African Journal of Microbiology Research

Volume 10 Number 15 21 April, 2016
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) is provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editors

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 15 21 April, 2016

ARTICLES

academicJournals

Vol. 10(15), pp. 486-504, 21 April, 2016
DOI: 10.5897/AJMR2015.7714
Article Number: D6694A058150
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens

Elshahat M. Ramadan[1]*, Ahmed A. AbdelHafez[1], Enas A. Hassan[1] and Fekria M. Saber[2]

[1]Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.
[2]Department of Biotechnology, Heliopolis University, Cairo, Egypt.

Received 10 August, 2015; Accepted 2 October, 2015

Growth promotion and disease control by rhizobacteria are complex interrelated processes that involve direct and indirect mechanisms. The mechanisms include synthesis of some metabolites (auxin, cytokinin and gibberellins), induction of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, production of siderophore, antibiotics, hydrogen cyanide hydrogen cyanide (HCN) and volatile compounds. They also include mineral solubilization competition, and induction of systemic resistance. These bacteria are suitable as soil inoculants because they have the potential for rapid and aggressive colonization. This feature alone is characterised as a disease control mechanism, which prevents the invasion of detrimental soil microorganisms onto the root surface. Inoculant-based plant growth-promoting rhizobacteria (PGPR) is applied extensively on agricultural crops to improve plants' growth and simultaneously reduce chemical inputs like fertilizer and pesticide which can cause environmental degradation. The structure of the rhizobacterial community is affected by several factors including plant genotype and is determined by the amount and composition of root exudates. In addition, soil type and fertility are the contributing factors that shape the community. This form of communication can affect plants' growth, nutrient status and also susceptibility to stress and pathogens in the host plant. PGPR inoculants cause diverse beneficial interactions among plants, which leads to sustainable and environment-friendly agriculture. The application of rhizosphere soil of agricultural crops with desirable bacterial populations is considered promising in both laboratory and greenhouse experiment. Further, a clearer understanding of the way PGPRs promote plants' growth can lead to expanded exploitation of these 'biofertilizers' in order to reduce the potential negative environmental effects associated with food and fiber production.

Key words: Rhizobacteria, plant growth-promoting rhizobacteria (PGPR), root microbione, phytohormones, biocontrol, soil-borne phytopathgen, fluorescent pseudomonads.

INTRODUCTION

Members of the genus *Pseudomonas* are rod-shaped Gram-negative bacteria that are characterized by metabolic versatility, aerobic respiration, motility owing to one or several polar flagella, and a high genomic G+C content (59–68%). The classification method divides all *Pseudomonas* spp. into five groups based on the relatedness of their rRNA genes, which undergo fewer changes than most other DNA sequences in the course of evolution (Von Graevenitz, 1977). Bacteria belonging to the genus *Pseudomonas* are effective root colonizers

and biocontrol agents. Growth promotion and disease control by *Pseudomonas* spp. are complex interrelated processes involving direct and indirect mechanisms that include synthesis of some metabolites (auxin, cytokinin and gibberellins), induction of ACC deaminase, production of siderophore, antibiotics, hydrogen cyanide HCN and volatile compounds. Others include mineral solubilization competition, and induced systemic resistance (Lucy et al., 2004; Adesemoye et al., 2008). These bacteria are used to improve vegetable crops yield and to reduce economic and environmental costs with mineral fertilizers (Dias et al., 2013).

This large and heterogeneous group comprises, most notably, *Pseudomonas aeruginosa*, *Pseudomonas putida, Pseudomonas fluorescens* and *Pseudomonas syringae*. They are found in soils, foliage, fresh water, sediments, and seawater (Von Graevenitz, 1977). Among PGPR, fluorescent pseudomonads are good rhizosphere colonizers, although they have also been found inside tissues of flowers and fruits (Compant et al., 2010). They possess high rhizosphere competence and they are recognized as one of the main groups of PGPR or plant-probiotic bacteria (Höfte and Altier, 2010). Whereas most studies have focused on suppression of fungi and oomycetes, others have shown that plant-pathogenic nematodes may also be suppressed by the production of various inhibitory compounds (Glick, 2010). Inoculant-based PGPRs are applied extensively on agricultural crops to improve plant growth and at the same time to reduce chemical inputs including fertilizer and pesticide which can cause environmental degradation.

The interaction or communication between plants and rhizobacteria occurs through chemical signals released by both partners. The structure of the rhizobacterial community is affected by several factors including plant genotype and is determined by the amount and composition of root exudates (Marschner et al., 2004). In addition, soil type and fertility are contributing factors that also shape the community (Innes et al., 2004). The rhizobacterial community may influence this interaction by exuding compounds as a means of communication recognized by neighbouring bacteria and root cells of host plants (Bais et al., 2004; Gray and Smith, 2005). This form of communication can affect plant growth, nutrient status and also susceptibility to stress and pathogens in the host plant (Morgan et al., 2005).

ROOT MICROBIOME

Rhizosphere is the thin layer of soil adjacent to plant roots that are influenced by root activities. This term was first introduced by Lorenz Hiltner, a soil microbiologist, in the early 1900's after years of studying the role of different plant (legumes and non-legumes) root exudates in attracting different bacterial communities surrounding the root zone. He also studied how the bacteria colonizing the root surface and epidermis influence plants' nutrient availability (Hartmann et al., 2008). Hiltner's original definition of rhizosphere now extends to the larger proportion of the soil around plant roots that are also affected by root growth and the physical, chemical and biological properties of the soil (McCully, 2005). The rhizosphere is an intense interactive zone as the root releases sugars, amino acids and other organic compounds that can be utilised by soil microorganisms, including bacteria, for their viability (Dobbelaere et al., 2003; Singh et al., 2004; Lambers et al., 2009).

This nutritious environment results in a much higher population of bacteria in the rhizosphere but lower diversity/species richness than the bulk soil (van Loon and Bakker, 2003; Lugtenberg and Kamilova, 2009). The bacteria that occupy the rhizosphere are collectively termed rhizobacteria. Rhizobacteria can have profound effects on plant health and nutrition.

Antoun and Prévost (2006) classified rhizobacteria as being neutral, deleterious or beneficial. The presence of the neutral group might be insignificant to the host plant, while deleterious rhizobacteria produce metabolites that are adverse to plant health. The concept of deleterious rhizobacteria is debatable because previous studies were mostly done in gnotobiotic and soil-less conditions without any challenge from native soil bacteria (Antoun and Prevost, 2006), and these conditions are unlikely to exist naturally. Glick et al. (1999) stated that more destructive effects on agronomically important crops are mostly caused by phytopathogenic fungi, such as *Fusarium* and *Phytium* genera; thus the negative effects of deleterious rhizobacteria on plant growth are rarely discussed in relation to this topic. The beneficial categories of rhizobacteria are able to promote plant growth and development, and are generally further grouped according to their physical interaction with the host plant (Glick et al., 1999). Beneficial rhizobacteria may form symbiotic interactions which involve modification of the morphology of the host plant root through nodule formation. Other beneficial rhizobacteria are free-living in the soil and employ associative relationships with the host plant. These free-living rhizobacteria are defined as PGPR and form associations with many different plant species (Kloepper et al., 1989).

The interaction or communication between plants and rhizobacteria occurs through chemical signals released

*Corresponding author. E-mail: elshahatramadan@hotmail.com. Tel: 020- 01223948901

by both partners. The structure of the rhizobacterial community is affected by several factors including plant genotype and is determined by the amount and composition of root exudates (Marschner et al., 2004).

Ashrafuzzaman et al. (2009) reported that PGPRs are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. Plant growth promoting rhizobacteria (PGPR) can be applied to a wide range of plant and would promote growth and disease control (Yan et al., 2003). Biocontrol effects are not only characterized by reductions of pathogen level, but also by an increase of tolerance and/or resistance, growth and yield of inoculated plants. To increase agricultural efficiency, increase in plant growth by using eco-friendly alternatives is essential for sustainable agricultural production. Thus, the use of natural processes to improve the quantity and quality of agronomics can result in development of expanded food production system, which will ultimately bring sustainability to the ecological systems (Avis et al., 2008; Berg, 2009).

Currently, there is increasing interest in the introduction of bacterial biocontrol agents for managing soil-borne pathogens, partly as a response to public concerns about deleterious effect of synthetic fungicides and also because of the lack of effective control for soil-borne pathogens (Cook, 1993; Schmiedeknecht et al., 2001). In this direction, biological control of soil-borne diseases and plant growth promotion by an application of specific microorganisms to seed or planting materials has been studied over the last years (Thomashow and Weller 1996; Kilian et al., 2000; Bochow et al., 2001; Schmiedeknecht et al., 2001; Swelim et al., 2003).

PLANT GROWTH PROMOTING RHIZOBACTERIA

This group of rhizobacteria is mostly Gram-negative and rod-shaped; a lower proportion is Gram-positive rods, cocci and pleomorphic. Examples include *Allorhizobium undicola* (de Lajudie et al., 1998a), *Azorhizobium caulinodans* (Dreyfus et al., 1988), *Bradyrhizobium japonicum* (Guerinot and Chelm, 1984), *Mesorhisobium chacoense* (Velazquez et al., 2001), *Mesorhizobium pluriforium* (de Lajudie et al., 1998b), *Rhizobium ciceri* (Nour et al., 1994), *Rhizobium etli* (Segovia et al., 1993), *Rhizobium fredii* (Scholla and Elkan, 1984), *Rhizobium galegae* (Lindstrom, 1989), *Rhizobium gallicum*, *Rhizobium giardinii* (Amarger et al., 1997), *Sinorhizobium arboris* (Nick et al., 1999), *Sinorhizobium fredii* (Chen et al., 1988) and *Sinorhizobium medicae* (Rome et al., 1996).

PGPRs were first defined by Kloepper and Schroth (1978) as root-colonizing bacteria that are beneficial for plant growth. Due to their importance in increasing seedling emergence, vigor, biomass, proliferation of root systems, and crop yield in many species, several studies have focused on identifying PGPR in natural systems and the development of these bacterial strains for commercial use (Podile and Kishore, 2006).

PGPRs inhabit the rhizosphere, the volume of soil under the immediate influence of the plant root system, and favor the establishment of a large amount of active microbial population. Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere microorganisms (Handelsman and Stabb, 1996). Rhizosphere is subject to dramatic changes, which create interactions that lead to biocontrol of diseases (Rovira, 1965, 1969, 1991; Hawes, 1991; Waisel et al., 1991). *Streptomyces* spp. have been described as rhizosphere-colonizing bacteria and antifungal biocontrol agents useful in controlling fungal root diseases, and able to work *in vitro* as producers of siderophore and plant growth-promoting hormones (Rothrock and Gottlieb, 1984; Miller et al., 1990). PGPRs are free-living bacteria that have beneficial effects on plants. PGPRs enhance emergence of seedlings, colonize roots and stimulate overall plant growth. They also improve seed germination, root development, mineral nutrition and water utilization. They can also suppress diseases of plants. The manipulation of the crop rhizosphere by inoculation with PGPR for biocontrol of plant pathogens has shown considerable promise (Handelsman and Stabb, 1996; Siddiqui and Mahmood, 1999; Berg et al., 2002; Nelson, 2004).

A diverse array of bacteria, including species of *Pseudomonas*, *Bacillus*, *Azospirillum* and *Azotobacter* has been shown to promote plant growth. The mechanism by which these rhizobacteria enhance plant growth are not clear, but it is postulated that they may produce phytohormones, suppress plant pathogens, fix nitrogen, mineralize organic phosphorus and/or enhance mineral uptake (Grayston et al., 1990; Joo et al., 2004).

The search for PGPRs and their mode of action is increasing at a rapid rate in order to use the best PGPR strains as commercial biofertilizer. Investigations into the mechanisms of plant growth promotion by PGPR strains indicated that effective PGPRs increased plant growth basically by changing the whole microbial community structure in rhizosphere (Kloepper and Schroth, 1981). According to Glick et al. (1999), the general mechanisms used for plant growth promotion by PGPR include associative nitrogen fixation, lowering of ethylene levels, production of siderophores and phytohormones, induction of pathogen resistance, solubilization of nutrients, promotion of mycorrhizal functioning, decreasing of pollutant toxicity etc. Castro et al. (2009) suggested that PGPR strains can promote plant growth and development

either directly and indirectly. Direct stimulation includes biological nitrogen fixation, producing phytohormones like auxins, cytokinins and gibberellins, solubilizing minerals like phosphorus and iron, production of siderophores and enzymes and induction of systemic resistance, while indirect stimulation is basically related to biocontrol, including antibiotic production, chelation of available Fe in the rhizosphere, synthesis of extracellular enzymes to hydrolyze the fungal cell wall and competition for niches within the rhizosphere (Zahir et al., 2004; van Loon 2007). PGPR strains, especially *P. fluorescens* and *Bacillus subtilis* are best recorded as the most promising candidates for indirect stimulation (Damayanti et al., 2007). Besides, nitrogen transformation, increasing bioavailability of phosphate, iron acquisition, exhibition of specific enzymatic activity and plant protection from harmful pathogens with the production of antibiotics can also successfully improve the quality of crops in agriculture (Spaepen et al., 2007). Thus, based on their mechanism of action, PGPRs can be categorized into three general forms: biofertilizer, phytostimulator and biopesticide. The phenomenon of quorum regulation can affect the expression of each of these traits as PGPRs are reported for their regular interactions with the resident microbial community in rhizosphere (Lugtenberg and Kamilova, 2009). PGPR may use more than one of these mechanisms to enhance plant growth as experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously (Martinez-Viveros et al., 2010).

Biochemical and molecular approaches are providing new insight into the genetic basis of these biosynthetic pathways, their regulation and importance in biological control (Joshi and Bhatt, 2011). However, to be more effective in the rhizosphere, PGPR must maintain a critical population density for a longer period, although inoculation of plants with PGPR can temporarily enhance the population size. Regarding *Pseudomonas* role in producing PGPs, Lifshitz et al. (1987) found that inoculation of canola (*Brassica compestris* seeds with a nitrogen-fixing strain of *P. putida* (GR122) drastically increased the root length of seedlings grown in sterile growth pouches. El-Khawas (1995) and Forlani et al. (1995) identified several bacteria strains of genera *Azotobacter, Azospirillum, Bacillus, Enterobacter, Klebsilla, Sarcina* and *Pseudomonas* isolated from the rhizosphere of various crops as auxins producer strains. El-Khawas et al. (2000) reported that the ability of *Pseudomonas* sp. and other isolates to produce auxins for different incubation periods was measured using spectrophotometer and determined as ug/ml minimal media supplemented with glucose and tryptophan. The amounts of auxins produced were greater after 72 h than 24 or 48 h. The amounts of auxins ranged from 33 to 75 ug/ml after 72 h.

Filamentous actinobacteria are also considered as one of the important community in rhizosphere microbiota. Garcia de Salamone et al. (2001) stated that five plant growth promoting rhizobacteria (PGPR) strains produced the cytokinin dihydrozeatin riboside (DHZR) in pure culture.Cytokinin produced by *P. fluorescens* G20-18.a rifampicin resistance mutant (RIF), and two *Tnpho*A-derived mutants (CNT1, CNT2), with reduced capacity to synthesize cytokinins, were further characterized in pure culture. G20-18 produced higher amounts of isopentenyl adenosine (IPA), trans-zeatin ribose (ZR), and DHZR than the three mutants during stationary phase. IPA was the major metabolite produced, but the proportion of ZR and DHZR accumulated by CNT1and CNT2 increased with time. No differences were observed between strain G20-18 and the mutants in the amounts of indole acetic synthesized; gibberellins were not detected in supernatants of any of the strains. Schmiedeknecht et al. (2001) studied the effect of different environmental conditions on plant growth promotion of two *B subtilis* strains. In these studies, culture solution and soil under different ecological factors were used. They pointed out that these bacterial strains may produce substances that enhance plant growth and yield of maize and sunflower.

Patten and Glick (2002) reported that indole acetic acid accumulates in the culture medium of the plant growth promoting bacterium *P. putida* Gr12-2 only when grown in the presence of oxogenous tryptophan. This suggests that the expression of indole pyruvate decarboxylase, a key enzyme in the IAA biosynthesis pathway in this bacterium, may be regulated by tryptophan. Bai et al. (2002) isolated plant growth promoting *Bacillus* sp. from surface sterilized soybean root nodules. Three isolates were found to increase soybean weight when soybean seedlings were co-inoculated with one of the isolates and *Bradyrhizobium japonicum* and / or nitrogen – free condition compared with plants inoculated with *B. japonicum*.

Bacillus is a Gram- positive aerobic organism that can resist environmental stress by forming endospores (Kumar et al., 2011); many strains of *Bacillus* and *Paenibacillus* are known to stimulate plant growth. Emmert and Handelsman (1999) highlighted the endospore forming character of *Bacillus* as important for a potential biocontrol inoculant. This is because the spore can endure heat and desiccation ensuring the formulation will be stable over time. This genus is considered non-rhizosphere competent, unlike *Pseudomonas*; but given that rhizospheric competency is strain-dependent, some strains of *Bacillus* may be rhizosphere competent (Kumar et al., 2011).

