expression. They described how protein kinase candidate identification and ranking can be made robust by cross-validation with phosphor-proteomics data, as well as, through a literature-based text-mining approach. In conclusion, data integration can produce robust candidate rankings for understanding cell regulation through identification of protein kinases responsible for gene expression changes, and thus rapidly advancing drug target discovery and unraveling drug mechanisms of action. Here they present a rational approach for identifying and ranking protein kinases that are likely responsible for observed changes in gene expression.

Sandhya *et al* (2010) showed that rhizobacterial populations of stressed soils are adapted and tolerant to stress and can be screened for isolation of efficient stress adaptive/tolerant, plant growth promoting rhizobacterial (PGPR) strains that can be used as inoculants for crops grown in stressed

ecosystems. The effect of inoculation of five drought tolerant plant growth promoting *Pseudomonas* spp. strains namely *P. entomophila* strain BV-P13, *P. stutzeri* strain GRFHAP-P14, *P. putida* strain GAP-P45, *P. syringae* strain GRFHYTP52, and *P. monteilli* strain WAPP53 on growth, osmoregulation and antioxidant status of maize seedlings under drought stress conditions was investigated. Drought stress induced by withholding irrigation had drastic effects on growth of maize seedlings. However maize seeds inoculation with *Pseudomonas spp.* bacteria improved plant biomass, relative water content, leaf water potential, root adhering soil/root tissue ratio, aggregate stability and mean weight diameter and decreased leaf water loss. The inoculated plants showed higher levels of proline, sugars, free amino acids under drought stress. However the protein and starch content was reduced under drought stress conditions. Inoculation decreased electrolyte leakage compared to the uninoculated seedlings under drought stress. As compared to uninoculated seedlings, inoculated seedlings showed significantly lower activities of antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) under drought stress, indicating that inoculated seedlings felt less stress as compared to uninoculated seedlings. The strain GAP-P45 was found to be the best in terms of influencing growth and biochemical and physiological status of the

seedlings under drought stress. The study reports the potential of rhizobacteria in alleviating drought stress effects in maize.

Ahmad *et al*. (2012) used *Bacillus thuringiensis* and *B. subtilis* isolated from rhizospheric soil and roots as bio-formulations in carrot plant. They noticed that, the plant these bio-fertilizers are caused plant growth promotion, especially when the crops grow in less fertilize soil regions.,

Sabir *et al*. (2012) studied the effects of the plant growth-promoting rhizobacteria (PGPR) strains Burkholderia gladii BA-7, *Bacillus subtilis* OSU-142, *Bacillus megatorium* M-3 and *Azospirillum brasilense* Sp 245 on vegetative development and minerals uptake. They found that vegetative development of grapevine rootstocks was obviously promoted by bacterial inoculation, with the maximum increase induced by Sp 245. Inoculation with Sp 245 also significantly improved the chlorophyll concentrations of the leaves of the two rootstocks. In addition, among the bacteria, OSU-142 significantly stimulated vegetative development and mineral acquisition of the plants. Nutrient contents of the leaf blades of the plants were generally higher than those of control plants. They concluded also that *A. brasilense* Sp 245 and *B. subtilis* OSU-142 performed

more efficiently than the other strains. Therefore, these bacteria seem to have a considerable potential in reducing the need for inorganic fertilizers.

Skorokhod *et al*. (2012) studied the influence of granulated bacterial preparation of complex action on the growth and yield of barley (*H. distichum* L.). They reported that, the treatment of barley seeds by this preparation has been established to have a very significant effect on the mass of 1000 grains, grain natural weight and to increase the yield of plants, but to different degree. Consequently, the interaction of certain barley varieties with bacteria-components of the preparation is rather specific. It has been displayed that the treatment of grains of different barley varieties by the bacterial preparation takes a very significant influence on the function of microbial associations in the rhizosphere.

MATERIALS AND METHODS

Sandy culture experiment

A greenhouse trial designed as a factorial experiment (completely randomized type) with four replications was conducted in the successive season of 2009/2010, including two wheat cultivars (*Triticum aestivum* L.) nominated (Beni swafe and Salambo) which were obtained from

Agriculture Research Center, Al-kriemia, Libya. The plants were grown up to one hundred and twenty days after germination as *Bacillus subtilis* treated and none treated ones (control).

***Bacillus subtilis* treatment:**

Wheat plants of the two aforementioned cultivars were grown in pots with thirty five cm diameter, each pot filled with water well washed sand (10-12 kg / pot) mixed with equal amounts of *Bacillus subtilis* inoculants for the treated plants. *Bacillus subtilis* inoculants were obtained from biofertilizers unit, Faculty of Agriculture, Ain Shams University. Four pots for each of the two cultivars under investigation. On the other hand, another four pots for each of the two cultivars were filled with similar amounts of water well washed sand (10-12 kg / pot) for control plants. four seedlings were grown in each pot. The experiment was terminated after four monthes of germination.

The effect of wheat roots inoculation with *Bacillus subtilis* on the plant growth was detected using six different yield related traits which were measured in the end of the experiment. The traits were plant height (P.H.), number of tillers (N.T.), shoot fresh weight (S.F.W.), number of Leaves / plant (N.L.P.), Total root length (T.R.L.), weight of the youngest elongate blade leaf (Y.E.B.) and number spikes of plant (N.S.P)

The weights were measured using two decimal, sensitive balances. Data were obtained and statistically analyses according to **Steel and Torrie (1980)**.

I- Molecular studies:

Samples of leafs of wheat plants were collected from *Bacillus subtilis* inoculated and non inoculated ones (control) of the two cultivars under investigation for the purpose of RNA extraction.

a) RNA extraction:

RNA was extracted from the four samples according to **Ashoub *et al.* (2006)** using one gm of leaf of each sample was grind with Tris saturated phenol dissolved in diethyl pyro carbamide solution (DEPC) to avoid the RNA destruction with RNAase enzyme during the extraction.

Purification of Total RNA from Plant Cells and Tissues:

This protocol requires the RNeasy Plant Mini Kit.

Determining the correct amount of starting material:

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 100 mg plant material or 1 x 107 cells can generally be processed. For most plant materials, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts.

Average of RNA yields from various plant materials was determined. If there is no information about the nature of your starting material, we recommend starting with no more than 50 mg plant material or 3–4 x 106 cells. Depending on RNA yield and purity, it may be possible to use up to 100 mg plant material or up to 1 x 107 cells in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality. Starting material, as a guide, is 1.5 cm diameter leaf disc weighs 25–75 mg.

Important points before starting:

Fresh or frozen tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.

The RNeasy Plant Mini Kit provides a choice of lyses buffers: Buffer RLT and buffer RLC, which contain guanidine thiocyanate and guanidine

hydrochloride, respectively. In most cases, Buffer RLT is the lyses buffer of choice due to the greater cell disruption and denaturation.

Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (20–25°C).

Perform all steps of the procedure at room temperature. During the procedure, work quickly. Perform all centrifugation steps at 20–25°C in a standard micro-centrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

β-Mercaptoethanol (β-ME) must be added immediately, to Buffer RLT or buffer RLC before use. Add 10 µl β-ME per 1 ml buffer RLT or buffer RLC. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT or Buffer RLC containing β-ME can be stored at room temperature for up to 1 month.

Buffer RPE is supplied as a concentrate. Before using for the first time, add four volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

RNA Extraction Procedure:

1. The amount of plant material was determined. Do not use more than 100 mg. Weighing tissue is the most accurate way to determine the amount.

2. Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 1.5 ml micro-centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3. RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. An amount of 450 µl Buffer RLT or Buffer RLC was Added (see "Important points before starting") to a maximum of 100 mg tissue powder. Vortex vigorously. A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, do not incubate samples with high starch content at elevated temperatures; otherwise swelling of the sample will occur.

4. The lysate to a QIA shredder spin column (lilac) placed in a 2 ml collection tube was transferred, and centrifuge for 2 min at full speed. Carefully it transferred the supernatant of the flow-through to a new

micro-centrifuge tube without disturbing the cell-debris pellet in the collection tube.

It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new micro-centrifuge tube.

5. Half volume of ethanol (96–100%) was Added to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately step 6.

Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. The sample (usually 650 µl) was transferred, including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15

seconds at (10,000 rpm). Discard the flow-through. Reuse the collection tube in step 7. If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

7. An amount of 700 µl Buffer RW_1 was added to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds at (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8. Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

8. An amount of 500 µl Buffer RPE was Add to the RNeasy spin column. Close the lid gently, and centrifuged for 15 seconds at (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9. Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to buffer RPE before use.

9. An amount of 500 µl Buffer RPE was Add to the RNeasy spin column, closed the lid gently, and centrifuged for 2 min at (10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin

column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. Optional: Placed the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuged at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11.T RNeasy spin column was Placed in a new 1.5 ml collection tube (supplied). An amount of 30–50 µl RNase-free water was added directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at (10,000 rpm) to elute the RNA.

Note:

12. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNasefree water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.

If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

RNA Cleanup:

Determining the correct amount of starting material:

A maximum of 100 µg RNA could be cleaned up in this protocol. This amount corresponds to the RNA binding capacity of the RNeasy spin column. Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (20–25°C).

Buffer RLT contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach.

Things to do before starting:

Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Procedure:

1. The sample was adjusted to a volume of 100 µl with RNase-free water. Add 350 µl buffer RLT, and mix well.

2. An amount of 250 µl ethanol (96–100%) was added to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.

3. The sample (700 µl) was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at (10,000 rpm). Discard the flow-through. Reuse the collection tube in step 4.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Empty the collection tube completely.

4. An amount of 500 µl Buffer RPE was added to the RNeasy spin column, the lid was closed gently, and centrifuged for 15 s at 8000 xg (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 5.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to buffer RPE before use.

5. An amount of 500 µl Buffer RPE was added to the RNeasy spin column, the lid was closed gently, and centrifuged for 2 min at (10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no

ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

6. Optional: The RNeasy spin column was placed in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 5.

7. The RNeasy spin column was placed in a new 1.5 ml collection tube (supplied). An amount of 30–50 µl RNase-free water was added directly to the spin column membrane. The lid was gently closed, and centrifuged for 1 min at (10,000 rpm) to elute the RNA.

Note:

8. If the expected RNA yield is >30 µg, repeat step 7 using another 30–50 µl RNasefree water, or using the eluate from step 7 (if high RNA concentration is required). Reuse the collection tube from step 7.

Glassware

Glassware treated before use to ensure that it is RNase-free. Glassware used for RNA work cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware treated with DEPC* (diethyl pyrocarbonate). Glassware was filled with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37 ° C and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks:

Electrophoresis tanks cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plastic ware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. 0.1 ml DEPC was added to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. The solution was incubated for 12 hours at 37°C, autoclaved for

15 minutes to remove any trace of DEPC. DEPC reacts with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and Co_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Residual DEPC eliminated from solutions by autoclaving or heating to 100°C for 15 minutes. Plastics used for some electrophoresis tanks are not resistant to ethanol.

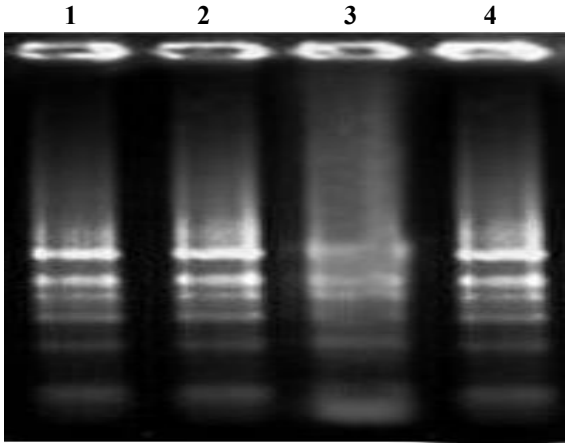


Figure (1): electrophoresis using FA denaturing gel for the RNA of the two cultivars as *Bacillus subtilis* inoculated and non inoculated plants.

Storage, Quantification, and of Quality of RNA determination

Storage of RNA

Purified RNA stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

Spectrophotometer quantification of RNA

To ensure significance, A260 readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml (A260=1 → 44 μg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNasefree water. Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification as follow:

Volume of RNA sample = 100 μl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution). Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free) A260 = 0.2. Concentration of RNA sample = 44 µg/ml x A260 x dilution factor = 44 µg/ml x 0.2 x 50 = 440 µg/ml

Purity of RNA:

The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A260/A280 ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A260/A280 ratio can vary greatly. Lower pH results in a lower A260/A280 ratio and reduced sensitivity to protein contamination. Calibrate the spectrophotometer with the same solution used for dilution. For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A260 reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA.

Formaldehyde Agarose Gel Electrophoresis:

The following protocol for formaldehyde agarose (FA) gel electro-phoresis is routinely used. A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the

gel than conventional protocols according to **Sambrook *et al*. (1989)** Molecular cloning a laboratory manual. 2nd ed. Disposable gloves is essential to use when deal with RNA.

Formaldehyde (FA) gel preparation:

FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, was prepared as follow:

1.2 g agarose*

10 ml 10x FA gel buffer, RNase-free water was added up to 100 ml

Heat the mixture to melt agarose. Cool it to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer for at least 30 min.

RNA sample preparation for FA gel electrophoresis:

Add 1 volume of 5x RNA loading buffer (see composition below) to 4 volumes of RNA sample (e.g., 10 µl of loading buffer and 40 µl of RNA) and mix. Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

Gel running conditions:

Run gel at 5–7 V/cm in 1x FA gel running buffer.

Composition of FA gel buffers:

10x FA gel buffer:

200 mM 3-[N-morpholino] propanesulfonic acid (MOPS)

50 mM sodium acetate

10 mM EDTA, pH was adjusted to 7.0 using (1N) NaOH

1x FA gel running buffer:

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

5x RNA loading buffer:

16 µl saturated aqueous bromophenol blue solution

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde

2 ml 100% glycerol

3.084 ml formamide

4 ml 10 x FA gel buffer

RNase-free water to 10 ml

Stability: approximately 3 months at 4°C

RT-PCR:

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can be carried out either sequentially in the same tube (one-step RT-PCR) or separately (two-step RT-PCR).

One-step RT-PCR requires gene-specific primers. For this application, QIAGEN offers the QIAGEN one step RT-PCR Kit, which enables one-step RT-PCR of any RNA template without optimization.

Two-step RT-PCR is generally carried out using oligo-dT primers in the RT step and gene-specific primers in the PCR step.

Taq DNA Polymerase for PCR without a hot start

Hot-Star Taq® DNA polymerase for PCR with a hot start

Hot-Star Taq plus DNA polymerase for PCR with a hot start.

Real-time RT-PCR:

The range of QuantiTect Kits and Assays guarantee highly specific and sensitive results in real-time RT-PCR on any real-time cycler and require no optimization of reaction and cycling conditions. QuantiTect Kits are available for two-step and one-step RT-PCR and are compatible with detection by SYBR® Green I dye or by sequence-specific probes

(e.g., Taq Man and FRET probes). Multiplex RT-PCR of up to 4 targets is also possible.