Nasr (2002) revealed that the highest levels of auxin were produced by *Bacillus cereus* and *P. fluorescens*, grown on shaker as a batch culture of 8.3 and 4.4 mg/L, respectively. Regarding the effect of different concentrations of tryptophan (TRP) and zinc (Zn) added to the culture media for maximizing the biosynthesis of

auxin, *P. fluorescens* recorded the highest amount of auxin among tested microorganisms. The higher auxin excreted was 8.3, 11.5, 10.3, and 13 mg/L on King's medium, medium supplemented with tryptophan, medium supplemented with zinc (on orbital shaker) and medium containing 0.1 mg/ml TRP + 0.001 mg/ml Zn (in fermentor), respectively.

The inoculation of *Pinus pinea* plants with plant growth promoting rhizobacteria of the genus *Bacillus* (*B. licheniformis* CECT5001 and *B*. *pumilus* ECT501s) promoted the growth of *P. pinea* seedling. This is probably caused by gibberellin production (Probanza et al., 2002). Joo et al. (2004) isolated *B. cereus* Mj-1, *B. macroides* Cj-29, and *B. pumilus* from the rhizosphere of red pepper which promoted the growth of seedlings.

Gibberellins (GAs), a well-known plant growth promoting hormone, were detected in the culture broth of their rhizobacteria. Khaled et al. (2003) noted that the inoculation of 4 cultivars of wheat with plant growth promoting rhizobacteria (*Pseudomonas* sp.) significantly increased plant height (up to 9.9%), number of tillers (up to 32.3%) and spike, spike length (up to 6.8%), straw and yields (up to 16.1, 29.0%), respectively in all tested cultivars of wheat with different degrees of efficiency. Lucas-Garcia et al. (2003) reported that the inoculation of seedling (*Capsicum annuum cv. Roxy*) with *P. fluorescans* Aur 6 as a plant growth promoting bacteria (PGPB) significantly enhanced all biometric parameters measured such as fresh weight, height, neck root diameter and slender index (height/neck root diameter). *P. fluorescans* strain Aur 6 effects could be related to auxin and siderophore production.

The colonization of plant rhizosphere by *Azospirillum* sp., *B. subtilis* sp., and *Pseudomonas* sp., has been well studied (Steenhoudt and Vanderleyden, 2000; Trivedi et al., 2005). Moreover, immobilized form of PGPR inoculants in comparison to free forms has greater ability of survival and plant root colonization. It has been reported that soil microorganisms, including free-living as well as associative and symbiotic rhizobacteria belonging to the genera like *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Proteus, Pseudomonas, Rhizobium, Serratia, Xanthomonas* in particular, are the integral parts of rhizosphere biota (Glick, 1995; Kaymak, 2011) exhibiting successful rhizosphere colonization. Lugtenberg et al. (2001) reported a large number of cell surface molecules as responsible for effective rhizosphere colonization. Rhizospheric colonization is thus considered as a crucial step in the application of microorganisms for beneficial purposes such as biofertilization, phyto-stimulation, biocontrol and phytoremediation; although the colonization of rhizosphere by PGPRs is not a uniform process. For example, *Kluyvera ascorbata* colonized the upper two-thirds of the surface of canola roots but no bacteria were detected around the root tips (Ma et al., 2001).

Phosphorus is one of the most essential nutrient requirements in plants. Ironically, soils may have large reservoir of total phosphorous (P) but the amounts available to plants are usually a tiny proportion of this total. This low availability of phosphorous to plants is because of the vast majority of soil P found in insoluble forms; and the plants can only absorb it in two soluble forms: mono-basic ($H_2PO_4^-$) and diabasic (HPO_4^{2-}) ions (Glass, 1989). Several phosphate solubilizing microorganisms (PSMs) are now recorded to convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons (Richardson et al., 2009) and chelation and exchange reactions (Hameeda et al., 2008). Saprophytic bacteria and fungi are reported in the chelation-mediated mechanisms (Whitelaw, 2000) for solubilizing phosphate in soil. Release of plant root exudates such as organic ligands can also alter the concentration of P in soil solution (Hinsinger, 2001).

According to Nahas (1996), phosphate solubilization takes place through various microbial processes including organic acid production and proton extrusion. In certain cases, phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al., 1999). Bacterial genera like *Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* are the most significant phosphate solubilizing bacteria (Sturz and Nowak, 2000; Sudhakar et al., 2000; Mehnaz and Lazarovits, 2006). Rhizobacteria can solubilize inorganic P sources and enhance growth and yield of crop plants. Besides, examples of some widely reported P solubilising microbial species intimately associated with a large number of agricultural crops like potato, tomato, wheat, radish, pulses etc., are *Azotobacter chroococcum*, *Bacillus circulans* and *Cladosporium herbarum* (Singh and Kapoor, 1999), *B. japonicum* (Antoun et al., 1998), *Enterobacter agglomerans* (Kim et al., 1998*), Pseudomonas chlororaphis* and *P. putida* (Cattelan et al., 1999) and *Rhizobium leguminosarum* (Chabot et al., 1998).The ability of PGPRs to solubilize mineral phosphate, therefore, has been of immense interest to agricultural microbiologists since it can enhance the availability of phosphorus for effective plant growth. PGPRs have been recorded to solubilize precipitated phosphates to plants, representing a possible mechanism of plant growth promotion under field conditions. Synthesis of organic acids by rhizosphere microorganisms could be the possible reason for solubilization of inorganic P sources (Verma et al., 2001).

Sumera et al. (2004) revealed that 12 plant growth promoting rhizobacteria *Bacillus* strains were isolated from rice. Nine isolates produced indole acetic acid

ranging from 20.0 - 90.8 mg/L. Most of the isolates showed resistance against environmental stresses like 10 - 40°C, 0.2 - 1.0 M salt concentration and 4.0 - 8.5 pH range. Inoculation with these bacterial isolates resulted in higher plant biomass, root area and total N and P contents in Tanzannian rice variety BKWPRAT 3036B under controlled condition. Concurrently, the bacterial enzyme ACC deaminase acts as an extracellular sink for plant-produced ethylene precursor, ACC. It metabolizes it into the inert byproducts ammonia and "ketobutyrate, reduces the amount of ACC available for conversion into ethylene and minimizes the stress response that is a result of increased ethylene concentration in the plant (Gamalero et al., 2009). Several direct and indirect mechanisms for growth-promotion have been documented. Direct mechanisms include nitrogen-fixation (Bashan et al., 2004), production of phytohormones such as the auxin indole-3-acetic acid (IAA), which stimulates cell growth and proliferation at low concentrations (Vessey, 2003), metabolism of the ethylene precursor 1, aminocyclepropane-1-carboxylate (ACC) through the enzyme ACC deaminase (Glick et al., 1998), and increased availability of iron through bacterial production of siderophores (Kloepper et al., 1991).

Ashrafuzzaman et al. (2009) observed that isolates PGB4, PGT1, PGT2, PGT3, PGG1 and PGG2 induced the production of IAA, whereas only PGT3 isolate was able to solubilize phosphorus. Most of the isolates resulted in a significant increase in plant height, root length, and dry matter production of shoot and root of rice seedlings. Furthermore, PGPR isolates remarkably increased seed germination of rice. Among the ten isolates, PGB4 and PGG2 were found almost equally better in all aspects such as dry matter production, plant height and root length of rice, and IAA production. Isolate PGT3 was also found to be promising in IAA production having an additional property of phosphate solubilization. Growth promoting substances are likely to be produced in large quantities by these rhizosphere microorganisms that influence indirectly the overall morphology of the plants. Recent progress in our understanding on the diversity of PGPR in the rhizosphere along with their colonization ability and mechanism of action should facilitate their application as a reliable component in the management of sustainable agricultural system (Bhattacharyya and Jha, 2012). There are some PGPRs that can exert a positive plant growth by direct mechanisms such as solubilization of nutrients, nitrogen fixation, production of growth regulators, etc., or by indirect mechanisms such as stimulation of mycorrhizae development, competitive exclusion of pathogens or removal of phytotoxic substances (Bashan and de-Bashan, 2010). However, in accordance with their degree of association with the plant root cells, PGPRs can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Martinez-Viveros et al., 2010). The ePGPRs may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex; on the other hand, iPGPRs locate generally inside the specialized nodular structures of root cells. Potential role of PGPRs in conferring resistance to water stress in tomatoes and peppers has been investigated (Mayak et al., 2004).

Fluorescent pseudomonads and species of *Bacillus* were reported with very high efficiency in host root colonization and production of growth metabolites resulting in improved strategic crop yield (Khalid et al., 2004). Plant-root interactions in rhizosphere may include root–root, root-insect and root-microbe interactions, resulting in the production of more root exudates that ultimately favour maximum microbial populations (rhizosphere engineering) in this ecologically significant region. Changes in rhizobacterial community structure have been reported with the application of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), resulting in significant alterations in plant–microbes interactions (Herschkovitz et al., 2005). However, successful root colonization and persistence of PGPRs in plant rhizosphere are required in order to exert their beneficial effect on the plant (Elliot and Lynch, 1984).

The intimacy between the plants and environment in rhizosphere is thus essential for better acquisition of water and nutrients by plants as well beneficial interactions of plants with soil-borne microorganisms (Ryan et al., 2009). According to Cardoso and Freitas (1992) the rhizosphere microbial communities are vigorously associated with the biogeochemical cycling of nutrients like C, P, N and S, removal of toxins and production of phytohormones or antibiotics etc. Rhizobacteria may depend on other microbes for nutrient sources as one microbe may convert plant exudates into a form that can be used by another microbe. Thus, rhizosphere has a versatile and dynamic ecological environment of intense plant-microbe interactions (Mayak et al., 2004) harnessing essential micro and macro-nutrients affecting plant growth; although, the process of root colonization is under the influence of various parameters such as bacterial traits, root exudates and several other biotic and abiotic factors (Benizri et al., 2001). PGPR can alter root architecture and promote plant development with the production of different phytohormones like IAA, gibberellic acid and cytokinins (Kloepper et al., 2007). Several PGPRs as well as some pathogenic, symbiotic and free living rhizobacterial species are reported to produce IAA and gibberllic acid in the rhizospheric soil and thereby play a significant role in increasing the root surface area and number of root tips in many plants (Han et al., 2005).

Recent investigations on auxin synthesizing rhizobacteria (Spaepen et al., 2007) as phytohormone

producer demonstrated that rhizobacteria can synthesize IAA from tryptophan by different pathways; although the general mechanism of auxin synthesis was basically concentrated on the tryptophan-independent pathways. Phytopathogenic bacteria rather use the indole acetamide pathway to synthesize IAA that has been implicated earlier in the tumor induction in plants. Swain et al. (2007) reported a positive effect of IAA producing strains of *B. subtilis* on *Dioscorea rotundata* L. They applied a suspension of *B. subtilis* on the surface of the plant, which resulted in an increase in the root: stem ratio as well as number of sprouts compared to the non-inoculated plants. Potentiality of *Azotobacter* spp. to produce high amount of IAA (7.3–32.8 mg/ml) in agriculture was reported by Ahamad et al. (2005). Similarly, significant shoot growths in maize and rice dwarf mutants were promoted by gibberellins-like substances excreted by *Azospirillum* spp. (Boiero et al., 2007).

Ribaudo et al. (2006) represented some of the efficient PGPR strains as the producer of different plant growth regulators. IAA-mediated ethylene production could increase root biomass, root hair number and consequently the root surface area of PGPR inoculated tomato plants. Involvement of PGPR formulated cytokinins was also observed in root initiation, cell division, cell enlargement and increase in root surface area of crop plants through enhanced formation of lateral and adventitious roots (Werner et al., 2003). It has been established that the working pathways of these phytostimulators leading to overall development in crop plants are differently regulated by catabolite repression as physiological regulator of biofilm formation (Zaied et al., 2009).

The discovery of rhizobacterial-produced volatile organic compounds (VOCs) constitutes an important mechanism for the elicitation of plant growth by rhizobacteria. Ryu et al. (2003) recorded some PGPR strains namely *B. subtilis* GB03, *B. amyloliquefaciens* IN937a and *Enterobacter cloacae* JM22 that released a blend of volatile components, particularly, 2, 3-butanediol and acetoin, which promoted growth of *Arabidopsis thaliana.* This suggests that synthesis of bioactive VOCs is a strain-specific phenomenon. Acetoin-forming enzymes have been identified earlier (Forlani et al., 1999) in certain crops like tobacco, carrot, maize and rice, although their possible functions in plants were not properly established in that period. It has now been established that the VOCs produced by the rhizobacterial strains can act as signaling molecule to mediate plant–microbe interactions as volatiles produced by PGPR colonizing roots are generated at sufficient concentrations to trigger the plant responses (Ryu et al., 2003). Farmer (2001) identified low-molecular weight plant volatiles such as terpenes, jasmonates and green leaf components as potent signal molecules for living organisms in different trophic levels. However, to acquire a clear appreciation on the mechanisms of VOCs in signaling plants to register plant defence more investigations into the volatile components in plant-rhizobacteria system should follow.

PGPRs comprise a broad range of soil bacterial taxa (Vessey, 2003; Lucy et al., 2004). Some common and well identified genera are *Azospirillum, Pseudomonas, Azotobacter*, and *Bacillus*. *Azospirillum* is a Gram negative, motile vibrio or *spirillum*, 1 µm in diameter, and is one of the most well studied genera since it is a free-living beneficial root associated bacterium (Morgan et al., 2005).The Bashan foundation, a non-profit scientific organization in Oregon, USA, has extensively studied and dedicated one of its major research programs to PGPR especially *Azospirillum*. The foundation provides a number of comprehensive papers on this particular genus, from the effective isolation and quantification methods from wheat roots, root colonization characteristics in different plant species, detailed PGP mechanisms, ecology, agricultural applications, physical and molecular studies and also the future challenges and potential use of *Azospirillum* as a commercial PGP inoculant (Bashan and Levanony, 1985; Bashan et al., 2004; Mayak et al., 2004; Bashan and de-Bashan, 2010).

Iron is abundant in the Earth's crust but most of it is in the highly insoluble from of ferric hydroxide and thus unavailable to organisms in soil solution. Some bacteria have developed iron uptake systems (Neilands and Nakamura, 1991). These systems involve a siderophore – an iron binding ligand – and an uptake protein, needed to transport iron into the cell. It has been suggested that the ability to produce specific siderophores, and/or to utilize a broad spectrum of siderophores, may contribute to the root colonizing ability of *Pseudomonas* strains. The production of siderophores that chelate, and thereby scavenge, the ferric iron in the rhizosphere, may result in growth inhibition of other microorganism whose affinity for iron is lower (Kloepper et al., 1988). Siderophore mechanisms will only be relevant under conditions of low iron availability. As soil pH decreases below 6, iron availability increases and siderophores become less effective (Neilands and Nakamura, 1991). Optimal suppression of pathogens occurred at levels between 10^{-9}-10^{-24} M. The critical level of iron at which a siderophore-producing strain of *P. putida* suppressed the growth of a fungal pathogen, *Fusarium oxysporium*, was found to be $< 10^{-16}$ M (Neilands and Nakamura, 1991). Since the synthesis of each siderophore generally requires the activity of several gene products (Mercado-Blanco et al., 2001), it is difficult to genetically engineer bacteria to produce modified siderophores. Complementation studies of siderophore-deficient mutants of *P. fluorescens* M114 indicated that at least five separate genetic loci are needed to encode the enzymes involved in the synthesis of the siderophore pseudobactin M114 (O'Sullivan et al.,

1990).

Zulfitri (2012) has shown the importance of selecting the most effective plant-microbe interactions by screening desired PGP traits and the most responsive host plant to ensure the most beneficial effects on plant growth. Sp245 culture grown with tryptophan and *L. stoechas cutting* was the most effective combination and showed comparable root growth parameters compared to commercial rooting hormone; it was superior to the control treatment. The IAA concentration and Sp245 cells contained in the immersion solution most likely contributed to the improved root growth. Peat cultures of Sp245 as possible inoculants formulation were ineffective for *L. stoechas* cutting-based propagation due to the low concentration of IAA. This study has contributed information to the application of IAA-producing rhizobacteria in plant propagation, specifically ornamental cutting production, and also has attempted to explore the possibility of reducing the synthetic root growth hormone in ornamental propagation.

ROLE OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN BIOLOGICAL CONTROL

Biological control plays an important role in suppression of soil-borne plant pathogens. It is defined as, the reduction of inoculum density in its active or dormant state, by one or more organism. Biological control and biological preparations occupy a very tiny place on the map of plant production (Harman, 1991). The rhizosphere of plants is the habitat of a community comprising many different organisms. Soil bacteria often possess traits that enable them to act as antagonists by suppressing soil-borne plant diseases, for example, by excreting antifungal metabolites that directly or indirectly support plant growth (Haas and Defago, 2005).

Rhizobacteria as biocontrol agents

Soil-borne pathogens are well known for their devastating effects on plant health and yield. For successful disease management, it is important to find the most effective and economical ways to protect the plant from various pests or diseases. The use of PGPR as inducers of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Wei et al., 1996). The use of natural PGPR strains in plant frontline defense may offer a practical way to deliver immunization. PGPRs have been reported to increase plant resistance to fungal, bacterial and viral diseases (Maurhofer et al., 1998), insects (Zehnder et al., 1997) and nematodes (Sikora, 1992). Mode of action studies has revealed that biological control by PGPR involves production of bacterial metabolites that reduce the population or activities of pathogens or deleterious rhizosphere microflora (Glick, 1995; Kloepper, 1996). These metabolites may include siderophores that bind Fe, making it less available to certain members of the native pathogenic microflora (Berthelin et al., 1991; Subba Rao, 1993).