Reverse transcription-PCR mixture for (CDPKs), (PEPCs) and (P5CS):

RNA of each of the four aforementioned samples was reverse transcribed (RT), to produce the first strand of cDNA in the presence of 5 mM MgCl2, 1X PCR Buffer, 1 mM dNTPs, 25 u MuLV Reverse Transcriptase, 4 u RNA-guard Ribonuclease inhibitor, the mixture prepared as described three times in three different PCR tubes and 2.5 µl of 20 P mol of CDPKs reveres primer with the following sequence (AAT TGA TGG CCA TGG CCT GAC TTT C) was added to the mixture in one of the three PCR tubes and to the second tube 2.5 µl of 20 P mol of PEPCs reveres primer with the following sequence (GCC GGC TTG CTC GTG TCC AT), finally .5 µl of 20 P mol of P5CS reveres primer with the following sequence (GTA AAG CGT ATC CGC ACT AAC GC) was added in a final reaction volume of 30 µl in each tube. Reactions were carried out at 42 °C for 30 min, followed by a 10 min step at 94 °C to denature the enzyme, then was cooling at 4 °C.

Real time PCR quantification of cDNA encoding for CDPKs, PEPCs and P5CS. All PCR processes were performed using commercially available reagents that included a thermostable DNA polymerase, dNTPs, MgCl2, and other salts and buffering agents necessary for optimum performance. One µl of cDNA of the four aforementioned samples was used as template in the reaction mix, in a final volume of 25 µl in all assays. Conventional PCR,

using CDPKs, PEPCs and P5CS forward and reverse primers with the following sequences [CDPKs forward (TGA GTA AGG CCG ACA AGG AGG ATA), reverse (AAT TGA TGG CCA TGG CCT GAC TTT C)] and [PEPCs forward (TGG CCC CAC TCA TCT TGC TAT CTT), reverse (GCC GGC TTG CTC GTG TCC AT)] and [P5CS forward (ATT CCG ACC TTG TGT AAC CGG C), reverse (GTA AAG CGT ATC CGC ACT AAC GC)], respectively, were employed to define the detection limit of the assay. Cycling was carried out in a Stratagene Mx-3000 Real-time PCR system which allows detection of most commercially available dyes including FAM, SYBR® Green I, TET, HEX™, JOE™, VIC™, TAMRA™, TexasRed®, ROX™, Cy5™, Cy3™ and ALEXA Fluor® 350. The system supports 96-well plate format and can perform multiple sub-experiments up to four dyes in the same well.

Bioron product, SYBR® Green I Real Time QPCR (cat No. 119205) master mix for (100 rcs) detection protocol was used in this investigation as described in Bioron manual. Data from fluorescence thresholds were statistically analyzed using Microsoft Excel (Office 2000). PCR products were dissolved in TBE agarose gel as described earlier.

RESULTS AND DISCUSSIONS

Assessment of Bacillus subtilis Effect on Plant Growth:

Bacillus subtilis causes changes in plant growth and its production. The growth of two cultivars under investigation Beni swafe and Salambo were evaluated as response for *Bacillus subtilis* inoculation and control (non inoculated plants) in two replications. The evaluation was performed in sand culture experiment under greenhouse conditions on the base of some yield related traits.

The following yield related traits were scored and used as detectors for plant growth changes as response for *Bacillus subtilis* inoculation, plant

height, number of tillers/plant, shoot fresh weight, number leaves/plant, total root length, and the weight of youngest elongate blade leaf (YEB).

In general, plant growth of the two cultivars under investigation was elevated as response for root inoculation with *Bacillus sabtilis*, as detected from the aforementioned yield related traits. As well as, too many changes on the molecular level were occurred in the inoculated plants as compared to the non inoculated ones. The plant response for the inoculation with *Bacillus subtilis* was varied, on the yield related traits level or on the molecular level, between the two cultivars under investigation.

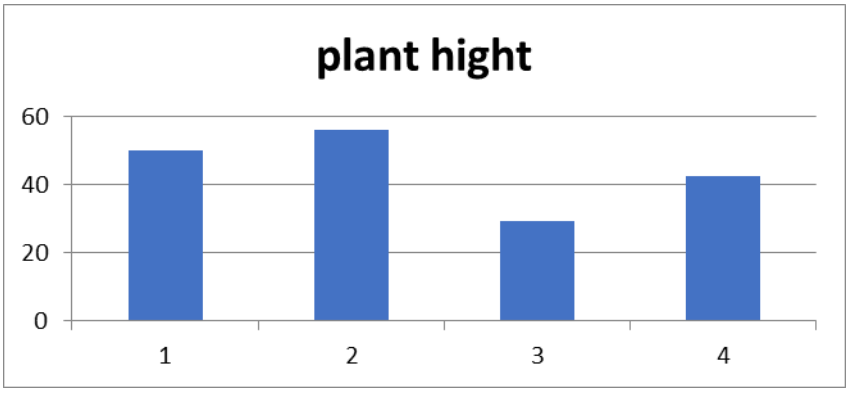
1. . Yield related traits

Plant height

Table (1): Plant heights in the two cultivars Beni swafe and Salambo, *Bascillus subtilis* inoculated and control

	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
Plant Height	Beni swafe	C	51	47	53	49	50	
		T	58	56	54	56	56	12.00
	Salambo	C	36	26	24	31	29.25	
		T	47	41	43	39	42.5	45.30

Figure (2): Average of plant heights in cultivars Beni swafe and, control and *Bascillus subtilis* inoculated, then Salambo, control and *Bascillus subtilis* inoculated, respectively.



Plant heights as shown in (Table 1) and (Figure 2) was significantly affected under *Bacillus subtilis* inoculation if compared with the non inoculated plants (control). The results showed that the average of plant height was increased from 50.00 in the non inoculated plants to 56.00 cm in the *Bacillus subtilis* inoculated plants of cultivar Beni swafe with increasing of 12.00% relative to the control. On the other hand, the trait was increased from 29.25 in the non inoculated plants to 42.50 cm in the *Bacillus subtilis* inoculated plants case of cv. Salambo with an increasing of 45.30%. These results indicated that Salambo plants had a greater response for *Bacillus subtilis* inoculation than the other cultivar Beni swafe in plant height.

1.1.2. Number of tillers/plant

Table (2): Number of tillers/plant in cultivars Beni swafe and Salambo, *Bascillus subtilis* inoculated and control

	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
Number of tillers /plant	Beni swafe	C	5	7	7	4	5.75	
		T	7	8	12	7	8.5	47.82
	Salambo	C	2	6	4	4	4	
		T	6	3	5	6	5	25.00

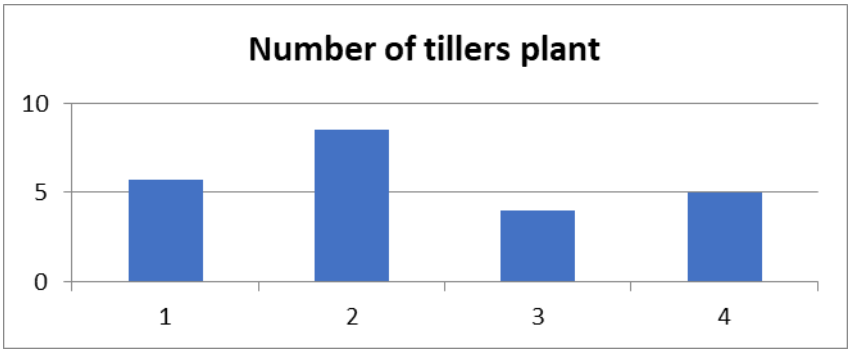


Figure (3): Average of number of tillers/plant in cultivars Beni swafe control and *Bascillus subtilis* inoculated, then Salambo, control and *Bascillus subtilis* inoculated, respectively.

Number of tillers/plant as shown in Table (2) and (Figure 3) was significantly increased under *Bacillus subtilis* inoculation if compared with non inoculated plants (control). The results showed that the average of number of tillers/plant was increased from 5.75 in the non inoculated plants to 8.50 tillers/plant in the inoculated plants of cultivar Beni swafe with an increasing of 47.82%. On the other hand, the number of tillers / plant was increased from 4.00 in the non inoculated plants to 5.00 tillers / plant in the *B. subtilis* inoculated plants of cultivar Salambo with an increasing of 25.00%. These results indicated that Beni swafe plants had a greater response for the inoculation than cultivar Salambo in this trait. This trait is highly correlated with the grain yield where the increasing of number of tillers/plant resulted in increasing the grain yield/plant.

1.1.3. Shoot fresh weight

Table (3): Shoot fresh weight in cultivars Beni swafe and Salambo, *Bascillus subtilis* inoculated and control

	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
Shoot fresh weight	Beni swafe	C	4.9	8.9	13.4	5.6	8.2	
		T	8.8	8.9	9.9	7.5	8.775	7.01
	Salambo	C	2.5	2.6	2.3	2.7	2. 53	
		T	7.2	3.8	4.2	3.6	4.7	85.77

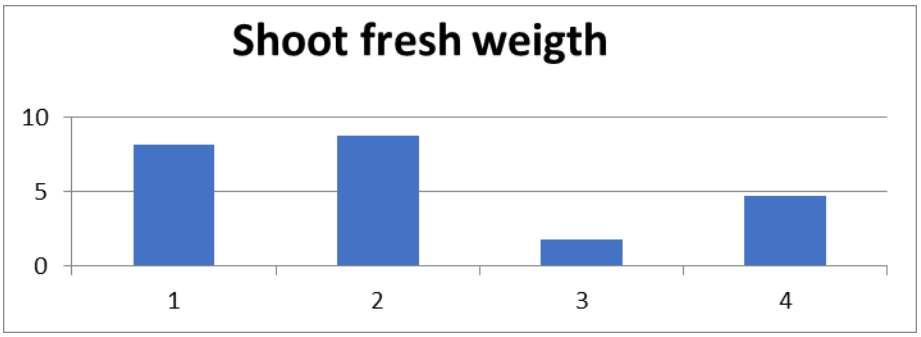


Figure (4): Average of shoot fresh weight gm in cultivars Beni swafe control and *Bascillus subtilis* inoculated, then Salambo, control and *Bascillus subtilis* inoculated, respectively.

Shoot fresh weight as shown in Table (3) and Figure (4) was significantly affected under inoculation as compared with the non inoculated plants (control). The results showed that the average of shoot fresh weight was increased from 8.20 gm in the non inoculated plants to 8.775 gm in the inoculated plants of cultivar Beni swafe with an increasing of 7.01%.

On the other hand, the trait was increased from 2.53 gm in the non inoculated plants to 4.70 gm in the inoculated plants in cultivar Salambo with an increasing of 85.77%. These results showed that both of Beni swafe and Salambo cultivars have a different response for the inoculation and that the increase in this trait was greater in cultivar Salambo than cultivar Beni swafe. This trait is highly correlated with total biomass which usually used to express the vegetative growth in plants.

1.4. Number of leaves/ plant

Table (4): number of leaves plant in cultivars Beni swafe and Salambo, *Bascillus subtilis* inoculated and control

Number of leaves /plant	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
	Beni swafe	C	15	27	35	20	24.25	
		T	37	42	42	32	38.25	57.73
	Salamo	C	9	18	12	11	12.5	
		T	31	17	29	28	26.25	110.00

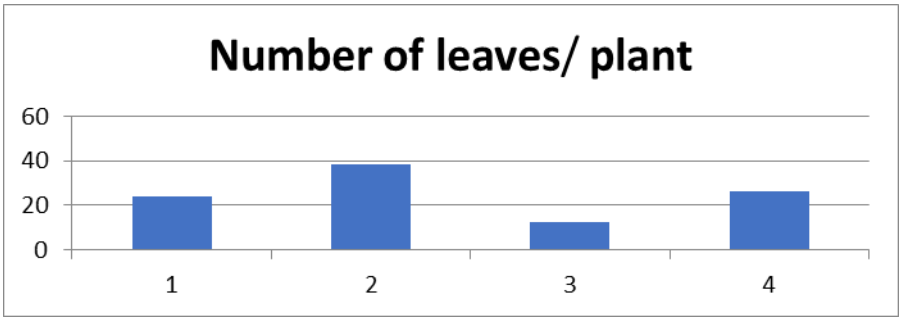


Figure (5): Average of Number of leaves plant in cultivars Beni swafe control and *Bascillus subtilis* inoculated, then Salambo, control and *Bascillus subtilis* inoculated, respectively

Numbers of leaves/plant are shown in Table (4) and Figure (5) was significantly increased as response for *Bacillus subtilis* inoculation if compared with the non inoculated plants (control). The results showed that the average of numbers of leaves/plant was increased from 24.25 in the non inoculated plants to 38.25 in the inoculated plants in cultivar Beni swafe with an increasing of 57.73%. On the other hand, the trait was increased from 12.50 in the non inoculated plants to 26.25 in *B. subtilis* inoculated plants of cultivar Salambo with an increasing of 110.00%. Data analyzed and showed that the trait was increased in both of Beni

swafe and Salambo plants and the cultivars have a different response for the inoculation and that the increase in number of Leaves/plant was significantly higher in cultivar Salambo than the increase of the trait in cultivar Beni swafe. This trait is highly related with total biomass which usually used to express the vegetative growth in plants.

1.1.5. Total root length

Table (5): Total root length in cultivars Beni swafe and Salambo, *Bacillus subtils* inoculated and control

	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
Total Root length	Beni swafe	C	25	38	34	22	29.75	
		T	46	42	24	32	36	21.01
	Salambo	C	11	8	7	9	8.75	
		T	12	11	16	15	13.5	54.29

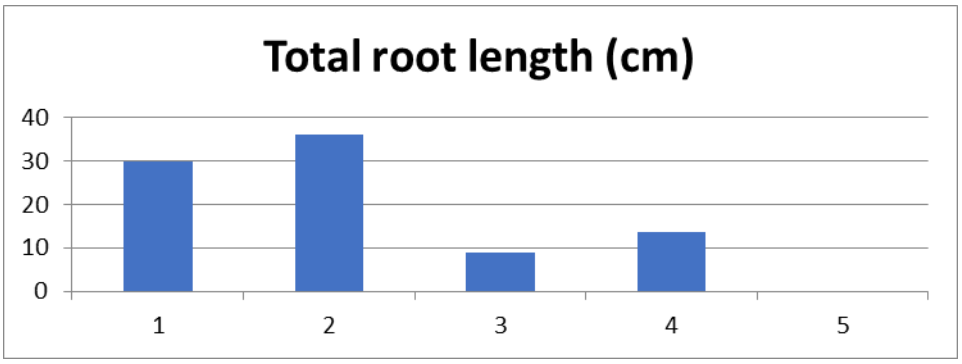


Figure (6): Average of total root length in cultivars Beni swafe control and *Bacillus subtilis* inoculated, then Salambo, control and *Bascillus subtilis* inoculated, respectively

Data of total root length are shown in Table (5) and Figure (6). It was noticed that there was significantly increasing as response for *Bacillus subtilis* inoculation as compared with the non inoculated plants (control). The results indicated that the average of total root length was increased from

29.75 cm in the non inoculated plants to 36.00 cm in the inoculated plants of cultivar Beni swafe with an increasing of 21.01%. On the other hand, the trait was increased from 8.75 cm in non inoculated plants to 13.50 cm in the inoculated plants of cultivar Salambo with an increasing of 54.29%. These results indicated that both cultivars Beni swafe and Salambo plants have different responses for *Bacillus subtilis* inoculation and that the increase in the trait was less in cultivar Beni swafe than the increases of the trait in cultivar Salambo as response for the inoculation with *Bacillus subtilis*. These results reflected the effects of *Bacillus subtilis* inoculation on wheat total root length, as known from previous plant physiology investigations that the increase of root growth combined with increasing in elements absorption by the plants.