Ghonim (1999) reported that *B. subtilis* reduced the harmful effect of *F. oxysporum*, the causative agent of tomato wilt disease. Tomato seeds treated with biocontrol agent, *B. subtilis* and sown in soil infested with *F. oxysporum* produced less infected plants compared to those treated with the pathogen only; and also had improved some growth parameters such as fresh and dry weights of shoots and roots. Kazmar et al. (2000) stated that *B. cereus* had beneficial effects on crop health including enhancement of soybean yield and nodulation, suppression of damping-off of tomato and suppression of damping-off alfalfa disease (Benizri et al., 2001), being able to influence the plant development as well to protect the plant roots against phytopathogens. Ezzat et al. (2001) studied microbial communities of the rhizosphere of peto-86, Pritchard and Super-Marmande tomato *(Lycopersicon esculentum Mill)* cultivars. The identified antifungal substances produced by rhizobacteria belonged to genera, *Bacillus, Enterobacter* and *Pseudomonas*. The most active antifungal producer was *Bacillus* sp. which was identified as *B. subtilis*. King's broth medium was the most suitable one for antifungal substances produced by *B. subtilis*.

Sarhan et al. (2001) observed that cell-free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, spore germination germ-tubes length of *F. oxysporum* LSP *lycopersici* and also fusaric acid decreased. They also found that treatment of tomato seedlings with *B. subtilis* spore suspension reduced tomato wilt disease index and fusaric acid content in tomato plants. On the other hand, treatment with *B. subtilis* spore suspension enhanced the growth parameters of tomato plants and inhibited the disruption of parenchymatous tissues of cortex of crown region of tomato seedlings. Volatile of *Stenotrophomonas*, *Serratia*, and *Bacillus* species inhibited mycelial growth of many fungi and *A. thaliana* (40 to 98%), and volatile of *Pseudomonas* species and *Burkholderia cepacia* retarded the growth to lesser extents. *Aspergillus niger* and *Fusarium* species were resistant, and *B. cepacia* and *Staphylococcus epidermidis* promoted the growth of *Rhizoctonia solani* and *A. thaliana*. Bacterial volatiles provide a new source of compounds with antibiotic and growth-promoting features (Berg et al., 2005; Vespermann et al., 2007).

Numbers of reports (Gomes et al., 2000; Sousa et al., 2008; Köberl et al., 2013; Köberl, 2013) are available on the potential of actinomycetes as plant growth-promoting agent. Actinomycetes strains like *Micromonospora* sp*.,* *Streptomyces* spp*., Streptosporangium* sp*.,* and

Thermobifida sp*.,* are recorded as best to colonize the plant rhizosphere, showing an immense potentiality as biocontrol agent against a range of root pathogenic fungi (Franco-Correa et al., 2010). Rhizosphere *streptomycetes* as potential biocontrol agent of *Fusarium* and *Armillaria* pine rot and as PGPR of *Pinus taeda* were reported (de Vasconcellos and Cardoso, 2009). Evidences are now available on actinobacteria used in the control of *R. solani and Pseudomonas solanacearum* in tomato and *Colletotrichum musae* in banana (Taechowisan et al., 2003). Soil actinomycetes are also an important source of diverse antimicrobial metabolites (Terkina et al., 2006). de Vasconcellos et al. (2010) isolated and screened antagonistic actinobacteria of *Araucaria angustifolia* rhizosphere for the production of active metabolites. The metabolites, especially, IAA and chitinase are recorded as responsible for the degradation of different complex and relatively recalcitrant organic compounds present in soil. Similar antagonistic activity of endophytic *Streptomyces griseorubiginosus* against *F. oxysporum* f. sp. *cubense* has been recorded by Cao et al. (2004).

Mode of action for the suppression of phytopathogens by PGPRs

The growth stimulation in plants by PGPR can be a direct effect of production of secondary metabolites such as auxins, IAA, cytokinins, riboflavin and vitamins (Dakora, 2003). These stimulate growth of plant organs via cell division and expansion (Campanoni et al., 2003) or by improving nutrient availability (Glick, 1995; Chabot et al., 1996; Yanni et al., 1997). They also release organic acids, which help to make available forms of nutrients (Biswas et al., 2000) and often lead to increase plant growth through uptake of water and mineral nutrients or indirectly when the rhizobia inhibits pathogens or deleterious microorganisms by producing siderophores, HCN (Vidhyasekaran and Muthamilan, 1999; Wei et al., 1996) and antibiotics (Glick, 1995) in the rhizosphere.

One of the major mechanisms postulated for the biological control of plant root diseases is the production of antimicrobial compounds by the disease control agent. Hanlon et al. (1994) revealed that *B. subtillus* inhibited phytopathogenic fungi by antibiosis mechanism; it produced a lipopeptide substance. Anjaiah et al. (1998) selected *P. aeruginosa* PNA1 from a total of 98 fluorescent pseudomonads isolated from chickpea rhizosphere in India. This strain was highly and widely effective against a number of phytopathogenic fungi and Oomycetes. Antagonism could be attributed to the production of 1-substituted phenazine, such as phenazine carboxylic acid (PCA) and ixychlororaphin (OCP). Shirifi et al. (1998) stated that the antimicrobial compound 2,4 diacetylphloroglucinol produced by fluorescent pseudomonads was used for protecting plant roots against fungal pathogens.

Anjaiah et al. (2003) reported that *P. aeruginosa* PNA1, an isolate from chickpea rhizosphere in India, protected pigeonpea and chickpea plants from *Fusarium* wilt disease, caused by *F. oxysporum* f. sp. *ciceris* and *Fusarium udum*. Inoculation with strain PNA1 significantly reduced the incidence of *Fusarium* wilt in pigenopea and chickpea on both susceptible and moderately tolerant genotypes. Root colonization of pigeonpea and chickpea showed ten-fold lower root colonization of susceptible genotypes than that of moderately tolerant genotypes. This indicates that this plant-bacteria interaction could be important for disease suppression in this plant. Strain PNA1 produced two phenazine antibiotics: phenazine-1-carboxiylic acid and oxychlororaphin, *in vitro*. It has been shown before that phenazines, mainly pyocyanin (De Vleesschauwer et al., 2006), and certain lipopeptides (massitolide) (Tran et al., 2007) are able to trigger an immune response in the plant which will lead to systemic disease resistance in leaves. Previous research has made it clear that *Pseudomonas* CMR12a produced two cyclic lipopeptides, one that is related to tolaasin, and another one related to the orfamides that are also produced by the well-known biocontrol agent, *P. fluorescens* Pf5. Phenazines and cyclic lipopeptides produced by *Pseudomonas* CMR12a are involved in biocontrol against *R. solani* on bean and cabbage (D'aes et al., 2011). Mutant analysis has revealed that phenazines can be active alone, while the two cyclic lipopeptides produced by *Pseudomonas* CMR12a act in concert and are both necessary for effective biocontrol. The various modes of action of a *B. subtilis* strain, FZB24 against phytopathogens are examined by Kilian et al. (2000), showing the role of the bacterium in plant vitality (Figure 1).

According to Cakmakci et al. (2006), soil rhizobacterial populations are capable of exerting beneficial effects on many plants like wheat, potato, maize, grasses, pea and cucumber by colonizing rhizosphere. Applications of PGPR increased the nodulation and nitrogen fixation of soya bean (*Glycine max* L. Merr.) over a wide range of root zone temperatures (RZTs). Thus, it has been established that the inoculation of PGPRs can increase nodulation, nitrogen uptake, growth and yield response of crop plants. In addition to this, employing microorganisms as co-culture in biotization is also another important area of research (Sekar and Kandavel, 2010) in recent decade.

Large numbers of PGPR strains of different bacterial classes and genera with multifunctional traits have, therefore, been described for their potent application in boosting plant activities in modern agriculture. However, it is equally important to study in detail the potentiality of this group of rhizospheric microbiota along with their mechanism of action involved in sustainable crop

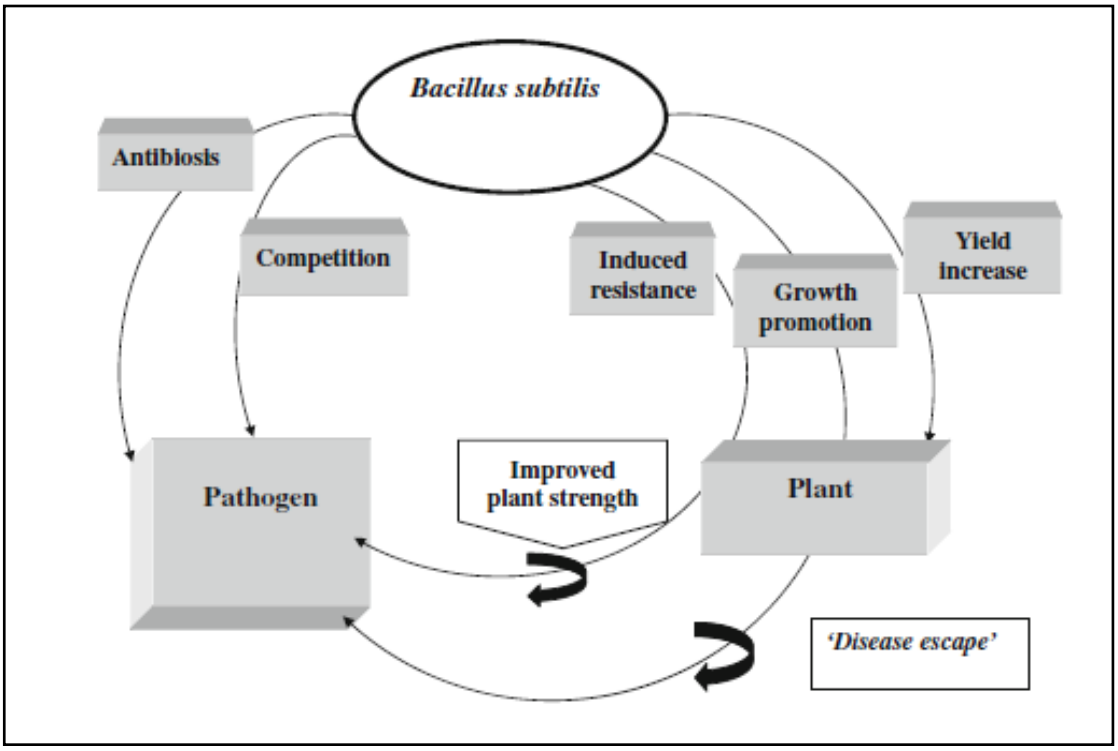

Figure 1. Modes of action of *Bacillus subtilis* strain, FZB24 promting plant growth (Adapted from Kilian et al., 2000).

production. There is need to improve the knowledge for the selection of potent microbial strains colonizing rhizosphere of growing plants for specific restoration programmes. PGPR can promote growth and yield of crop plants by direct and indirect mechanisms. In some PGPR species, plant growth promotion dominates with nitrogen fixation, phosphate solubilization and production of phytohormones like auxin and cytokinin and volatile growth stimulants such as ethylene and 2,3-butanediol (Ryu et al., 2003; Vessey, 2003).

Secretion of inhibitory substances against plant pathogens by PGPR

Production of siderophore compounds

Siderophores play an important role in the biocontrol of some soil-borne plant diseases and in plant iron nutrition (Loper and Buer, 1991). Siderophores are low molecular weight, high affinity iron (III) chelators that transport iron into bacterial cells (Leong, 1986). These systems are composed of ferric-specific ligands (siderophores) and their cognate membrane receptors as chelating agents in bacteria (Neilands, 1989). Subsequently, siderophores have been shown to be involved in the suppression of *F. oxysporum* (Baker et al., 1986). Because siderophores sequester the limited supply of iron (III) in the rhizosphere, they limit its availability to pathogens and ultimately suppress their growth (Schroth et al., 1984).There are two strategies for acquiring iron (Römheld, 1987). Strategy I is characterised by an increase in the activity of a NADPH-dependent 'reductase' and an increase in H^+ release. Strategy II is characterised by enhanced release of phytosiderophores and by a highly specific uptake system for Fe (III) phytosiderophores. Both activities are thought to enhance the solubilisation of Fe (III).

Numerous bacterial and fungal species have been shown to produce siderophores compounds. Carson et al. (2000) reported that two major types of siderophoric compounds were produced by microorganisms: hydroxamate and catechol compounds. Hydroxamate siderophores usually contain N hydroxyrnithine as the ligand involved in the chelation of iron. De Bellis and Ercolani (2001) determined rootlet elongation and bacterial growth on rootlets after inoculation of cucumber and spinach seedlings with *Pseudomonas* strains; they differ in the production of siderophores and HCN. Siderophore producers grew more profusely on cucumber. Sharma and Johri (2003) bacterized maize seeds with siderophore producing pseudomonads to

develop a system suitable for better iron uptake under iron-stressed conditions. Siderophore production was compared in fluorescent pseudomonads sp. GRP3A, PRS, and *P. chlororaphis* ATCC 9446 in standard succinate (SSM) and citrate (SCM) media. Succinate was more suitable for siderophore production; however, deferentiation of media resulted in increased siderophore production in all the strains. Maximum siderophore level (216. 23 µ g/ml) was observed in strain PRS, in SSM after 72 h of incubation. Strains GRP3A and PRS were also antagonistic against the phytopathogens, *Colletrichum dematium*, *R. solani* and *Sclerotium rolfsii*. Bacterization of maize seeds with strains GRP3A and PRS_9 showed a significant increase in germination percentage and plant growth.

Siderophore production for rhizosphere colonization has also been recorded as one of the important mechanism by certain PGPRs (*B. japonicum*, *R. leguminosarum* and Sinorhizobium meliloti) (Carson et al., 2000; El-Tarabily and Sivasithamparam, 2006) with plant growth promoting activity. Besides, iron-chelating siderophores (Schippers et al., 1988), antibiotics (Weller, 1988) and hydrogen cyanides (Stutz et al., 1986) are also likely to be produced by PGPR strains, participating tremendously in the reduction of phytopathogens and deleterious rhizobacteria with a corresponding improvement in plant health. However, regardless of the mechanism of plant growth promotion, PGPR must colonize the rhizosphere or root itself (Glick, 1995).

Production of hydrogen cyanide

Thomashow and Weller (1996) found that biocontrol mechanisms of bacteria, such as certain *Pseudomonas* strains, were usually based on secreted bioactive factors that attack the pathogen, e.g. antibiotics, exoenzymes, or HCN. Dekkers et al. (2000) showed that phenazine-1-carboxamide (oxychlororaphin, or OCP), a phenazine produced by *P. chlororaphis* PCL1391, suppressed tomato root rot caused by *F. oxysporum f. sp. radicis-lycopersici*. Lugtenberg et al. (2001) revealed that fluorescent pseudomonads frequently have been considered effective biological control agents against soil-borne plant pathogens because of their rapid and aggressive colonization of plant roots. They added that competition for nutrients in the rhizosphere at preferred colonization sites was one mechanism, while others include the production of metabolites, such as antibiotics, siderophores, and hydrogen cyanide. Kremer and Souissi (2001) reported that rhizobacteria strains were characterized by the ability to synthesize hydrogen cyanide and having effects on seedling root growth of various plants. They found that approximately 32% of bacteria from a collection of over 2000 isolates were cyanogenic, evolving HCN from trace concentrations to >30 nmoles/mg cellular protein. Cyanogensis was predominantly associated with pseudomonads and was enhanced when glycine was provided in the culture medium.

Production of antibiotics

In many biocontrol systems, one or more antibiotics have been shown to play a role in disease suppression. Molecular tools have been effective here, because mutants defective in antibiotic production are easily obtained, and in vitro assays are useful tests. The most widely studied group of rhizospheric bacteria with respect to the production of antibiotics is that of the fluorescent pseudomonads. The first antibiotics described as being implicated in biocontrol were phenazine derivatives produced by fluorescent pseudomonads (Weller and Cook, 1983). Their role has been elucidated by transposon insertion mutations which result in a defect in production of phenazine-1-carboxylate, thus reducing disease suppressive activity (Pierson and Pierson, 1996).

The genes encoding the enzymes responsible for synthesis of the metabolites have been isolated and their regulation studied (Bangera and Thomashow, 1996; Pierson et al., 1995). Global regulatory elements have been shown to coordinate the production of these metabolites (Pierson et al., 1994). The presence of other bacteria can influence phenazine production by *P. aureofaciens*, since mutants cannot be produced by other (related) rhizosphere inhabitants (Pierson and Pierson, 1996; Wood and Pierson, 1996). Also, other environmental sensors such as the regulatory proteins GacA and ApdA can influence the production of secondary metabolites involved in pseudomonads biocontrol (Corbell and Loper, 1995; Haas et al., 2002). In addition, sigma factors are important for $sigma^{70}$ and the stress-related $sigma^{S}$ have critical roles in the production of antibiotic metabolites in disease suppression (Schnider et al., 1995). Paul and Banerjee (1986) mentioned that soluble antibiotics produced by *Streprtomyces galbus* could inhibit spore germination of *Alternaria solani, A. niger, Curvularia pallescense* and *Helminthosporium oryzae.*

Antibiotic production is one of the most intensively studied aspects of biocontrol, but in many cases it is difficult to distinguish between antibiosis and competition. Several studies have demonstrated that production of antibiotics (Pyrrolnitrin, phycocyanin, 2,4-diacetylphloroglucinol) by microbial inoculants can cause suppression of pathogens (Subba Rao, 1993; Glick, 1995). Glick (1995) was of the view that the most effective mechanism that a PGPR can employ to prevent proliferation of phytopathogens is the synthesis of antibiotics.