1.1.6 Weight of youngest elongate blade leaf

Table (6): YEB leaf in cultivars Beni swafe and Salambo, bacillus subtils inoculated and control

	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
YEB Leaf	Beni swafe	C	0.2	0.15	0.3	0.3	0.24	
		T	0.3	0.3	0.4	0.2	0.30	25.00
	Salambo	C	0.1	0.1	0.1	0.1	0.10	
		T	0.1	0.1	0.1	0.1	0.10	00.00

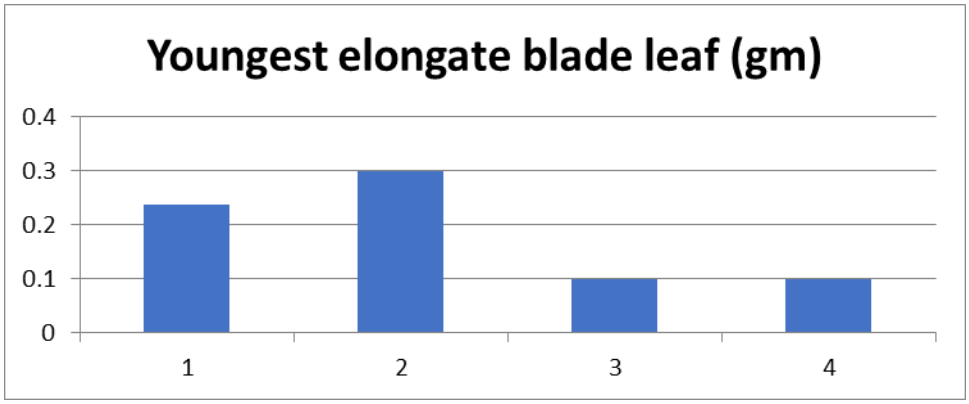


Figure (7): Average of YEB leaf in cultivars Beni swafe control and *Bacillus subtilis* inoculated, then Salambo, control and *Bacillus subtilis* inoculated, respectively

Youngest elongate blade leaf as shown in Table (6) and Figure (7) was increased under *Bacillus subtilis* inoculation as compared with the non inoculated plants (control). The results showed that the average of youngest elongate blade leaf was increased from 0.24 in non inoculated plants to 0.30gm in the inoculated ones of cultivar Beni swafe with an increasing of 25.00%. On the other hand, the trait was the same in the non inoculated plants and in the inoculated ones of cultivar Salambo with value of 0.1gm. These results indicated that both cultivars Beni swafe and Salambo have a different response for the inoculation and that the increasing in the trait was only occurred in cultivar Beni swafe as response for the inoculation with *Bacillus subtilis*.

1.1.7 Number of spikes/plant

Table (7): Number of spikes/plant in cultivars Beni swafe and Salambo, *Bacillus subtilis* inoculated and control

No.	Cv.		$R_1 P_1$	$R_1 P_2$	$R_2 P_1$	$R_2 P_2$	Average	% increase
	Beni	C	2	4	6	3	3.75	

Spikes \plant	swafe	T	6	8	8	6	7.00	86.67
	Salambo	C	2	1	2	1	1.50	
		T	5	2	5	6	4.50	200.00

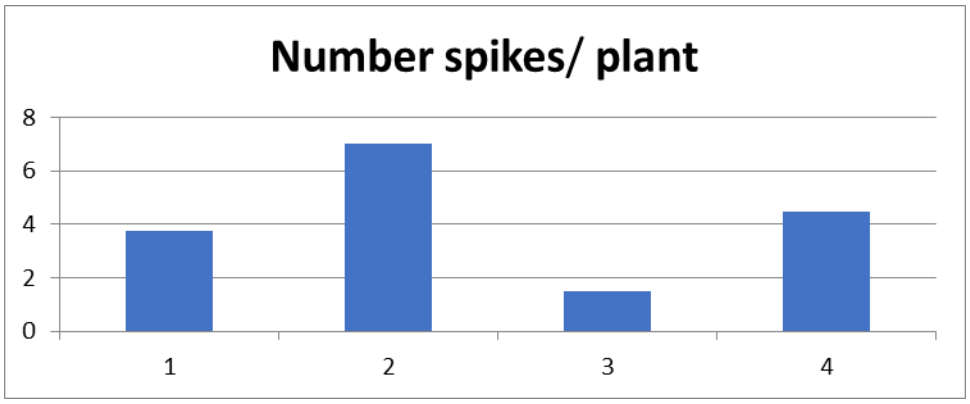


Figure (8): Average of number spikes plant in cultivars Beni swafe control and *Bacillus subtilis* inoculated, then Salambo, control and *Bacillus subtilis* inoculated, respectively

Data of Number of spikes/plant are shown in Table (7) and Figure (8). It was noticed that there was significantly increasing as response for *Bacillus subtilis* inoculation if compared with the non inoculated plants (control). The results indicated that the average of Number of spikes/plant was increased from 3.75 in the non inoculated plants to 7.00 in the inoculated plants of cultivar Beni swafe with an increasing of 86.67%. On the other hand, the trait was increased from 1.50 in non inoculated plants to 4.50 in the inoculated plants of cultivar Salambo with an increasing of 200.00%. These results indicated that both cultivars Beni swafe and Salambo plants have different responses for *Bacillus subtilis* inoculation and that the increase in

the trait was less in cultivar Beni swafe than the increases of the trait in cultivar Salambo as response for the inoculation with *Bacillus subtilis*. These results reflected the effects of *Bacillus subtilis* inoculation on number of wheat spicks/plant, as known previously that the increase of this trait resulted in increasing in seed yield production in wheat.

Generally, it possible to conclude that, wheat root inoculation with *Bacillus subtilis* resulted in an increase of plant growth in form of increase the plant height, number of tillers/plant, shoot fresh weight, root dry weight, number of spicks/plant and the weight of youngest elongate blade leaf. Although the inoculation with *Bacillus subtilis* resulted in increase of all the investigated traits, the response was varied from one trait to another. Moreover, the response was varied from cultivar Beni swafe to cultivar Salambo. Commonly, the incensement in the rest of the traits under investigation were greater in cultivar Salambo but some of the traits were greater in cultivar Beni swafe indicating that the growth increased as response for root inoculation with *Bacillus subtilis* was greater in cultivar Salambo than cultivar beni swafe. The obtained results are in agreement with the findings of **de Melo Pereira *et al.* (2012)** where they used a multiphasic approach, characterized by the simultaneous use of culture-dependent and culture-independent methods, to investigate endophytic bacterial communities in

strawberry (*Fragaria ananassa*) fruit. They extracted the nucleic acids from the strawberry fruit and subjected to 16S rRNA gene directed polymerase chain reaction denaturing gradient gel electrophoresis (16S rRNA PCR-DGGE). The species *B. subtilis*, Enterobacter sp., and Pseudomonas sp. were detected both by isolation and DGGE. The DGGE fingerprints of total bacterial DNA did not exhibit bands corresponding to several of the representative species isolated in the extinction dilution (*L. plantarum, C. citreum, and P. punctata*). In contrast, bands in the DGGE profile that were identified as relatives of Arthrobacter sp. and one uncultivable Erythrobacter sp. were not recovered by cultivation techniques. After isolation, the nitrogen fixation ability and the in vitro production of indole-3-acetic acid (IAA) equivalents and siderophores were evaluated. A high percentage of isolates were found to possess the ability to produce siderophores and IAA equivalents; however, only a few isolates belonging to the genera Pseudomonas and Enterobacter showed the ability to fix nitrogen. Plant growthpromotion was evaluated under greenhouse conditions and revealed the ability of the Bacillus strains to enhance the number of leaves, shoot length, root dry weight, and shoot dry weight. The activity of the bacterial isolate identified as B. subtilis NA-108 exerted the greatest influence on strawberry growth and showed a 42.8% increase in number of

leaves, 15.26% for high shoot, 43.5% increase in root dry weight, and a 77% increase in shoot dry weight when compared with untreated controls.

II) MOLECULAR STUDIES

The recorded changes in plant growth and productivity as response for root inoculation with *Bacillus subtilis* switch the authors mined to study the internal changes in the plant on the molecular level. The bacteria, as detected from the previous studies, effects extended to the whole plant. This fact leads to study the way by which the effects of *Bacillus subtilis* inoculation transferred from roots to different parts of the plant. Cell signaling is the way by which the effects of *Bacillus subtilis* inoculation transferred from roots to different part of the plant. Calcium dependent protein kinase

(CDPKs) is a membranous protein which acts as secondary messenger. It is activated by receipting calcium and then it is activates different protein molecules by phosphorelation. The activated proteins could affect finally the expression of different genes. The affected genes could lead to increase photosynthesis rate in the inoculated plants and thus increase plant growth. Other genes could increase the plant tolerance for abiotic stress by increasing the solid substances in plant cells of the inoculated plants.

II- a) Calcium dependent protein kinase (CDPKs)

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of calcium dependent protein kinase (CDPKs) was performed as described previously. The products of RT-PCR were resolved in 1.4% agarose / TBE gel. The results were recorded in Table (8) and Figure (9), showed that the gene expression of the gene conferring calcium dependent protein kinases exhibited two different lengths 860 and 780 bp. The band with length of 860 bp was newly synthesized band under inoculation in both cultivars under investigation, but it was not exhibited in control plants of the two cultivars. The results indicated that this band posses a higher intensity in the treated plants of cultivar Salambo and semi quantitative RT-PCR results confirm this results where it showed the higher concentration (19.81 pg/µL). The second band with length of 780 bp was almost similar in the control and

inoculated plants of the two cultivars indicating no response for root inoculation with *Bacillus subtilis* for this band. In general, CDPKs gene expression increased in the inoculated plants as compared to non inoculated ones in both cultivars Beni swafe and Salambo but with a little higher increase in Salambo plants in Beni swafe cultivar reflecting a higher response in cultivar Salambo than in cultivar Beni swafe.

The results were in agreement with the findings of **Bulavenko and Kurdysh (2005)** who studied phosphatase activity of two strains of bacteria - *Bacillus subtilis* IMV B-7023 and *B. megaterium* 12. He reported that phosphatase activity is found to reach 260 mk mol/g x hour for B. subtilis IMV B-7023 and 12-100 mk mol/g x hour for *B. megaterium* 12 at optimal temperature (55 degrees C) and pH (9.5-10.0). Synthesis of alkaline phosphatase is shown to reach its maximum values at the end of logarithmic phase of the culture growth. It is revealed that Mg2+, Ca2+ cations increase phosphotase activity of *B. subtilis* IMV B-7023. They also reported that the increase of phosphate in the form of phosphoric acid in the soil resulted decreasing of rhizospher pH and thus increase the availability of different elements specially $Ca^{2+.}$

Using an RNA interference–based screen for gene that is involved in root development in *Medicago truncatula*, *Ca^{2+}-dependent protein kinase1*

(*CDPK1*) were identified. It is predicted to encode a Ca^{2+}-dependent protein kinase, resulted in significantly reduced root hair and root cell lengths. Inactivation of *CDPK1* is also associated with significant diminution of rhizosphere microbes symbiotic colonization. Additionally, microarray analysis revealed that silencing *CDPK1* alters cell wall and defense related gene expression. Moreover, CDPK1 is a key component of one or more signaling pathways that directly or indirectly modulates cell expansion or cell wall synthesis in *M. truncatula*, **Sergey *et al.* (2005)**.

Kurdish *et al*. (2009) investigated the ability of phosphate-mobilizing bacteria, related to four species of Bacillus genus, to colonize the cucumber root zone. They reported that, all the studied strains can adapt to the root zone of those plants colonizing its parts to different extent. Essential non-uniformity of the root surface populating with bacteria is noted. Microcolonies of these organisms are formed in some root zones. They also concluded that, the four different strains increased the plant growth with different rates.

Sandhya *et al.* (2010) reported that maize seeds inoculation with *Pseudomonas spp.* bacteria improved plant biomass, relative water content, leaf water potential, root adhering soil/root tissue ratio, aggregate stability and mean weight diameter and decreased leaf water loss. The inoculated

plants showed higher levels of proline, sugars, free amino acids. However, inoculation decreased electrolyte leakage compared to uninoculated seedlings. As compared to uninoculated seedlings, inoculated seedlings showed significantly lower activities of antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), indicating that inoculated seedlings felt less stress as compared to uninoculated seedlings. The strain GAP-P45 was found to be the best in terms of influencing growth and biochemical and physiological status of the seedlings.

Skorokhod *et al*. (2012) studied the influence of granulated bacterial preparation of complex action on the growth and yield of barley (*H. distichum* L.). They reported that, the treatment of barley seeds by this preparation has been established to have a very significant effect on the mass of 1000 grains, grain natural weight and to increase the yield of plants, but to different degree. Consequently, the interaction of certain barley varieties with bacteria-components of the preparation is rather specific. It has been displayed that the treatment of grains of different barley varieties by the bacterial preparation takes a very significant influence on the function of microbial associations in the rhizosphere.

Table (8): Quantification of calcium dependent protein kinase gene expression in two wheat cultivars under *Bascillus subtilis* inoculation and non inoculated plants

Cultivars	Band M.W.	control	inoculated	Times of control
Beni swafe	860 bp.	00.00	11.38	A
Salambo		00.00	19.82	A
Beni swafe	740bp.	13.34	13.47	1.010
Salambo		12.89	13.01	1.009

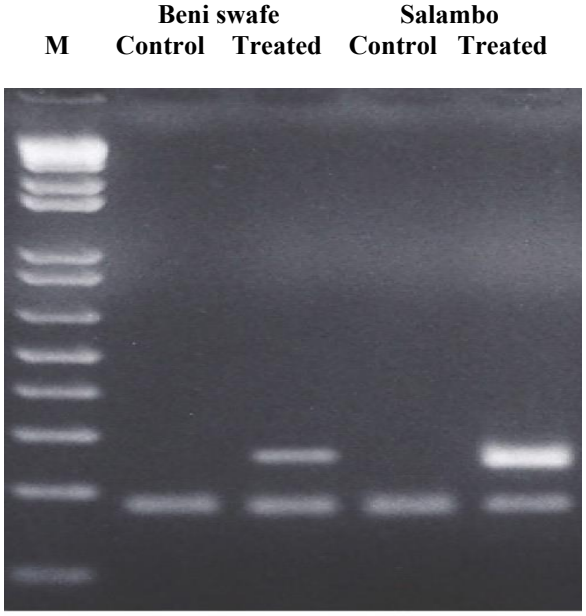


Figure (9): Quantification of calcium dependent protein kinase gene expression using RT-PCR in two wheat cultivars under *B. subtilis* inoculation and non inoculated plants