Streptomyces lydicus WYEC108 showed strong *in vitro*

antagonism against various fungal plant pathogens in plate assays by producing extracellular antifungal metabolites. When *Pythium ultimum* or *R. solani* was grown in liquid medium with *S. lydicus* WYEC108, inhibition of growth of the fungi was observed. When *S. lydicus* WYEC108 spores or mycelia were used to coat pea seeds, the seeds were protected from invasion by *P. ultimum* in an oospore-enriched soil. While 100% of uncoated control seeds were infected by *P. ultimum* within 48 h after planting, less than 40% of coated seeds were infected. When the coated seeds were planted in soil 24 h prior to introduction of the pathogen, 96 h later, less than 30% of the germinating seeds were infected. Plant growth chamber studies were also carried out to test for plant growth effects and suppression by *S. lydicus* WYEC108 of *Pythium* seed rot and root rot. When *S. lydicus* WYEC108 was applied as a spore-peatmoss-sand formulation (108 CFU/g) to *P. ultimum*-infested sterile or nonsterile soil planted with pea and cotton seeds, significant increases in average plant stand, plant length, and plant weight were observed in both cases compared with untreated control plants grown in similar soils. *S. lydicus* WYEC108 hyphae colonized and were able to migrate downward with the root as it elongated**.** The potential of microbial antagonism was explored in the control of sugar beet disease caused by *Fusarium*. *In vitro* studies showed that 70% concentration of the culture filtrate of *Streptomyces aureofaciens* significantly inhibited the spore germination, mycelial growth and sporulation of *Fusarium solan*i. The studies *in vivo* involved different treatments: seed coating treatment was the most effective in controlling *F. solani* at all cultivation periods in all the three-sugarbeet cultivars Raspoly, TOP and Tribel. The former cultivar showed the highest growth response compared to the other two cultivars. Soil pre-inoculation was less effective whereas seed-soaking treatment was the least effective in this respect (Moussa and Rizk, 2002).

Streptomyces spp., isolated from the rhizosphere soils of various crops, were screened by dual culture and cell free culture filtrate techniques against *F. oxysporum* f.sp. *dianthi* and *F. oxysporum* f.sp. *gladioli*, causing wilt in carnation (*Dianthus caryophyllus*) and gladiolus (*Gladiolus hortulanus*), respectively. Results indicated that *Streptomyces* sp. isolate CAAC-Banuri exerted maximum inhibition against *F. oxysporum* f.sp. *dianthi* and GLAC-Kotli was highly effective in inhibiting the growth of *F. oxysporum* f. sp. *gladioli* (Shanmugam et al., 2004). The culture filtrate and crude extract from *S. aureofaciens* CMUAc130 were all inhibitory to *C. musae* and *F. oxysporum*. The culture filtrate and crude extract from this strain were all inhibitory to tested phytopathogenic fungi. The major active ingredients from the culture filtrate of *S. aureofaciens* CMUAc130 were purified by silica gel-column chromatography and identified to be (i) 5,7-dimethoxy-4-*p*-20 methoxylphenylcoumarin and (ii) 5,7-dimethoxy-4-phenylcoumarin by NMR and mass-spectral data, respectively. Bioassay studies showed that compounds (i) and (ii) had antifungal activities against tested fungi, and their minimum inhibitory concentrations were found to be 120 and 150 µg ml^{-1}, respectively (Thongchai et al., 2005). Cao et al. (2005) also studied the controlling of *F. oxysporium* f.sp *cubense in vitro* of banana plants grown in pots by *Streprtomyces* sp.

Plant hormone production

Plant growth hormones are organic compounds that influence the physiological processes in plants at extremely low concentrations. Production of phytohormones by inoculants has been suggested as one of the most plausible mechanisms of action affecting plant growth. There are five classes of well-known phytohormones, namely auxins, IAA, cytokinins, ethylene and abscisic acid. Soil microbiotas, particularly the rhizosphere microflora, are potential sources of these phytohormones (Frankenberger and Arshad, 1995; Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Arshad and Frankenberger, 1998). Plant growth regulators help to solublise nutrients so that they can easily be taken up by plant via activating the roots and stimulating cell division of root tissues. Solubilisation of nutrients such as phosphorus and iron by rhizobia makes them more readily available for plant uptake, as demonstrated by Belimov et al. (1995), Noel et al. (1996), Glick et al. (1998) and Biswas et al. (2000). They suggested that production of organic acids was the major mechanism of action by which insoluble phosphorus compounds were converted to more soluble forms. Other scientists reported that rhizobia can create an acidic environment to promote mineral nutrient solubilisation (Alexander, 1977). The rhizobia influence crop growth and development by changing the physiological status (Glick and Bashan, 1997) and morphological characteristics of inoculated roots (Noel et al., 1996; Yanni et al., 1997), which favours improved nutrient uptake (Okon and Kapulnik, 1986). The ability of rhizobia to solubilise both inorganic and organic phosphate has been the subject of many investigations (Abd-Alla, 1994; Martin et al., 2002).

Other potential mechanisms

Other mechanisms for biological control of disease may include competition for infection sites and nutrients, parasitism on pathogens, that is, destruction of fungal pathogens by the action of lytic enzymes (e.g. chitinase and ß-1, 3-glucanase) that degrade fungal cell walls, and uncharacterised antifungal factors (Fridlender et al., 1993;

Kloepper, 1996). Buchenauer (1998) reported various mechanisms for biological control such as competition for space and nutrients in the rhizosphere and spermosphere, lytic enzymes, HCN and many other metabolites produced by rhizobia. A consortium of PGPR may often have more influence on biological control and plant growth than a single strain (Krishnamurthy and Gnanamanickam, 1998; Bapat and Shah, 2000). However, in some cases, mixtures of different strains had no synergistic effect. Recent work on the broad spectrum of PGPR-mediated induced systemic resistance against different pathogens in different crops has gained importance (Ramamoorthy et al., 2001).

FLUORESCENT PSEUDOMONADS

The genus *Pseudomonas*, firstly described by Migula in 1894, is characterized as straight or slightly bent Gram-negative rods with one or more polar flagella, not forming spores. Its metabolism is chemoorganotrophic and strictly aerobic with a respiratory type in which oxygen is used (Fuchs et al., 2001). *Pseudomonas* is an aerobic Gram negative, fast growing, competitive root colonist, and is commonly found in the rhizosphere (Weller, 2007). Lugtenberg and Dekkers (1999) reviewed molecular based studies on identifying traits responsible for effective colonization of *Pseudomonas* by screening impaired mutants on different plants, then comparing their colonization ability with the wild type. The authors noted that slow growth and an inability to biosynthesize essential amino acids are among factors affecting the rhizosphere competence of PGPR. Kumar et al. (2011) found that effective root colonization and survival in the presence of indigenous soil inhabitants determine the rhizospheric competency of a PGPR. Some *Pseudomonas* strains have been shown to improve plant growth by releasing a wide range of antifungal metabolites that suppress the growth of pathogens of agronomically important crops in both laboratory and field trials (Haas and Keel, 2003). Amein et al. (2008) reported that a strain of *P. fluorescens* provided consistent protection to field grown winter wheat seedlings from blight disease over two growing seasons. A considerable increase in plant survival rate and yield was also reported. *Pseudomonas* is the largest of the groups, and includes both fluorescent and non-fluorescent ones. The most important fluorescent species are *P. aeruginosa*, *P. fluorescens*, *P. putida* and plant pathogenic species (*P. syringae*) (Scarpellini et al., 2004). Several species of rRNA group I pseudomonads have the ability to produce and excrete, under condition of iron limitation, soluble yellow green pigments that fluorescence under UV light named pyoverdines (PVDs) or pseudobactins, which act as siderophores for these bacteria (Meyer, 2000). These molecules are thought to be associated with biocontrol of fungal pathogens in the biosphere (Fuchs et al., 2001).

The abundance of literature on genus *Pseudomonas* is due to their elevated metabolic versatility capable of utilizing a wide range of simple and complex organic compounds and holding an important position in biosphere ecology (Scarpellini et al., 2004). Consequently, they are isolated from a variety of natural sources including soil, plants and mineral waters and from clinical specimens and they are characterized by a high level of metabolic diversity (Moore et al., 1996). Often, they are able to survive and multiply in poor nutrient conditions. Fluorescent pseudomonads have been considered as an important bioinoculants due to their innate potential to produce plant growth promoting hormones and antimicrobial secondary metabolites (Costa et al., 2006; Dong and Zhang, 2005).

Fluorescent pseudomonads are considered to be the most promising group of plant growth promoting rhizobacteria involved in biocontrol of plant diseases (Gardner et al., 1984; Moeinzadeh et al., 2010). They produce secondary metabolites such as antibiotics (Keel et al., 1992), Phytohormones (Keel et al., 1992), Volatile compound Hydrogen Cyanide (HCN) and siderophores (Defago and Haas, 1990). Plant growth-promoting ability of these bacteria is mainly because of the production of IAA (Patten and Glick, 2002), siderophores (Schippers et al., 1987) and antibiotics (Colyer and Mount, 1984). Production of antibiotics such as phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, phenazine-1-carboxylic acid, 2,4-diacetylphloro-glucinol, viscosinamide and tensin in different species of pseudomonads has been reported (Sunish Kumar et al., 2005). Production of siderophores has also been linked to the disease suppressing ability of certain fluorescent *Pseudomonas* species (Loper and Buyer, 1991). The control of *Phytophthora* root rot of soybean (Lifshitz et al., 1987), tobacco black root rot (Keel et al., 1989), fungal diseases of orange, lemon citrus roots (Gardner et al., 1984), and ornamental plants (Yuen and Schorth, 1986) has been demonstrated with fluorescent pseudomonads. A soil isolate CV6 was identified according to chemotaxonomic characterizations as well as 16S rDNA gene sequence analysis. The possible growth-promoting and biocontrol potential of the fore mentioned strain has been investigated by determining the secondary metabolites, viz. IAA, siderophore, and HCN production. Mezaache-Aichour et al. (2012) isolated fluorescent Pseudomonads bacteria from rhizosphere of potato plants in Algeria and identified it as *Ps. chlororaphis* and found that this isolate was capable of inhibiting the growth of phytopathogenic fungi *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *albedinis*, *F. solani* and *R. solani* and the oomycete *P. ultimum*. Extracts of supernatants from liquid cultures of this *Ps. chlororaphis* isolate completely inhibited these organisms when incorporated into potato dextrose agar

at a rate equivalent to 0.31 ml culture filtrate/ml, or greater.

Conflict of interest

The authors have not declared any conflict of interests.

REFERENCES

Abd-Alla MH (1994). Use of organic phosphorus by *Rhizobium leguminosarum*bv. *viciae* phosphatises. Biol. Fertil. Soils 8: 216-218.

Ahamad F, Ahmad I, Saghir KM (2005). Indole acetic acid production by the indigenous isolates of Azatobacter and fluorescent pseudomonas in the presence and absence of tryptophan. Turk. J. Bio. 29: 29-34.

Adesemoye AO, Obini M, Ugoji EO (2008). Comparison of plant growth-promotion with *Pseudomonas aeruginosa* and *Bacillus subtilis* in three vegetables. Braz. J. Microbiol. 39:423-426.

Alexander M (1977). "Introduction to Soil Microbiology" (2nd ed.). Wiley, New York, USA. P 467.

Amarger N, Macheret V, Laguerre G (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. 47(4):996-1006

Amein T, Omer Z, Welch C (2008). Application and evaluation of *Pseudomonas* strains for biocontrol of wheat seedling blight. Crop Prot. 27: 532-536.

Anjaiah V, Koedam N, Thompson BN, Loper JE, Höft M, Tambong JT, Cornelis P (1998). Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5-derivatives towards *Fusarium* sp. and *Pythium* sp. Mol. Plant- Microbe Interact. 11: 847-854.

Anjaiah V, Cornelis P, Koedam N (2003). Effect of genotype and root colonization in biological control of *fusarium* wilts in pigeonpea and chickpea by *Pseudomonas aeruginosa* PNA1. Can. J. Microbiol., 49:85-91.

Antoun H, Prevost D (2006). Ecology of plant growth promoting rhizobacteria. In: SIDDIQUI, Z. A. (ed.) PGPR: Biocontrol and Biofertilization. Springer Netherlands.

Antoun H, Beauchamp CJ, Goussard N, Chabot R, Lalande R (1998). Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (*Raphanus sativus* L.). Plant Soil 204:57–67.

Arshad M, Frankenberger WT (1998). Plant growth regulating substances in the rhizosphere: Microbial production and functions. Adv. Agron. 62: 46-151.

Ashrafuzzaman M, Hossen FA, Razi Ismail M, Anamul Hoque MD, Zahurul Islam M, Shahidullah SM, Meon S (2009). Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. Afr. J. Biotechnol. 8(7): 1247-1252.

Avis TJ, Gravel V, Antoun H, Tweddell RJ (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil. Biol. Biochem. 40:1733-1740.

Bai Y, D'Aoust F, Smith DL, Driscoll BT (2002). Isolation of plant growth promoting *Bacillus* strains from soybean root nodules. Can. J. Microbiol. 48: 230-238.

Bais H, Park PSW, Weir TL, Callaway RM, Vivanco JM (2004). How plants communicate using the underground information superhighway. Trends Plant Sci. 9: 26-32

Baker PA, Weisheek PJ, Schippers B (1986). The role of siderophores in plant growth stimulation by *fluorescent Pseudomonas* sp. Med. Fac. Landboucow Rijksumiv. Gent. 51(31): 1357-1362.

Bangera MG, Thomashow LS (1996). Characterization of a genomic locus required for synthesis of the antibiotic 2-4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. Mol. Plant Microbe Interact. 9: 83-90.

Bapat S, Shah AK (2000). Biological contact of *Fusarium* wilt of pigeon pea by *Bacillus* brevis on wheat. Can. J. Microb. 46: 125-132.

Bashan Y, Levanony H (1985). An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can. J. Microbiol. 31: 947952.

Bashan Y, de-Bashan LE (2010). Chapter two - How the plant growth-promoting bacterium *Azospirillum* promotes plant growth a critical assessment. Adv. Agron. 108:77–113.

Bashan Y, Holguin G, de-Bashan LE (2004). *Azospirillum* plant relationships: Physiological, molecular, agricultural, and environmental advances (1997-2003). Can. J. Microbiol. 50: 521-577.

Belimov AA, Kojemiakov AP, Chuvarliyeva CV (1995). Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. Plant Soil 173: 29-37.

Benizri E, Baudoin E, Guckert A (2001). Root colonization by inoculated plant growth-promoting rhzobacteria. Biocontrol Sci. Technol. 11: 557-574.

Berg G (2009). Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 84: 11-18.

Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A, Hallmann J (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiol. Ecol. 51: 215-2291.

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl. Environ. Microbiol. 68:3328-3338.

Berthelin J, Leyrol C, Laheurt F, Degiudici P (1991). Some considerations on the relations between phosphate solubilizing rhizobacteria and their effect on seedling and plant growth related to phosphorus solubilizing. In "Growth Promoting Rhizobacteria: Progress and Prospects" (C. Keel, B. Koller, and G. Defago, Eds.), IOBC, Switzerland. pp. 359-364.

Bhattacharyya PN, Jha DK (2012).Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327-1350.

Biswas JC, Ladha JK Dazzo FB (2000). Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci. Soc. Am. J. 64:1644-1650.

Bochow H, El-Sayed SF, Junge H, Stavropoulou A, Schmiedeknecht G (2001). Use of *Bacillus subtilis* as biocontrol agent. IV Salt-stress tolerance induction by *Bacillus subtilis* FZB 24 seed treatment in tropical vegetables field crops, and its mode of action. Z. PfiKramkh. PflSchuez 108: 21-30.

Boiero L, Perrig D, Masciarelli O, Penna C, Cassan F, Luna V (2007). Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl. Microbiol. Biotechnol. 74:874–880.

Buchenauer H (1998). Biological control of soil-borne diseases by rhizobacteria [Review]. Z. Pflanzenk, Pflanzens. 105: 329-348.

Cakmakci R, Donmez F, Aydin A, Sahin F (2006). Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol. Biochem. 38(6):1482–1487.

Campanoni P, Blasius B, Nick P (2003). Auxin transport synchronizes the pattern of cell division in a tobacco cell line. Plant Phys. 133: 1251-1260.

Cao J, Jiang W, He H (2005). Induced resistance in Yali Pear (*Pyrus bertschneideri* Rehd.) fruit against infection by *Penicillium expensum* by postharvest infiltration of acibenzolar-s-methyl. J. Phytopathol. 153:640-646.