II-b) Phosphoenol Pyruvate Carboxylase (PEPCs)

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of Phosphoenol Pyruvate Carboxylase (PEPCs) was performed as described previously. The products of RT-PCR were resolved in 1.4% agarose / TBE gel. Bands with length of 320 bp were resulted in the four samples with different

intensities. The results were recorded in Table (9) and Figure (10). The results showed that the gene expression of the gene conferring phosphoenol pyruvate carboxilase (PEPCs) increased in the inoculated plants as compared to non inoculated ones in both cultivars Beni swafe and Salambo. The results also showed that the increase in gene expression of the gene conferring phosphoenol pyruvate carboxilase (PEPCs) was greater in cultivar Salambo than Beni swafe, reflecting a kind of higher response for *B. subtilis* inoculation in Salambo cultivar than Beni swafe. Quantification of the gene expression of this gene using semi quantitative RT-PCR protocol revealed that, in Beni swafe cultivar the gene expression increased from 6.17pg/µl in non inoculated plants to 15.62pg/µl in the inoculated ones, (2.532 times control). On the other hand, in cultivar Salambo the gene expression increased from 6.83pg/µl in non inoculated plants to 21.15pg/µl in inoculated ones, (3.101 times control). These results indicated that B. subtilis inoculation stimulates the photosynthesis in form of the electron transmitter compounds which their synthesis conferred by phosphoenol pyruvate carboxilase (PEPCs). That's finally reflected on the rate of photosynthesis and thus plant growth. The results were in agreement with the findings of **(Vance and Gantt, 1992),** they previously described phosphoenol pyruvate carboxilase (PEPCs) to be induced in nodules, and a corresponding gene is found here to be transcriptionally activated. A carbonic anhydrase gene (MtC00156), is highly up-

regulated at early and late symbiotic stages, as already documented **(Coba de la Pena *et al*., 1997; Galvez *et al*., 2000)**, which could related to the control of osmolarity.

Table (9): Quantification of phosphoenol pyruvate carboxilase (PEPCs) gene expression in two wheat cultivars under *B. subtilis* inoculation and non inoculated plants

Cultivars	Non inoculated	inoculated	Times of increasing
Beni swafe	6.17	15.62	2.532
Salambo	6.82	21.15	3.101

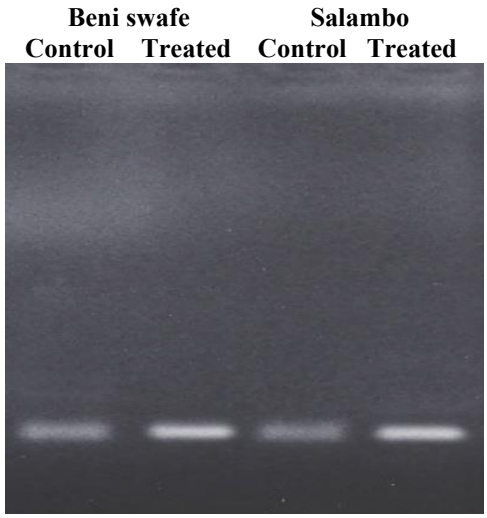


Figure (10): Quantification of phosphoenol pyruvate carboxilase (PEPCs) gene expression using RT-PCR in two wheat cultivars under *B. subtilis* inoculation and non inoculated plants.

II-c) Proline-5 Carboxylate Synthetase (P-5CS):

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of Proline-5 Carboxylate Synthetase (P-5CS) was performed as described previously. The products of RT-PCR were resolved in 1.4% agarose / TBE gel. One band with length of 570 bp was resulted. This band was presented in the four samples with different intensities as response for *B. subtilis*

inoculation as well as, it produced in the inoculated plants of the two cultivars with higher but intensities than in the non inoculated ones. The results were recorded in Table (10) and Figure (11). The results indicated that the gene expression of the gene conferring proline-5 Carboxylate Synthetase (P-5CS) increased in the inoculated plants as compared to non inoculated ones in both cultivars Beni swafe and Salambo. The results also showed that the increase in gene expression of the gene conferring Proline-5 Carboxylate Synthetase (P-5CS) was greater in cultivar Salambo than Beni swafe, reflecting a kind of higher response for *B. subtilis* inoculation in Salambo cultivar than Beni swafe. Quantification of the gene expression of this gene using semi quantitative RT-PCR protocol revealed that, in Beni swafe cultivar the gene expression increased from 8.75 pg/µl in non inoculated plants to 17.83 pg/µl in the inoculated ones, (2.038 times control). On the other hand, in cultivar Salambo the gene expression increased from 7.11 pg/µl in non inoculated plants to 23.87 pg/µl in inoculated ones, (3.357 times control). These results indicated that *B. subtilis* inoculation stimulates the bio-synthesis pass way of proline in form of P5CS which conferring the production of proline. That's finally reflected on the plant tolerance for abiotic stresses. The results were in agreement with the findings of **Waller *et al*. (2004)** report on the potential of *P. indica* to induce

resistance to fungal diseases and tolerance to salt stress in the monocotyledonous plant barley. The beneficial effect on the defense status is detected in distal leaves, demonstrating a systemic induction of resistance by a root-endophytic fungus. The systemically altered "defense readiness" is associated with an elevated antioxidative capacity due to an activation of the glutathione ascorbate cycle and results in an overall increase in grain yield. They concluded that the fungus could be exploited to increase salt tolerance and yield in crop plants.

Ouziad *et al*. (2005) reported that quantitative Real-Time RT-PCR-experiments performed with Lemt2, LeNramp1 and LeNramp3 largely corroborated the Northern analysis data. In situ hybridization experiments with Lemt2 and LeNramp1 showed that both genes were strongly expressed throughout the plant cells in non-colonized roots, whereas colonized roots revealed only few signals restricted to some parenchyma cells.

Baltruschat *et al*. (2008) investigated the root colonization with endophytic basidiomycete Piriformospora indica they reported that colonization caused an increase in resistance against biotic stress and tolerance to abiotic stress in many plants. Biochemical mechanisms underlying P. indica-mediated salt tolerance were studied in barley, *Hordeum vulgare* with special focus on antioxidants. Physiological markers for salt stress, such as metabolic

activity, fatty acid composition, lipid peroxidation, ascorbate concentration and activities of catalase, ascorbate peroxidase, dehydroascorbate reductase, monodehydro-ascorbate reductase and glutathione reductase enzymes were assessed. They reported that root colonization by P. indica increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux and fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid. The endophyte significantly elevated the amount of ascorbic acid and increased the activities of antioxidant enzymes in barley roots under salt stress conditions. Likewise, a sustained up-regulation of the antioxidative system was demonstrated in NaCl-treated roots of the salt-tolerant barley cultivar California Mariout, irrespective of plant colonization by P. indica. These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

De Melo Pereira *et al.* (2012) used a multiphasic approach, characterized by the simultaneous use of culture-dependent and culture-independent methods, to investigate endophytic bacterial communities in strawberry (*Fragaria ananassa*) fruit. They extracted the nucleic acids from the strawberry fruit and subjected to 16S rRNA gene directed polymerase chain reaction denaturing gradient gel electrophoresis (16S rRNA PCR-DGGE). The species *B. subtilis*, Enterobacter sp., and Pseudomonas sp. were detected

both by isolation and DGGE. The DGGE fingerprints of total bacterial DNA did not exhibit bands corresponding to several of the representative species isolated in the extinction dilution (L. plantarum, C. citreum, and P. punctata). In contrast, bands in the DGGE profile that were identified as relatives of Arthrobacter sp. and one uncultivable Erythrobacter sp. were not recovered by cultivation techniques. After isolation, the nitrogen fixation ability and the in vitro production of indole-3-acetic acid (IAA) equivalents and siderophores were evaluated. A high percentage of isolates were found to possess the ability to produce siderophores and IAA equivalents; however, only a few isolates belonging to the genera Pseudomonas and Enterobacter showed the ability to fix nitrogen.