Cao L, Qiu Z, Dai X, Tan H, Lin Y, Zhou S (2004). Isolation of endophytic actinomycetes from roots and leaves of banana (*Musa acuminata*) plants and their activities against *Fusarium oxysporum* f. sp. *cubense*. World J. Microbiol. Biotechnol. 20:501–504.

Cardoso EJBN, and Freitas SS (1992). A rizosfera. In: Cardoso EJBN, Tsai SM, Neves PCP (eds) Microbiologia do solo. Sociedade Brasileira de Ciencia do Solo, Campinas. pp. 41–57.

Carson KC, Meyer JM, Dilworth MJ (2000). Hydroxamate siderophores of root nodule bacteria. Soil Biol. Biochem. 32:11–21.

Castro RO, Cornejo HAC, Rodriguez LM, Bucio JL (2009). The role of

microbial signals in plant growth and development. Plant Sig. Behav 4(8):701–712.
Cattelan AJ, Hartel PG, Fuhrmann JJ (1999). Screening for plant growth-promoting rhizobacteria to promote early soybean growth. Soil Sci. Soc. Am. J. 63:1670–1680
Chabot R, Beauchamp CJ, Kloepper JW, Antoun H (1998). Effect of phosphorus on root colonization and growth promotion of maize by bioluminescent mutants of phosphate-solubilizing *Rhizobium leguminosarum* biovar phaseoli. Soil Biol. Biochem. 30:1615-1618.
Chabot R, Antoun H, Kloepper JW, Beauchamp CJ (1996). Root colonization of maize and lettuce by bioluminescent *Rhizobium leguminosaurm* biovar phaseoli. Appl. Environ. Microbiol. 62: 2767-2772.
Chen WX, Yan GH, Li JL (1988). Numerical taxonomic study of fast growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. 38(4):392–397.
Colyer PD, Mount MS (1984). Bacterization of potatoes with *Pseudomonas putida* and its influence on post harvest soft rot diseases. Plant Dis. 68: 703-706.
Cook RJ (1993). Making greater use of introduced micro-organisms for biological control of plant pathogens. Ann. Rev. Phytopathol. 31: 53-80.
Compant S, Clément C, Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42: 669-678.
Corbell N, Loper JE (1995). Aglobal regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 177: 6230-6236
Costa R, Gomes NCM, Peixoto RS, Rumjanek N, Berg G, MendonçaHagler LCS, Smalla K (2006). Diversity and antagonistic potential of *Pseudomonas* spp. associated to the rhizosphere of maize grown in a subtropical organic farm. Soil Biol. Biochem. 38: 2434-2447.
Costacurta A, Vanderleyden J (1995). Synthesis of phytohormones by plant-associated bacteria. Crit. Rev. Microb. 21: 1-18.
D'aes J, Gia KHH, De Maeyer K, Pannecoucque J, Forrez I, Ongena M, Dietrich LEP, Thomashow LS, Mavrodi DV, Hofte M (2011). Biological control of *Rhizoctonia* root rot on bean by phenazine and cyclic lipopeptide-producing *Pseudomonas* CMR12a. Phytopathology 101:996-1004.
Dakora FD (2003). Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. New Phytol. 158: 39–49.
Damayanti TA, Pardede H, Mubarik NR (2007). Utilization of root colonizing bacteria to protect hot-pepper against *Tobacco Mosaic* Tobamovirus. Hayati J. Biosci. 14(3): 105-109.
De Bellis P, Ercolani GL (2001). Growth interactions during bacterial colonization of seedling rootlets. Appl. Environ. Microbiol. 67: 1945-1948.
de Lajudie P, Laurent-Fulele E, Willems A (1998a). *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Bacteriol. 48:1277–1290
de Lajudie P, Willems A, Nick G, Moreira F (1998b). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. Int. J. Syst. Bacteriol. 48:369–382.
de Vasconcellos RLF, Cardoso EJBN (2009). Rhizospheric streptomycetes as potential biocontrol agents of *Fusarium* and *Armillaria* pine rot and as PGPR for *Pinus taeda*. Biocontrol 54:807-816.
de Vasconcellos RLF, da Silva MCP, Ribeiro CM, Cardoso EJBN (2010). Isolation and screening for plant growth-promoting (PGP) actinobacteria from *Araucaria angustifolia* rhizosphere soil. Sci. Agric. 67:743-746.
De Vleesschauwer D, Cornelis P, Höfte M (2006). Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to Magnaporthe grisea but enhances *Rhizoctonia solani* susceptibility in rice. Mol. Plant Microbe Interact. 19(12): 1406-1419.
Defago G, Haas D (1990). Pseudomonads as antagonists of soil borne plant pathogens: Mode of action and genetic analysis. Soil Biochem. 6: 249-291.
Dekkers LC, Mulders IHM, Phoelich CC, Chin-A-Woemg TFC, Wijfjes AHM, Lugtennberg BJJ (2000). The colonization gene of the tomato *Fusarium oxysporum* f. sp. Radicis- lycopersici biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. Bacteria. Mol. Plant-Microbe Interact. 13: 1177-1183.
Dias A, Santos SG, Vasconcelos VGS, Radl V, Xavier GR, Rumjanek NG, Ribeiro RL (2013). Screening of plant growth promoting rhizobacteria for the development of vegetable crops inoculants. Afr. J. Microbiol. Res. 7(19):2087-2092.
Dobbelaere S, Vanderleyden J, Okon Y (2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22: 107-149.
Dong YH, Zhang LH (2005). Quorum sensing and quorum-quenching enzymes. J. Microbiol. 43: 101-109.
Dreyfus B, Garcia JL, Gillis M (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. 38:89-98.
El-Khawas HM (1995). Indole acetic acid production by natural soil microresidents. Egypt. J. Appl. Sci., 10:575-582.
El-Khawas H.M, Ibrahim IA, Anwar HM, Hegazi NA (2000). Isolation and characterization of plant growth promoting rhizobacteria producing indole-3-acetic acid from plants growing in Egypt. J. Agric. Sci. Mansoura Univ. 25: 6493-6502.
Elliot LF, Lynch JM (1984). *Pseudomonads* as a factor in the growth of winter wheat (*Triticum aestivum* L.) Soil Biol. Biochem. 16:69-71.
El-Tarabily KA, Sivasithamparam K (2006). Non-streptomycete actinomycetes as biocontrol agent of soil-borne fungal plant pathogens and as plant growth promoters. Soil Biol. Biochem. 38:1505-1520.
Emmert EA, Handelsman J (1999). Biocontrol of plant diseases: A (Gram-) positive perspective. FEMS Microbiol. Lett. 171: 1–9.
Ezzat SM, Sarhan MM, Tohamy MRA, El-Essawy AA (2001). Isolation, optimization and characterization of an antifungal substance from *Bacillus subtilis* against *Fusarium oxysporum* f. sp. *Lycopersici*. Egypt. J. Microbiol. 36: 191-209.
Farmer EE (2001). Surface-to-air signals. Nature 411:854-856.
Forlani G, Mantelli M, Branzoni M, Nielsen E, Favilli F (1995). Root colonization efficiency, plant growth promoting activity and potentially related properties associated bacteria. J. Gene Breed. 49: 343-351.
Forlani GM, Mantelli M, Nielsen E (1999). Biochemical evidence for multiple acetoin-forming enzymes in cultured plant cells. Phytochemistry 50: 255-262.
Franco-Correa M, Quintana A, Duque C, Suarez C, Rodriguez MX, Barea JM (2010). Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. Appl. Soil Ecol. 45:209-217.
Frankenberger WT, Arshad M (1995). Phytohormones in Soil: Microbial Production and Function. Dekker, New York, USA. P 503.
Fridlender M, Inbar J, Chet I (1993). Biological control of soil-borne plant pathogens by a ß-1, 3-glucanase producing *Pseudomonas cepacia*. Soil Biol., Biochem. 25: 1211-1221.
Fuchs R, Schäfer M, Geoffroy V, Meyer JM (2001). Siderotyping - A powerful tool for the characterization of pyoverdines. Curr. Trop. Med. Chem. 1:31-35.
Gamalero E, Lingua G, Berta G, Glick BR (2009). Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress. Can. J. Microbiol. 55: 512-514
Garcia de Salamone IE, Hynes RK, Nelson LM (2001). Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can. J. Microbol. 47:404-411.
Gardner JM, Chandler L, Feldman AW (1984). Growth promotion and inhibition by antibiotics producing fluorescent Pseudomonads on citrus root. Plant Soil 77:103-113.
Ghonim MI (1999). Induction of systemtic resistance against *Fusarium*

wilt in tomato by seed treatment with the biocontrol agent *Bacillus subtilis*. Bull. Fac. Agric., Cairo Univ. 50: 313-328.
Glass ADM (1989). Plant nutrition: An introduction to current concepts. Jones and Bartlett Publishers, Boston. P 234.
Glick BR (1995). The enhancement of plant growth promotion by free living bacterial. Can. J. Microb. 41: 109-117.
Glick BR (2010). Using soil bacteria to facilitate phytoremediation. Biotechnol. Adv. 28:367-374.
Glick BR, Bashan Y (1997). Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. Biotechnol. Adv.15: 353-378.
Glick BR, Patten CL, Holguin G, Penrose DM (1999). Biochemical and genetic mechanisms used by plant growth-promoting bacteria. Imperial College Press, London.
Glick BR, Penrose DM, Li JP (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190: 63-68.
Gomes RC, Semedo LTAS, Soares RMA, Alviano CS, Linhares LF, Coelho RRR (2000). Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Lett. Appl. Microbiol. 30:146-150.
Gray EJ, Smith DL (2005). Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol. Biochem. 37: 395-412.
Grayston SJ, Stephens JH, Nelson LM (1990). Field and green house studies on growth promoting of spring wheat inoculated with co-existent rhizobacteria. Second International Workshop on PGPR, Interlaken, Switzerland, pp. 88-96.
Guerinot ML, Chelm BK (1984). Isolation and expression of the *Bradyrhizobium japonicum* adenylate cyclase gene (cya) in *Escherichia coli*. J. Bacteriol. 159:1068-1071.
Gyaneshwar P, Parekh LJ, Archana G, Poole PS, Collins MD, Hutson RA, Naresh Kumar G (1999). Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. FEMS Microbiol. Lett. 171: 223-229.
Haas D, Defago G (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3:307-319.
Haas D, Keel C (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 41: 117-153.
Haas D, Keel C, Reimmann C (2002). Signal transdction in plant-beneficial rhizobacteria with biocontrol properties. Antonie Van Leeuwenhoek 81: 385-395.
Hameeda B, Harini G, Rupela OP Wani SP, Reddy G (2008). Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiol. Res. 163:234-242.
Han J, Sun L, Dong X, Cai Z, Sun X, Yang H, Wang Y, Song W (2005). Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. Syst. Appl. Microbiol. 28(1):66-76.
Handelsman J, Stabb EV (1996). Biocontrol of soil-borne plant pathogens. Plant Cell 8: 1855-1869.
Hanlon D, Rosario ML, Venema GWG, Van Sinderen D (1994). Identification of T1 pC.a noval 62 Kda McP- like protein from *Bacillus subtilis*. Microbiology 140: 1847-1856.
Harman EG (1991). Seed treatments for biological control of plant disease. Crop Prot. 10: 166-171.
Hartmann A, Rothballer M, Schmid M (2008). Lorenz Hiltner: A pioneer in rhizosphere microbial ecology and soil bacteriology research. Plant Soil 312: 7-14.
Hawes MC (1991). Living plant cells released from the root cap: A regulator of microbial populations in the rhizosphere? In The Rhizosphere and Plant Growth, D.L. Keister and P.B. Cregan, eds, Kluwer Academic Publishers, Boston, MA, pp. 51-59.
Herschkovitz Y, Lerner A, Davidov Y, Rothballer M, Hartmann A, Okon Y, Jurkevitch E (2005). Inoculation with the plant-growthpromoting rhizobacterium *Azospirillum brasilense* causes little disturbance in the rhizosphere and rhizoplane of maize (*Zea mays*). Microb. Ecol. 50(2):277-288.
Hinsinger P (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root induced chemical changes: A review. Plant Soil 237:173-195.
Höfte M, Altier N (2010). Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. Res. Microbiol. 161:464-471.
Innes L, Hobbs PJ, Bardgett RD (2004). The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. Biol. Fertil. Soils 40: 7-13.
Joo GJ, Kim YM, Lee IJ, Song KS, Rhee IK (2004). Growth promotion of red pepper plug seedlings and the production of gibberllins by *Bacillus cereus*, *Bacillus macroides* and *Bacillus Pumilus*. Biotechnol. Lett. 26: 487-491.
Joshi P, Bhatt AB (2011). Diversity and function of plant growth promoting rhizobacteria associated with wheat rhizosphere in North Himalayan region. Int. J. Environ. Sci. 1(6): 1135-1143.
Kaymak HC (2011). Potential of PGPR in Agricultural Innovations. In: Maheshwari DK (ed) Plant growth and health promoting bacteria. Microbiol monographs. Springer, Berlin. 18: 45-79.
Kazmar ER, Goodman RM, Grau CR, Johnson DW, Norrdheim EV, Undersander KJ, Handelsman J (2000). Regression analyses for evaluatging the enfluence of *Bacillus cereus* on alfalfa yield under variable disease indensity. Phytopathology 90: 657-665.
Keel C, Voisard C, Berling CH, Kadr G, Defago G (1989). Iron sufficiency, a prerequisite for the suppression of tobacco root rot by *Pseudomonas fluorescens* strain CHAO under gnotobiotic conditions. Phytopathology 79: 584-589.
Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U, Wirthner P, Haas D, Defago G (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHAO: Importance of bacterial secondary metabolite, 2,4-diacetylphoroglucinol. Mol. Plant-Microbe Interact. 5:4-13.
Khalid A, Arshad M, Zahir ZA (2004).Screening plant growthpromoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96(3):473–480.
Kilian M, Steiner U, Krebs B, Junge H, Schmiedeknecht G, Hain R (2000). FZB24 *Bacillus subtilis* mode of action of a microbial agent enhancing plant vitality. Pflanzenschutz Nachr Bayer 1: 72-93.
Kim KY, Jordan D, McDonald GA (1998). Effect of phosphatesolubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol. Fertil. Soils 26: 79-87.
Kloepper JW (1996). Biological control agents vary in specificity for host, pathogen control, ecological habitat and environmental conditions. Biol. Sci. 46: 406-409.
Kloepper JW, Schroth MN (1978).Plant grotwh-promoting rhizobacteria in radish in Proceedings of 4th International Conference on Plant Pathogenic Bacteria. Gilbert-Clarey, Tours, France, pp. 879-882.
Kloepper JW, Schroth MN (1981). Relationship of *in vitro* antibiosis of plant growth promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology 71: 1020-1024.
Kloepper JW, Gutierrez-Estrada A, Mclnroy JA (2007). Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. Can. J. Microbiol. 53(2): 159-167.
Kloepper JW, Lifshitz R, Zablotowicz R (1989). Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7: 39-44.
Kloepper JW, Zablokovicz RM, Tipping EM, Lifshitz R (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. In: The rhizosphere and plant growth. Keister, DL, and Cregan, PB. (ads) Kluwer Academic Publishers, Netherlands. pp. 315-326.
Klopper JW, Lifshitz R, Novacky A (1988). *Pseudomonas* inoculation to benefit plant production. Anim. Plant Sci. 8: 60-64.
Köberl M (2013). The microbiome of medicinal plants and its potential for biocontrol and promotion of plant growth and quality. Ph.D. Thesis, Environmental Biotech. Dept. Graz Univ. Graz, Austuria.
Köberl M, Ramadan EM, Adam M, Cardinale M, Hallmann J, Heuer H, Smalla K, Berg G (2013). *Bacillus* and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt. FEMS Microbiol.Lett. 342: 168-178.