Table (10): Quantification of proline5 carboxylate synthetase (P5CS) gene expression in two wheat cultivars under B. subtilis inoculation and non inoculated plants

Cultivars	Non inoculated	inoculated	Times of increasing
Beni swafe	8.75	17.83	2.038
Salambo	7.11	23.87	3.357

Beni swafe Salambo
Control Treat. Control Treat

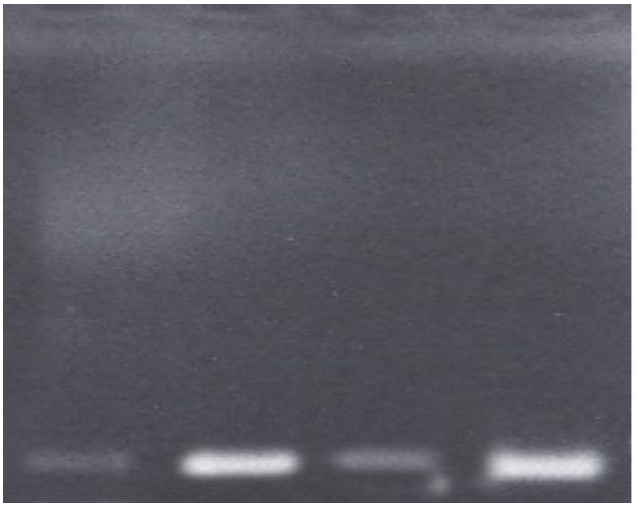


Figure (11): Quantification of proline5 carboxylate synthetase (P5CS) gene expression using RT-PCR in two wheat cultivars under *B. subtilis* inoculation and non inoculated plants.

Bacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can act as signalling compounds that are perceived by neighbouring cells within the same micro-colony, by cells of other bacteria that are present in the rhizosphere, or by root cells of the host plant **(Van Loon and Bakker 2003; Bais *et al*. 2004; Gray and Smith 2005; Kiely *et al*. 2006)**.

The best-studied example of signal exchange is the Rhizobium-legume symbiosis, in which the plant releases flavonoid compounds that act as signals for the bacterium to secrete Nod factors. Nod factors are perceived by plant root hairs and function in a hormone-like fashion to induce root nodules in which the Rhizobium bacterium can fix atmospheric nitrogen.

The bacterium grows at the expense of carbohydrates from the host, but provides fixed nitrogen for amino acid biosynthesis in return **(Brencic and Winans 2005; Gray and Smith 2005)**.

This symbiosis is a prime example of an intimate relationship between a soil bacterium and its host plant, and illustrates the concept behind the term 'plant growth promoting rhizobacteria' (PGPR): in nitrogen-poor environments the Rhizobium bacterium promotes legume plant growth by providing a limiting nutrient. Growth promotion by soil microorganisms is far from uncommon **(Glick *et al*. 1999; Ryu *et al*. 2005)** and can be considered part of a continuum in which interactions between plants and microorganisms range from deleterious (pathogens) to beneficial (PGPR).

Van Loon (2007) reported that non-pathogenic soil-borne microorganisms can promote plant growth, as well as suppress diseases. Plant growth promotion is taken to result from improved nutrient acquisition or hormonal stimulation. Several rhizobacterial strains have been shown to act as plant growth-promoting bacteria through both stimulation of growth and induced systemic resistance (ISR), but it is not clear in how far both mechanisms are connected. Such reduced disease susceptibility can be local or systemic, result from developmental or environmental factors and depend on multiple mechanisms. The spectrum of diseases to which PGPR elicited ISR confers

enhanced resistance overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal resistance of the plant that depends on the signalling compounds jasmonic acid and salicylic acid, respectively, and pathogens are differentially sensitive to the resistances activated by each of these signalling pathways. Root- colonizing Pseudomonas bacteria have been shown to alter plant gene expression in roots and leaves to different extents, indicative of recognition of one or more bacterial determinants by specific plant receptors. Conversely, plants can alter root exudation and secrete compounds that interfere with quorum sensing (QS) regulation in the bacteria. Such two-way signalling resembles the interaction of root-nodulating Rhizobia with legumes and between mycorrhizal fungi and roots of the majority of plant species. Although ISR-eliciting rhizobacteria can induce typical early defense-related responses in cell suspensions, in plants they do not necessarily activate defense-related gene expression. Instead, they appear to act through priming of effective resistance mechanisms, as reflected by earlier and stronger defense reactions once infection occurs.

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التوصيات

أولا: يعتبر نبات القمح من المحاصيل ذات الأهمية الكبيرة لغذاء الإنسان لذلك نوصى بدراسات مستفيضة على مكونات محصوله من شأنها أن تؤدى إلى زيادة المحصول و تحسين مواصفات الإنتاج و زيادة قدرة تحملة للإجهاد البيئى الحيوى و غير الحيوى.

ثانيا: التسميد بالمركبات الكيماوية و الأملاح غير أمنة على صحة الإنسان و كذلك فإنها مرتفعة التكاليف لذلك نوصى بعدم المغالاة فى إستخدام التسميد الكيميائى.

ثالثا: فى ظل الحاجة الملحة لزيادة الإنتاج لسد الفجوة الغذائية الكبيرة بين الإنتاج و الإستهلاك فلا بد من إيجاد بدائل للتسميد الكيماوى مثل التسميد الحيوى ببعض أنواع البكتيريا الجذرية مثل *B. Subtilis* و غيرها من الكائنات الحية التى تستخدم كسماد حيوى للنبات من خلال معيشتها التكافلية معه.

رابعا: من الثابت خلال الدراسة إختلاف إستجابة الأصناف تحت الدراسة للتسميد الحيوى ببكتيريا *B. Subtilis*. لذلك نوصى بدراسة التوليفات المختلفة لإختيار أفضل تركيب وراثى يعطى أعلى إستجابة للتسميد بالأسمد الحيوية لإستخدامها على المستوى الإقتصادى.

خامسا: تحتاج طبيعة العلاقة التكافلية بين النبات العائل و الكائن الحى المستخدم كسماد حيوى لمزيد من الدراسات لزيادة فهم طبيعة هذه العلاقة من أجل الوصول لمعظمة الإستفادة من الأسمدة الحيوية و مردودها على الإنتاج النباتى.

سادسا: دلت العديد من المراجع و البحوث المنشورة على تميز بكتيريا *B. Subtilis* بتنوعها الوراثى كثيرا و كذلك على تميزها بقدرة فائقة على إكساب النباتات المختله قدرة عالية على مقاومة العديد من الأمراض المختلفة و لذلك نوصى بالمزيد من الدراسة لهذة الظاهرة لفهم الميكانيكيات المختلفة لمقاومة هذة الأمراض التى يكتسبها النبات عند معاملتة ببكتيريا *B. Subtilis*.