Kremer RJ, Souissi T (2001). Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. Curr. Microbiol. 43: 182-186.
Krishnamurthy K, Gnanamanickam SS (1998). Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. Biol. Control 13: 158-165.
Kumar A, Prakash A, Johri BN (2011). *Bacillus* as PGPR in Crop Ecosystem. In: MAHESHWARI, D. K. (ed.) Bacteria in Agrobiology: Crop Ecosystems. Springer Berlin Heidelberg.
Lambers H, Mougel C, Jaillard B, Hinsinger P (2009). Plant-microbe-soil interactions in the rhizosphere: An evolutionary perspective. Plant Soil 321: 83-115.
Leong J (1986). Siderophores: Their biochemistry, and possible role in the biocontrol of plant pathogens. Ann. Rev. Phytopathol. 24: 187-209.
Lifshitz R, Kloepper JW, Mozlowski M, Simonson C, Carlson J, Tipping EM, Zaleska I (1987). Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can. J. Microbiol. 33: 390-395.
Lindstrom K (1989).*Rhizobiumgalegae*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. 39:365-367.
Loper JE, Buyer JW (1991). Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Int. 4: 5-13.
Lucas-Garcia JA, Schloter M, Durkaya T, Hartman A, GutierrezManero FJ (2003). Colonization of pepper roots by plant growth promoting *pseudomonas fluorescens* strain. Boil. Fertil. Soils 37: 381-385.
Lucy M, Reed E, Glick BR (2004). Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 86: 1-25.
Lugtenberg B, Kamilova F (2009). Plant-growth-promoting rhizobacteria. Ann. Rev. Microbiol. 63: 541-556.
Lugtenberg BJ, Dekkers L, Bloemberg GV (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu. Rev. Phytopathol. 39:461-490.
Lugtenberg BJJ, Dekkers LC (1999). What makes *Pseudomonas* bacteria rhizosphere competent? Environ. Microbiol. 1: 9-13.
Ma W, Zalec K, Glick BR (2001). Biological activity and colonization pattern of the bioluminescence-labeled plant growth-promoting bacterium *Kluyvera ascorbata* SUD165/26. FEMS Microbiol. Ecol. 35:137–144.
Marschner P, Crowley D Yang CH (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261: 199-208.
Martin L, Peix A, Mateos PF, Martinez-Molina E, Rodriguez-Barrueco C, Velazquez E (2002). Mobilization of phosphorus from soil to strawberry plants by a strain of *Rhizobiumleguminosarum*bv. *trifolii*. In First International Meeting on Microbial phosPhate Solubilization, Abstract Salamanca, Spain: Universidad de Salamanca, IRNA-CSIC.
Martinez-Viveros O, Jorquera MA, Crowley DE, Gajardo G, Mora ML (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J. Soil Sci. Plant Nutr. 10: 293-319.
Maurhofer M, Reimann C, Sacherer SP, Heebs S, Haas D, Defago G (1998). Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against necrosis virus. Phytopathology 88: 678-684.
Mayak S, Tirosh T, Glick BR (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol. Biochem. 42(6): 565-572.
McCully M (2005). The rhizosphere: The key functional unit in plant/soil/microbial interactions in the field. Implications for the understanding of allelopathic effects. Proceedings of the 4th World Congress on Allelopathy, "Establishing the Scientific Base", Wagga Wagga, New South Wales, Australia. pp. 43-49.
Mehnaz S, Lazarovits G (2006). Inoculation effects of *Pseudomonasputida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. Microb. Ecol. 51(3):326-335.
Mercado-Blanco J, van der Drift KM, Olsson PE, Thomas-Oates JE, van Loon LC, Bakker PA (2001). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. J. Bacteriol. 183: 1909-1920.
Meyer JM (2000). Pyoverdines: Pigments, siderophores and potential taxonomic markers of *fluorescent Pseudomonas* species. Arch. Microbiol. 174: 135-142.
Mezaache-Aichour S, Guechi A, Nicklin J, Drider D, Prevost H, Strange RN (2012). Isolation, identification and antimicrobial activity of pseudomonads isolated from the rhizosphere of potatoes growing in Algeria. J. Plant Pathol. 94(1):89-98.
Miller JR, Hare EW, Wu J (1990). Quantitative characterization of the vegetation red edge reflectance. I. An inverted-Gaussian reflectance model. Int. J. Rem. Sens. 11:1755-1773.
Moeinzadeh A, Sharif-Zadeh F, Ahmadzadeh M, Heidari Tajabadi F (2010). Biopriming of sunflower (*Helianthus annuus* L.) seed with *Pseudomonas fluorescens* for improvement of seed invigoration and seedling growth. Aust. J. Crop Sci. 4:564-570.
Moore ERB, Mau M, Arnscheidt A, Böttger EC, Hutson RA, Collins MD (1996).The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu strictu) and estimation of the natural intrageneric relationships. Syst. Appl. Microbiol. 19: 478-492.
Morgan JAW, Bending GD, White PJ (2005). Biological costs and benefits to plant microbe interactions in the rhizosphere. J. Exp. Bot. 56: 1729-1739.
Moussa TAA, Rizk MA (2002). Biocontrol of sugarbeet pathogen *Fusarium solani* (Mart.) Sacc.by*Streptomyces aureofaciens*. Pak. J. Biol. Sci. 5: 556-559.
Nahas E (1996). Factors determining rock phosphate solubilization by microorganisms isolated from soil. World J. Microbiol. Biotechnol. 12: 567-572.
Nasr SA (2002). Stimulation of auxin biosynthesis by some bacterial, and yeast strains, Arab Univ. J. Agric. Sci. Ain Shams Univ., Cairo, 10:89-107.
Neilands JB (1989). Siderophore systems of bacteria and fungi, Metal Ions and Bacteria (Beveridge, T. J. and Doyle, R. J., eds), pp. 141-164, Wiley, Somerset, NJ, USA.
Neilands JB, Nakamura K (1991). Detection, determination, isolation, characterization and regulation of microbial iron chelates. In CRC Handbook of Microbial Iron Chelates. G. Winkelmann. London: CRC Press.
Nelson LM (2004). Plant growth promoting rhizobacteria (PGPR): Prospects for new inoculants. Online.Crop Management doi:101094/Cm-2004-0301-05-RV.
Nick G, de Lajudie P, Eardly BD (1999). *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. Int. J. Syst. Bacteriol. 49: 1359-1368.
Noel TC, Sheng C, Yost CK, Pharis RP, MF Hynes (1996). *Rhizobium leguminosarum* as a plant growth promoting rhizobacterium: Direct growth promotion of canola and lettuce. Can. J. Microbiol. 42: 279-293.
Nour SM, Fernandez MP, Normand P, Cleyet-Marel JC (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum L*.). Int. J. Syst. Bacteriol. 44:511-522.
O'Sullivan DJ, Morris J, O'Gara F (1990). Identification of an additional ferric-siderophore uptake gene clustered with receptor, biosynthesis and fur-like regulatory genes in *fluorescent Pseudomonas* sp. Strain M114. Appl. Environ. Microbiol. 56: 2056-2064.
Okon Y, Kapulnik Y (1986). Development and functions of *Azospirillum* inoculated roots. Plant Soil 90:3-16.
Patten C, Glick B (2002). Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. Appl. Environ. Microbiol. 68:3795-3801.
Patten CL, Glick BR (1996). Bacterial biosynthesis of indole-3-acetic acid. Can. J. Microb. 42:207-220
Paul AK, Banerjee AK (1986). *In vitro* effect of antifungal antibiotic produced by *Streptomyces galbus* 5 ME-14. Hindustan Antibiot. Bull. 28: 15-19.
Pierson LS, Pierson EA (1996). Phenazine antibiotic production in *Pseudomonas aureofaciens*: Role in rhizosphere ecology and

pathogen suppression FEMS Microbiol. Lett. 136: 101-108.
Pierson LS, Gaffiney T, Lam S, Gong F (1995). Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30-84, FEMS Microbiol. Lett. 134: 299-307.
Pierson LS, Keppenne VD, Wood DW (1994).Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. J. Bacteriol. 176: 3966-3974.
Podile AR, Kishore GK (2006). Plant growth-promoting rhizobacteria. In: Plant Associated Bacteria. Gnanamanickam, SS(ed.). Springer, Dordrecht, Netherlands. pp. 195-230.
Probanza A, Garcia JAI, Palomino MR, Ramos B, Manero FJG (2002). *Pinus pinea* L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. Licheniformis* CECT 5106 and *B. pumilus* CECT 5105). Appl. Soil Ecol. 20: 75-84.
Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R (2001). Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot. 20: 1-11.
Ribaudo C, Krumpholz E, Cassan F, Bottini R, Cantore M, Cura A (2006). *Azospirillum* sp. promotes root hair development in tomato plants through a mechanism that involves ethylene. J. Plant Growth Regul. 24:175-185
Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305-339.
Rome S, Fernandez MP, Brunel B, Normand P, Cleyet-Marel JC (1996). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. Int. J. Syst. Bacteriol. 46: 972-980.
Römheld V (1987). Different strategies for iron acquisition in higher plants. Phys. Plant. 70: 231-234.
Rothrock CS, Gottlieb D (1984). Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var *geldanus* to *Rhizoctonia solani* in soil. Can. J. Microbiol. 30: 1440-1447.
Rovira AD (1965). Interactions between plant roots and soil microorganisms. Ann. Rev. Microbiol. 19: 241-266.
Rovira AD (1969). Plant root exudates. Bot. Rev. 35: 35-57.
Rovira AD (1991). Rhizosphere research-85years of progress and frustration. In: The Rhizosphere and Plant Growth, D.L. Keister and P.B. Cregan, (eds) Kluwer Academic Publishers, Boston, MA, pp. 3-13.
Ryan PR, Dessaux Y, Thomashow LS, Weller DM (2009). Rhizosphere engineering and management for sustainable agriculture. Plant Soil 321: 363-383.
Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003). Bacterial volatiles promote growth in Arabidopsis. Proc. Natl. Acad. Sci. USA 100: 4927-4932.
Sarhan MM, Ezzat SM, Tohamy MRT, El-Essawy AA, Mohamed FA (2001). Biocontrol of *Fusarium* tomato wilt disease by *Bacillus subtilis*. Egypt. J. Microbiol. 36: 103-118.
Scarpellini M, Franzetti L, Galli A (2004).Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiol.Lett. 236: 257-260.
Schippers B (1988). Biological control of pathogens with rhizobacteria. Philos. Trans. R. Soc. B-Biol. Sci. 318: 283-293.
Schippers B, Bakker AW, Bakker PAHM (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25: 339-358.
Schmiedeknecht G, Issoufou I, Junge H, Bochow H (2001). Use of *Bacillus subtilis* as biocontrol agent. V. Biological control of diseases on maize and sunflowers. J. Plant Dis. Prot. 108: 500-512.
Schnider U, Keel C, Troxier J, Defago G, Haas D (1995). Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. 177: 5387-5392.
Scholla MH, Elkan GH (1984). *Rhizobium fredii* sp. nov., a fastgrowing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. 34:484-486.
Schroth MN, Loper JE, Hildebrand DC (1984). Bacteria as biocontrol agents of plant disease.In Current Perspectives in Microbial Ecology ed. Klug, M.J. and Reddy, C.A. pp. 362-369. Washington, DC: American Society for Microbiology.
Segovia L, Young JPW, Matinez-Romero E (1993). Reclassification of American *Rhizobium leguminosarum* Biovar Phaseoli type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. 43:374-377.
Sekar S, Kandavel D (2010). Interaction of plant growth promoting rhizobacteria (PGPR) and endophytes with medicinal plants - New avenues for phytochemicals. J. Phytol. 2:91-100.
Shanmugam V, Vaidya M, Gulati A (2004). Evaluation of bio-control agents against *Fusarium* isolates infecting carnation and gladiolus. Ann. Plant Prot. Sci. 12(2): 314-320.
Sharma A, Johri BN (2003). Growth promoting influence of siderophore producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. Microbiol. Res. 158: 243-248.
Shirifi A, Zala M, Natsch A, Dejaga G (1998). Biocontrol of soil borne fungi plant diseases by 2,4 – diacetlyphloroglucinol producing *Pseudomonas fluorescence.* Eur. J. Plant Pathol. 104: 631-643.
Siddiqui ZA, Mahmood I (1999). Role of bacteria in the management of plant parasitic nematodes: A review. Bioresour. Technol. 69: 167-179.
Sikora RA (1992). Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Ann. Rev. Phytopat. 30: 245-270.
Singh BK, Millard P, Whiteley AS, Murrell JC (2004). Unravelling rhizosphere microbial interactions: Opportunities and limitations. Trends Microbiol. 12: 386-393.
Singh S, Kapoor KK (1999). Inoculation with phosphate-solubilizing microorganisms and a vesicular- arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. Biol. Fertil. Soils 28: 139-144.
Sousa CS, Soares ACF, Garrido MS (2008). Characterization of streptomycetes with potential to promote plant growth and biocontrol. Sci. Agric. 65:50-55.
Spaepen S, Vanderleyden J, Remans R (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. In: Unden F (ed) FEMS microbiol rev. Blackwell Publishing Ltd., New York. pp. 1-24.
Steenhoudt O, Vanderleyden J (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: Genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24: 487-506.
Sturz AV, Nowak J (2000). Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. J. Appl. Soil Ecol. 15: 183-190.
Stutz EW, Defago G, Kern H (1986). Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181-185
Subba Rao NS (1993). Biofertilizers in Agriculture and Forestry.Oxford and IBH Publishing Co. Pvt. Ltd, New Delhi 242 p.
Sudhakar P, Chattopadhyay GN, Gangwar SK, Ghosh JK (2000). Effect of foliar application of *Azotobacter*, *Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). J. Agric. Sci. 134:227-234.
Sumera Y, Bakar MAR, Malik KA, Hafeez FY (2004). Isolation, characterization and beneficial effects of rice associated plant growth promoting bacteria from Zanzibar soils. J. Basic Microbiol. 44: 241-252 (www CAB Abstracts 20043145747).
Sunish Kumar R, Ayyadurai N, Pandiaraja P, Reddy AV, Venkateswarlu Y, Prakash O, Sakthivel N (2005). Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broadspectrum antifungal activity andvbiofertilizing traits. J. Appl. Microbiol. 98: 145-154.
Swain MR, Naskar SK, Ray RC (2007). Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisetts by *Bacillus subtilis* isolated from culturable cowdung microflora. Pol. J. Microbiol. 56: 103-110.
Swelim MA, Amer MM, Abd El-Ghany BF, and Omer AM (2003). Role of some soil bacteria and actinomycetes in controlling cucumber root-rot disease. Egypt. J. Microbiol. 38: 217-228.
Taechowisan T, Peberdy JF, Lumyong S (2003). Isolation of endophytic actinomycetes from selected plants and their antifungal activity.

World J. Microbiol. Biotechnol. 19: 381–385.
Terkina IA, Parfenova VV, Ahn TS (2006). Antagonistic activity of actinomycetes of Lake Baikal. Appl. Biochem. Microbiol. 42: 173-176.
Thomashow LS, Weller DM (1996). Current concepts in the use of introduced bacteria for biological disease control: Mechanisms and antifungal metabolites, in: Plant-Microbe Interactions, Vol. I, G. Stacey and N. Keen, eds., Champman and Hall, New York, N.Y. pp. 187-235.
Thongchai T, Chunhua L, Yuemao S (2005). Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc 130 and their antifungal activity. J. Microbiol. 151: 1691-1695.
Tran H, Ficke A, Asiimwe T, Höfte M, Raaijmakers JM (2007). Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol. 175: 731-742.
Trivedi P, Pandey A, Palni LMS (2005). Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. World J. Microbiol. Biotechnol. 21:941-945.
van Loon LC (2007). Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol. 119:243–254.
van Loon LC, Bakker PAHM (2003). Signalling in rhizobacteria-plant interactions. In: Root ecology. H. DE KROON and E. J. W. VISSER (eds.) Berlin-Heidelberg: Springer-Verlag, Berlin Heidelberg. 287-330
Velazquez E, Igual JM, Willems A (2001). *Mesorhizobium chacoense* sp. nov.: A novel species that nodulates *Prosopis albain* the Chaco Arido region (Argentina). Int. J. Syst. Evol. Microbiol. 51: 1011-1021.
Verma SC, Ladha JK, Tripathi AK (2001). Evaluation of plant growth promoting and colonization from deep water rice. J. Biotechnol. 91: 127-141.
Vespermann A, Kai M, Piechulla B (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Appl. Environ. Microbiol. 73(17): 5639-5641.
Vessey JK (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255: 571-586.
Vidhyasekaran P, Muthamilan M (1999). Evaluation of powder formulation of *Pseudomonas fluorescens* Pf1 for control of rice sheath blight. Biocontrol Sci. Technol. 9: 67-74.
Von Graevenitz A (1977). The role of opportunistic bacteria in human disease. Ann. Rev. Microbiol. 31:447-471.
Waisel Y, Eshel A, Katkafl U (1991). Plant Roots: The Hidden Half. Marcel Dekker, New York Inc. New York. 948 p. ISBN 0-8247-8393-X.
Wei G, Kloepper JW, Tuzun S (1996). Induced systemic resistance to cucumber diseases and increased plant growth by Plant growth promoting rhizobacteria under field conditions. Phytopathology 86: 221-224.
Weller DM (1988). Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. 26:379-407.
Weller DM (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathology 97: 250-256.
Weller DM, Cook RJ (1983). Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology 73: 463-469.
Werner T, Motyka V, Laucou V, Smets R, Onckelen HV, Schmulling T (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15: 2532-2550.
Whitelaw MA (2000). Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv. Agron. 69:99-151.
Wood DW, Pierson LS (1996). The *phzI* gene of *Pseudomonas aureofaciens* (Pau) 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene 168(1): 49-53.
Yan Z, Reddy MS, Kloepper JW (2003). Survival and colonization of rhizobacteria in tomato transplant system. Can. J. Microbiol. 49: 383-389.
Yanni YG, Rizk RY, Corich V, Squartini A, Corich V, Mateos P, Ladha JK, Dazzo FB (1997). Natural endophytic association between *Rhizobium leguminosarum* bv*trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil 194: 99-114.
Yuen GY, Schroth MN (1986). Interaction of *Pseudomonas fluorescens* strains E6 with ornamental plants and its effect on the composition of root colonization microflora. Phytopathology 76: 176-179.
Zahir ZA, Muhammad A, Frankenberger WT (2004). Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Adv. Agron. 81:97-168.
Zaied KA, El-Diasty ZM, El-Rhman MMA, El-Sanossy ASO (2009). Effect of horizontal DNA transfer between *Azotobacter* strains on protein patterns of *Azotobacter* transconjugants and biochemical traits in bioinoculated Okra (*Abelmoschus esculentus*, L.). Aust. J. Basic. Appl. Sci. 3(2): 748-760.
Zehnder G, Kloepper JW, Yao C, Wei G (1997). Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria. J. Econ. Entomol. 90: 391-396.
Zulfitri A (2012). Pant growth promotion by IAA-producing rhizobacteria in ornamental plant propagation. A thesis Master of Science in Agriculture, Faculty of Agriculture and Environment, University of Sydney, New South Wales, Australia.

academicJournals

Vol. 10(15), pp. 505-510, 21 April, 2016
DOI: 10.5897/AJMR2016.7956
Article Number: DB8ECAE58164
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Asymptomatic bacteriuria: Occurrence and antibiotic susceptibility profiles among students of a tertiary institution in Ile-Ife, Nigeria

Osungunna Michael Oluwole* and Adeyemi Adenike Victoria

Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Received 8 February, 2016, Accepted 29 March, 2016

Asymptomatic bacteriuria, a form of urinary tract infections that involves isolation of a specified quantitative count of bacteria in an appropriately collected urine specimen obtained from a person without symptoms or signs referable to urinary infection is common, but the prevalence in population varies widely with age, sex and the presence of genitourinary abnormalities. This study therefore aimed at investigating the prevalence of asymptomatic bacteriuria among selected students of Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife Nigeria as well as the antibiotic resistance patterns of the bacterial isolates associated with the condition. Urine samples were collected from one hundred apparently healthy students and processed within two hours of collection. The isolates were identified by conventional biochemical tests while antibiotic susceptibility of the isolates were carried out according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. Results revealed 77% prevalence (49 females, 27 males) among the sample studied with the presence of *Staphylococcal* spp., *Klebsiella* spp., *Citrobacter* spp. and *Bacillus* spp. *Staphylococcus aureus* is the predominant species accounting for 54.5% of the isolates. Antibiotic susceptibility study revealed all the isolates to be multidrug resistant. However, resistance to ofloxacin by Gram-negative bacterial isolates was least while resistance to streptomycin by the Gram-positive isolates was least in this study. The study concluded that multidrug resistant bacteria can be associated with asymptomatic bacteriuria while the patients may not develop any sign or symptom referable to urinary tract infection.

Key words: Asymptomatic bacteriuria, urinary tract infections, genitourinary abnormalities, prevalence, multidrug resistance.

INTRODUCTION

Urinary tract infections (UTIs) have been defined as the condition in which actively-multiplying bacteria persist in the urine at any point from kidney to the urethral meatus (Perrin, 1974). Hilbert (2011), however, defined urinary tract infection as the presence of a significant level of bacteria in urine typically 10^4 to 10^6 colony forming units (CFU/mL).

*Corresponding author. E-mail: yomosun2002@yahoo.co.uk, mowole@oauife.edu.ng. Tel: +5516982135050.

Urinary tract infections (UTIs) are among the most frequent bacterial infections in human beings, affecting millions of people each year (Roos et al., 2006). They are the second most common type of infections in the body. (Stamm et al., 2001). UTI usually starts as a bladder infection but often evolves to encompass the kidneys and ultimately can result in renal failure or dissemination to the blood. Studies suggest that up to 95% of all UTIs develop by an ascending route of infection (Bacheller and Bernstein, 1997), meaning that infection begins by colonizing the periurethral area, followed by an upward progression to infect the bladder and, in some cases, continued progression of the bacteria through the ureters to infect the kidneys if conditions of infection allow. Transmission occurs in four ways; namely through sexual intercourse, from mother to the foetus via placenta, through poor personal hygiene and via communal sponge and towel usage (Onifade et al., 2011). UTIs may be with symptoms, that is, symptomatic or without symptom, that is, asymptomatic. Symptomatic UTIs can be classified as uncomplicated or complicated depending on patient comorbidities and the presence of anatomic or physiologic abnormalities that predispose to UTI (Mittal et al., 2009; Nicolle, 2008). It can also be classified into disease categories by the site of infection: Cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine) (Foxman, 2002). Paradoxically, the most frequent form of UTI is asymptomatic bacteriuria (Roos et al., 2006). Asymptomatic bacteriuria (ABU) has been defined as colonization of urine by microorganisms in the absence of clinical symptoms (Roos et al., 2006). ABU patients may carry large number of bacteria, more than 10^5 CFU/ml, for months or years without developing symptoms. This condition is benign and does not cause renal injury (Oyelami et al., 2005). Most patients with ABU do not need treatment and in many cases the colonizing organism actually helps to prevent infection by other more virulent bacteria (Hull et al., 2000; Darouiche et al., 2001). ABU occurs in up to 6% of healthy individuals and 20% of elderly individuals (Roos et al., 2006).

However, although not in all cases, there is propensity for asymptomatic bacteriuria to become symptomatic if left untreated. *Escherichia coli* has been reported as the most prominent bacteria associated with asymptomatic bacteriuria but an increased percent of *Proteus, Klebsiella, Enterococcus*, and *Pseudomonas* has also been identified (Katsarolis et al., 2010). This study therefore aimed at investigating the occurrence of asymptomatic bacteriuria among some students of Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife as well as the antibiotic susceptibility profiles of isolated organisms to commonly used antibiotics.

MATERIALS AND METHODS

After ethical approval for the study was obtained from the OAUTHC Research and Ethical Committee, urine samples were collected from one hundred (45 males and 55 females) apparently healthy students of Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria. Prior to sample collection, consent forms were given to the students. The first part contained the Biodata of the students e.g. name, sex, age, past medical history as well as history of UTI. The participating students were instructed to wash their external genital with mild toilet soap and rinse thoroughly with clean water. Thereafter, midstream urine was to be collected into the sterile specimen bottles, provided. The samples were labelled against the name of student in accordance with the supplied biodata. However, within two hours of collection, the samples were transported into the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutics for culturing. Students who were currently on antibiotics or have been on antibiotics treatments in the last three months were excluded from the study.

A loopful of the urine samples was streaked onto the surface of oven-dried Cysteine-Lactose Electrolyte Deficient (CLED) (Lab M, Bury, UK) agar and incubated at 37°C for 24 h. The colonies were counted and colony forming unit per millilitre (cfu/mL) calculated with a view to ascertaining if the growth was significant as to qualify for classification as asymptomatic bacteriuria or not.

The isolated colonies were identified by conventional biochemical tests (Barrow and Feltham, 2003).

Antibiotic susceptibility test

Susceptibility of both the Gram-negative and Gram-positive isolates to eight antimicrobial agents each was tested by the disc diffusion technique according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The Gram-negative antibiotic disc contained augmentin (30 µg); ofloxacin (5 µg); gentamycin (10 µg); nalidixic acid (30 µg); nitrofurantoin (200 µg); cotrimoxazole (25 µg); amoxycillin (25 µg) and tetracycline (25 µg) while the Gram-positive antibiotic disc contained cotrimoxazole (25 µg); cloxacillin (5 µg); erythromycin (5 µg); gentamicin (10 µg); augmentin (30 µg); streptomycin (10 µg); tetracycline (10 µg) and chloramphenicol (10 µg).

Four or five colonies of each test organism taken from a nutrient agar culture plate were inoculated into 10 ml of sterile distilled water using a sterile loop. The suspension was thoroughly mixed with a spin mixer. The resulting suspension was adjusted to a turbidity of 0.5 McFarland standard (A_{625nm} = 0.09). This was then applied to the surface of over-dried Mueller Hinton agar and spread evenly with a sterile swab stick. The inoculated plates were incubated at 37°C for 20 min for acclimatization and growth of the inocula. Antibiotic discs (Abtek, Liverpool, UK) were then lightly but firmly pressed onto the surface of the plates using a pair of sterile forceps. The plates were then refrigerated at 4°C for thirty minutes to ensure adequate diffusion of antibiotics. *E. coli* ATCC 25922 was used as control strain. All plates were incubated at 37°C for 18 h. The diameters of inhibition zones were measured in millimetres and interpreted according to CLSI manual.

RESULTS

Hundred samples were collected out of which 77 had significant growth after inoculating and incubating on the C.L.E.D agar. Of these 77 isolates, 49 (63.6%) were from the female subjects while 28 (36.4%) were from the male subjects.

In this study, four genera of bacteria were isolated. These include *Staphylococcal* spp*., Klebsiella* spp*., Citrobacter* spp*.* and *Bacillus* spp*.* The percent distribution of each spp. is as shown in Table 1.

Table 1. Percentage distribution of the isolates associated with asymptomatic urinary tract infections.

Bacterial isolates	Composition	Percentage	Total Number (n = 77)	Total percentage
Staphylococcal spp.	*S. aureus*	54.5 (N=42)	56	72.7
	S. saprophyticus	5.2 (N =4)		
	S. xylosus	11.7 (N =9)		
	S. hyicus	1.3 (N =1)		
Klebsiella spp.	*Klebsiella pneumoniae*	2.6 (N =2)	3	3.9
	Klebsiella oxytoca	1.3 (N =1)		
Bacillus spp.	*B. subtilis*	1.3 (N =1)	13	16.9
	B. flexus	11.7 (N =9)		
	B. cereus	1.3 (N =1)		
	B. licheniformis	2.6 (N =2)		
Citrobacter spp	*Citrobacter freundi*	6.5 (N = 5)	5	6.5

Table 2. Percentage distribution of resistance pattern for each gram-negative isolates to commonly used antibiotics.

Antibiotics	*Citrobacter freundi* % resistance (*n=5)*	*Klebsiella pneumoniae* % resistance (*n*=2)	*Klebsiella oxytoca* % resistance (*n*=1)
Gentamicin	100	100	100
Nalidixic Acid	100	100	100
Ofloxacin	0	0	100
Augmentin	100	100	100
Tetracycline	100	100	100
Amoxycillin	100	100	100
Cotrimoxazole	100	100	100
Nitrofurantoin	80	100	100

The percentage distribution of resistance patterns of Gram-negative bacterial isolates is shown in Table 2. The resistance to ofloxacin by all Gram-negative bacterial isolates is the least among the antibiotics used except the *Klebsiella oxytoca* that was resistant to all the antibiotics tested in the study while resistance to streptomycin is the least by Gram-positive bacterial isolates with the exception of *Staphylococcus hyicus* and *Bacillus licheniformis* that have 100% resistance to streptomycin as shown in Table 3.

DISCUSSION

Stamm and Hooton (1993) referred to UTI as a clinical (symptomatic) or subclinical (asymptomatic) disease that may involve just the lower tract or both the lower and upper tracts. “Asymptomatic bacteriuria,” or asymptomatic urinary infection, has been defined as isolation of a specified quantitative count of bacteria in an appropriately collected urine specimen obtained from a person without symptoms or signs referable to urinary infection (Rubin et al., 1992).

Asymptomatic bacteriuria is common, but the prevalence in populations varies widely with age, sex, and the presence of genitourinary abnormalities (Nicolle et al., 2005). In this study, the prevalence of asymptomatic bacteriuria in the population of students studied was 77%. This was significantly higher than those earlier reported (Onifade et al., 2011; Ngwai and Bakare, 2012; Adekunle et al., 2013; Ayoade et al., 2013). This may be attributed to unhygienic living conditions students were subjected to on campus and /or poor personal hygiene on the part of the students. In this study, more females than males (63.6% vs 36.4%) had asymptomatic bacteriuria. This, while in agreement with those earlier reported (Onifade et al., 2011; Adekunle et al., 2013) however contradicts the findings of Ngwai and Bakare (2012). This may be attributed to anatomical differences between male and female urinary tracts. For instance, the urethra of female is shorter than in male thus allowing bacteria a more ready access to the bladder. Also, the urethral opening in women is closer to sources of bacteria from the anus and the vagina (Lipsky, 1989).

Table 3. Percentage distribution of resistance pattern of each gram-positive isolates to commonly used antibiotics.

Antibiotics	*S. aureus* (n =46	*S. saprophyticus* (n=4)	*S. xyloses* (n=9)	*S. hyicus* (n=1)	*B. flexus* (n=9)	*B. subtilis* (n=1)	*B. licheniformis* (n=2)	*B. cereus* (n=1)
Gentamicin	100	100	100	100	100	100	100	100
Augmentin	100	100	100	100	100	100	100	100
Streptomycin	87.5	0	88.9	100	88.9	0	100	0
Tetracycline	100	100	100	100	100	100	100	100
Chloramphenicol	92.5	100	88.9	100	100	0	100	100
Cotrimoxazole	97.5	100	88.9	100	100	0	100	100
Cloxacillin	100	100	100	100	100	100	100	100
Erythromycin	100	100	100	100	100	100	100	100

One other reason for lower incidence in male than female is the antibacterial property of prostatic fluid (Onifade et al., 2011). However, the prevalence of bacteriuria among young women is strongly associated with sexual activity. It was 4.6% among premenopausal married women but only 0.7% among nuns of similar age (Kunin and McCormack, 1968).

Escherichia coli remains the single most common organism isolated from bacteriuric women (Kunin and McCormack, 1968; Evans et al., 1978; Bengtsson et al., 1998), although this happens proportionally less frequently than for women with acute uncomplicated urinary tract infection. Other Enterobacteriaceae (such as *Klebsiella pneumoniae*) and other organisms (including coagulase-negative staphylococci, Enterococcus species, group B streptococci, and *Gardnerella vaginalis*) are common as well. For men, coagulase-negative staphylococci are also common, in addition to gram-negative bacilli and Enterococcus species (Lipsky et al., 1984; Mims et al., 1990). In this study, four genera of bacteria were isolated. They include *Staphylococcal* spp., *Klebsiella* spp., *Bacillus* spp. and *Citrobacter* spp. Of these, *Staphylococcal* spp. was the predominant genera accounting for 72.7%. *Staphylococcus aureus*, among others as *S. saprophyticus S. hyicus* and *S. xylosus,* has the highest occurrence of 54.5%. This agrees with the report of Frank-Peterside and Wokoma (2009). The findings however contradicts the reports of Adekunle et al. (2013) and Onifade et al. (2011) where *E. coli* was reported the predominant species of bacteria isolated.

S. aureus is a common pathogen in the community and in hospitals. *S aureus* causes significant mortality and morbidity but is an infrequent cause of urinary tract infection (Demuth et al., 1979). In patients with *S. aureus* bacteremia, a positive urine culture is typically attributed to ascending infection or to hematogenous spread. Coagulase positive Staphylococci may have invaded the UTI through hematogenous routes while the coagulase negative Staphylococci may invade the urinary tract through sexual activity especially in females (Frank-Peterside and Wokoma, 2009).

It is however possible that the *S. aureus* isolated in this study may have gotten into the urinary tract by the individuals inadvertently touching their genitalia with hands that have been used to touch infected surfaces since *S. aureus* infections are spread by contact.

All the isolates in this study are multidrug resistant with many resistant to four or more antibiotics tested. This may be attributed to the isolates possessing genes that code for multidrug resistance. Some of the ways by which bacteria can develop resistance to antibiotics include: (i) Antibiotic inactivation- direct inactivation of the active antibiotic molecule (Wright, 2005); (ii) Target modification- alteration of the sensitivity to the antibiotic by modification of the target (Lambert, 2005); (iii) Efflux pumps and outer membrane (OM) permeability changes-reduction of the concentration of drug without modification of the compound itself (Kumar and Schweizer, 2005); or (iv) Target bypass- some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme. This mode of resistance is observed in many trimethoprim- and sulfonamide-resistant bacteria. This observation corroborates the postulation that healthy members of the community are the highest reservoir of antibiotic resistant bacteria (Lester et al., 1990; Lamikanra et al., 1996). Ofloxacin, a fluoroquinolone, and streptomycin, an aminoglycoside, are the drugs of choice with the least resistance by Gram-negative and Gram-positive isolates respectively in this study. Ofloxacin exerts its antibacterial effect by disrupting DNA synthesis through interference with type II topoisomerases DNA gyrase and topoisomerase IV during replication and by causing double strand breaks (Strohl, 1997). Resistance could be developed to ofloxacin by alterations in drug target enzymes and alterations that limit the permeability of the drug to the target (Hooper, 1999).

The bactericidal activity of streptomycin is attributed to the irreversible binding to the ribosomes. Resistance to streptomycin is through modification of the target sites by enzymes as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) (Shaw et al.,

1993). The activity of these enzymes is not restricted to streptomycin alone but other members of the aminoglycosides group.

Aminoglycosides modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described (Quintiliani and Courvalin, 1995).

Conclusion

The high prevalence of asymptomatic bacteriuria among apparently healthy students as obtained in this study is of public health concern and need to be addressed. The high prevalence may be attributed to poor public and personal hygiene going by the nature of the isolates in this study hence the need for public enlightenment to promote good personal hygiene and hand washing among others. Also living and environmental conditions of the students should be improved through regular sanitation and regular provision of potable water.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

The authors are grateful for the role played by Mr Salau A. O., the Principal Technologist in Pharmaceutical Microbiology Unit of the Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria in the course of this work.

REFERENCES

Adekunle OC, Olowe OA, Jagunmolu O (2013). Bacteriuria in apparently healthy individual in Osogbo. Int. J. Appl. Biol. Pharm. Technol. 4(3):56-59.

Ayoade F, Osho A, Fayemi SO, Oyejide NE, Ibikunle AA (2013). Unusually High Prevalence of Asymptomatic Bacteriuria among male University Students on Redemption Camp, Ogun State, Nigeria. Afr. J. Clin. Exp. Microbiol 14(1):19-22.

Bacheller CD, Bernstein JM (1997). Urinary tract infections. Med. Clinic North Am. 81:719-730.

Bengtsson C, Bengtsson U, Bjorkelund C, Lincoln KM, Sigurdson JA (1998). Bacteriuria in a population sample of women: 24-year follow-up study. Results from the prospective population-based study of women in Gottenburg, Sweden. Scand. J. Urol. Nephrol. 32:284-289.

CLSI (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA: Clinical and Laboratory Standards Institute.

Darouiche RO, Donovan WH, Del Terzo M, Thornby JI, Rudy DC, Hull RA (2001). Pilot trial of bacterial interference for preventing urinary tract infection. Urology 58:339-344.

Demuth PJ, Gerding DN, Crossley K (1979). *Staphylococcus aureus* bacteriuria. Arch. Int. Med. 139(1):78-80.

Evans DA, Williams DN, Laughlin LW, Miao L, Warren JW, Hennekens CH, Shimada J, Chapman WG, Rosner B, Taylor JO (1978). Bacteriuria in a population-based cohort of women. J Infect. Dis. 138(6):768-773.

Foxman B (2002) Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. Am. J. Med. 113(1):5-13.

Frank-Peterside N, Wokoma EC (2009). Prevalence of asymptomatic bacteriuria in students of University of Port Harcourt Demonstration Secondary School. J. Appl. Sci. Environ. Manage. 13(2):55-58

Hilbert DW (2011). Antibiotic Resistance in Urinary Tract Infections: Current Issues and Future Solutions, Urinary Tract Infections, Dr. Peter Tenke (Ed):,

Hooper DC (1999). Mechanisms of fluoroquinolone resistance. Drug Resist. Updates 2:38-55.

Hull R, Rudy D, Donovan W, Svanborg C, Wieser I, Stewart C, Darouiche R (2000). Urinary tract infection prophylaxis using *Escherichia coli* 83972 in spinal cord injured patients. J. Urol 163:872-877.

Katsarolis I, Poulakou G, Athanasia S, Kourea-Kremastinou J, Lambri N, Karaiskos E, Panagopoulos P, Kontopidou FV, Voutsinas D, Koratzanis G, Kanellopoulou M (2010). Acute uncomplicated cystitis: From surveillance data to a rationale for empirical treatment. Int. J. Antimicrob. Agents 35(1):62-67.

Kumar A, Schweizer HP (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake, Adv. Drug Deliv. Rev. 57:1486-1513.

Kunin CM, McCormack RC (1968). An epidemiologic study of bacteriuria and blood pressure among nuns and working women. N Eng. J. Med. 278:635-642.

Lambert PA (2005). Bacterial resistance to antibiotics: Modified target sites, Adv. Drug Deliv. Rev. 57:1471-1485.

Lamikanra A, Ako-Nai, Oguniyi DA (1996). Transferable antibiotic resistance in *Escherichia coli* isolated from healthy Nigerian children. pp. 59-64

Lester SC, Pla MD, Wang F, Schael IP, Jiang H, O'Brien TF (1990). The carriage of *Escherichia coli* resistant to antimicrobial agents by healthy children in Boston, Caracas, Venezuela and in Qin Pu, China. New Engl. J. Med. 323(5):285-289

Lipsky BA, Inui TS, Plorde JJ, Berger RE (1984). Is the clean-catch midstream void procedure necessary for obtaining urine culture specimens from men. Am. J. Med. 76:257 62.

Mims AD, Norman DC, Yamamura RH, Yoshikawa TT (1990). Clinically inapparent (asymptomatic) bacteriuria in ambulatory elderly men: Epidemiological, clinical, and microbiological findings. J Am. Geriatr. Soc. 38:1209-1214.

Mittal R, Aggarwal R, Sharma S, Chhibber S, Harjaib K (2009). Urinary tract infections caused by *Pseudomonas aeruginosa*: A mini review. J. Infect. Public Health 2:101-111.

Ngwai YB, Bakare OR (2012). Symptomatic bacteriuria and antimicrobial susceptibility of *Staphylococcus aureus f*rom University Students in Keffi, Nigeria. Int. J. Microbiol. Bioinform. 2(1):5-8.

Nicolle LE (2008). Uncomplicated urinary tract infection in adults including uncomplicated pyelonephritis. Urol. Clin. North Am. 35:1-12.

Onifade AK, Anibijuwon II, Azariah EJ (2011). Urinary tract infection in apparently healthy individuals in Ile-Ife, Nigeria: Detection of predominant microorganisms and antibiotics susceptibility profile. Afr. J. Microbiol. Res. 5(20):3233-3236.

Oyelami OA, Agbakwuru EA, Adeyemi LA, Adedeji GB (2005). The Effectiveness of Grapefruit (*Citrus paradisi*) Seeds in Treating Urinary Tract Infections. J. Altern. Complement. Med. 11(2):369-371

Perrin EC (1974). Urinary Tract Infections in Children. J. Postgrad. Gen. Pract. Update 9(11):1471-1478.

Quintiliani R, Courvalin P (1995). Mechanisms of resistance to antimicrobial agents, In Manual of Clinical Microbiology, (ed) Murray PR, Baron EJ, Pfaller MA, Tenover FR, Yolken RH. pp. 1308-1326.

Roos V, Nielsen EM, Klemm P (2006). A symptomatic bacteriuria *Escherichia coli* strains: Adhesins, growth and competition. FEMS Microbiol Lett 262:22-30.

Rubin RH, Shapiro ED, Andriole VT, Davis RJ, Stamm WE (1992). Evaluation of new anti-infective drugs for the treatment of urinary tract infection. Clin. Infect. Dis. 15(Suppl 1):S216-27.

Shaw KJ, Rather PN, Have RS, Miller GM (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside modifying enzymes. Microbiol. Rev. 57:138-163.
Stamm WE, Norby SR (2001). UTI: Diagnosis, Panorama and Challenges. J. Infect. Dis. 183(1):S1-S4.
Strohl WR (1997). Biotechnology of Antibiotics, Marcel Dekker Inc., New York, USA.
Svanborg C, Godaly G (1997);. Bacterial virulence in urinary tract infection. Infect. Dis. Clin. North Am. 11:513-530.
Wright GD (2005). Bacterial resistance to antibiotics: Enzymatic degradation and modification. Adv. Drug Deliv. Rev. 57:1451-1470.

academicJournals

Vol. 10(15), pp. 511-520, 21 April, 2016
DOI: 10.5897/AJMR2016. 7917
Article Number: 7B52CDC58166
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

An overview of food safety knowledge and practices in selected schools in the city of Al Ain, United Arab Emirates

Aisha Abushelaibi*, Baboucarr Jobe, Fatima Al Dhanhani, Sharifa Al Mansoori and Feryal Al Shamsi

Department of Food Science, College of Food and Agriculture, United Arab Emirates University. P. O. Box 15551 Al Ain, United Arab Emirates.

Received 11 January, 2016; Accepted 15 March, 2016

Safe food handling in school canteens is an important practice to protect the students from food borne illnesses. The main purpose of this study was to present an overview of food safety knowledge and hygienic practices in selected schools in Al Ain city in the Emirate of Abu Dhabi, United Arab Emirates. The study consisted of three parts. The first part tackled students' and teachers' behavior during breakfast snack in addition to a quick assessment of premises from a food safety point of view. The second part focused on food safety knowledge and practices among students and teachers while the third part dealt with assessment of microbiological contamination of food contact surfaces and hands of students, teachers and food handlers. Results showed that only four school out of eight (50%) keep foods at 5°C or below. With respect to cleanliness of refrigerators, six out of eight schools visited (75%) do not clean their refrigerators enough. For personal hygiene, it was observed that 37.5% of respondents do not wash their hands properly after using toilet. This behavior may expose students to bacterial food poisoning such as salmonella and staphylococcus, which result into vomiting and severe abdominal pain. For the second part, results showed that 60.3% of respondents eat their meal in school playground. Utilization of playground for eating and physical activity simultaneously may expose the area to more dust, which serves as a potential source of contamination. For the microbiological evaluation of surfaces and hands of students and food handlers, the study revealed that although the general microbiological conditions were normal, the presence of *Escherichia coli* and *Staphylococcus aureus* on hands of students, food handlers and even teachers was worrying. In survey questionnaire to assess food safety knowledge, 87% of the participants expressed their appreciation of the importance of food safety.

Key words: Food Safety, personal hygiene, *Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

According to the World Health Organization, 1.8 million people died from diarrheal diseases in 2005 alone and most of the cases were associated with ingestion of contaminated food and drinking water (WHO, 2007). Since food is one of the primary sources of disease transfer, meals prepared and supplied in schools require great attention because the intended users are young children who are more susceptible to food-borne illnesses

with limited choices (Soares et al., 2012). Aziz and Dahan (2013) described school children as captive consumers who are usually not able to buy food from external sources during school hours. Despite the greater care needed, several food-borne disease outbreaks in schools continue to be reported (Nicholas et al., 2002). Marzano and Balzaretti (2013) reported a Salmonella outbreak which occurred in schools in France, involving 544 adolescents (<20 years old). Further investigations identified frozen beef burger as the cause of the outbreak. Marzano and Balzaretti (2013) also reported an outbreak in Germany involving more than 11200 students. The outbreak was described as one of the largest foodborne gastroenteritis in Germany with several hundred schools affected. Laboratory analysis on patients showed that many of the victims were infected with noroviruses where the source of the norovirus was identified in deep-frozen Chinese strawberries (Robert Koch Institute, 2012). From epidemiological point of view, there are several causes of food borne disease outbreaks. Su et al. (2005) reviewed the food borne disease outbreaks due to bacteria in Taiwan from 1995 to 2001 and found that most common bacteria involved were *Vibrio parahemolyticus, Staphylococcus aureus,* and *Bacillus cereus*. Paulson (2000) and Green et al. (2006) reported that hygiene of food workers could be a critical factor in spreading food-borne illnesses in the food service environments. Therefore, in order to minimize the chance of food borne disease occurrences, Gibson et al. (2002) reported that hygienic food preparation and education of those involved in the preparation, processing and serving of meals are crucial. In the kitchen, cutting boards may cause cross-contamination if not adequately sanitized (Sneed et al., 2004; Staskel et al., 2007). The sink drain area of the dishwashing sink, the hand-washing sink, spout handles and the handle of garbage lid are also among the most common sources of bacterial contamination (Staskel et al., 2007). From the literature reviewed, it can been seen that food safety handling practices at various establishments including schools have been well studied in many places, however in the United Arab Emirates, no such study has been done to the best of our knowledge. For this reason, the first objective of this study was to generate a general idea about food safety handling and practices in schools of the city of Al Ain in the Emirate of Abu Dhabi; while the second objective was to use this information as a base to promote food safety attitudes in the school system in the Emirate of Abu Dhabi in future.

MATERIALS AND METHODS

Eight public schools from the city of Al Ain were randomly selected for the survey. After selection of schools, a letter of consent was written to each school administration seeking for approval to participate in the study. The chosen schools provide foods in the form of snacks to their students such as cheese croissants, chips, chocolate and juice. These foods are usually served between 8 to 9 am. The foods are considered low risk foods. However, if not handled properly, they can pose considerable risk to consumers. For this reason, the assessment was made specifically for personal hygiene of students, teachers, handlers and serving conditions in which foods are handled before delivering to consumers.

Design of the study

The design of the study consisted of three sections. The first section was to observe food safety behavior of school children, teachers and workers handling food in addition to the assessment of general conditions of school environment. The second section was designed to evaluate food safety knowledge and practices among students and teachers in the selected schools. Finally, the third section dealt with the assessment of microbiological contamination of food contact surfaces and hands of students, teachers and food handlers.

Observation of food safety behavior in schools

Here, observations were made about food safety practices and personal hygiene adopted by students and teachers during their breakfast snack. Conditions of facilities were also observed. In this part, four observable practices were used to evaluate temperature time control during storage and serving food. Seven elements were directed to personal hygiene of food handlers and students. Two elements evaluated cafeteria design while utensils used for serving food were evaluated in two practices. Pest control method being applied was evaluated in three observable practices. The list of observable points is detailed in Table 1.

Assessment of food safety knowledge and practices

Based on the recommended food handling practices, a set of questionnaire was prepared and administered in selected schools by a team of three female students. The questionnaire consisted of demographic information, interest in acquiring food safety knowledge, attitude towards hand washing and adopted food-handling practices. The details of questionnaire are shown in Tables 2, 3 and 4.

Microbiological assessments

The microbiological cleanliness of hands of teachers, students and other workers along with surfaces such as those of tables and windows was evaluated by swabbing as described in Yousef and Carlstrom (2003).

Media preparation

Phosphate buffer saline (PBS), egg yolk tellurite, violet red bile agar and MUG supplements were manufactured by Oxoid, (USA). Plate count agar, potato dextrose agar and Baird Parker agar were

*Corresponding author. E-mail: a.abushelaibi@uaeu.ac.ae. Tel: +97137136565. Fax +971 - 3 -713-6902.

Table 1. Food safety and hygienic practices observed in the selected schools.

Observed practice	Yes	No	% of Yes	% of No
Temperature time control (a)				
Cold food held at 5°C or below	4.0	4.0	50.0	50.0
Refrigerator freed from dirt	2.0	6.0	25.0	75.0
Food stored in proper containers	5.0	3.0	62.5	37.5
Food is stored at least 6 inches off the floor	4.0	4.0	50.0	50.0
Personal hygiene of workers (b)				
Hands washed after use of the toilet	40.0	24.0	62.5	37.5
Workers wear clean and appropriate uniform	24.0	40.0	37.5	62.5
Gloves/utensils used/changed as needed	24.0	40.0	37.5	62.5
Worker observed washing hands as needed	32.0	32.0	50.0	50.0
Students wash their hands before and after meal	40.0	24.0	62.5	37.5
Worker take appropriate action when coughing or sneezing	40.0	24.0	62.5	37.5
Perfect conditions of hygiene, cleanliness and organization: With suitable products for personal hygiene	32.0	32.0	50.0	50.0
Cafeteria design (C)				
Suitable localization: Area free of unsanitary condition; absence of trash, old objects, pets, insects, animals, rodents.	4.0	4.0	50.0	50.0
Suitable door, windows: Smooth surface ,impermeable ,washable ,easy to clean	4.0	4.0	50.0	50.0
Services (d)			*0.0*	*0.0*
Dishes are dry and clean	4.0	4.0	50.0	50.0
Smooth surface, easy to clean and disinfect and properly maintained in good working order	3.0	5.0	37.5	62.5
Pest control (e)				
Screens are on open windows and doors in good condition	5.0	3.0	62.5	37.5
Evidence of presence of pest	3.0	5.0	37.5	62.5
Foods protected against waste, spit ,insects and rodents	5.0	3.0	62.5	37.5

Table 2. Eating place, time and source of information of food safety knowledge.

Food safety knowledge	Score	%
Where are you eating your meal? n =146		
Class	23	15.8
School ground	88	60.3
Cafeteria	13	8.9
Teachers room	22	15.1
Total	146	100.0
When you eat your meal? n =146		
8-9 AM	16	11.0
9-10 Am	28	19.2
10-11 AM	102	69.9
Total	146	100.0
Have you ever read an article, magazines, or books on food safety? n = 153		
Yes	122	79.7
No	31	20.3
Total	153	100.0
Have you ever been suffered from food poisoning? n=149		
Yes	40	26.8
No	109	73.2
Total	149	100.0

Table 3. Hand washing attitude of students and teachers.

Hand washing attitude	Score	%
Do you wash your hands after coming out from toilet?		
Yes	143	96.0
No	1	0.7
Sometimes	4	2.7
Often	1	0.7
Total	149	100.0
Do you wash your hand with water only		
Yes	22	14.8
No	96	64.4
Sometimes	30	20.1
Often	1	0.7
Total	149	100.0
Do you wash your hand with water and soap		
Yes	125	83.9
No	3	2.0
Sometimes	20	13.4
Often	1	0.7
Total	149	100.0

Table 4. Food safety knowledge of students, teachers and sellers.

Food safety knowledge	Score	%
Meal should not be left more than 4 h at room temperature		
Accept	100	74.6
Do not Accept	8	6.0
I do not know	26	19.4
Total	134	100.0
Insects and cockroaches are food contaminants.		
Accept	140	95.2
Do not accept	5	3.4
I do not know	2	1.4
Total	147	100.0
Meal handling practices		
6-7 AM	3	6.1
7-8 AM	4	8.2
8-9 AM	42	85.7
Total	49	100.0
Where do you store the meal?		
Cafeteria	32	56.1
Refrigerator	24	42.1
Closet	1	1.8
Total	57	100.0
Do you wear gloves in the process of selling?		
Yes	10	16.7
No	47	78.3
Sometimes	3	5.0
Total	60	100.0

Table 4. Contd.

What action do you take on remaining meal?		
Throw it	32	65.3
Give it to students	13	26.5
Store it for next day	4	8.2
Total	49	100.0
Response of students and teachers to the descriptive questions		
What are the causes of food poisoning?		
Uncovered food	5	6.3
Uncooked food	3	3.8
Expired food	10	12.7
Unclean tools	5	6.3
Bad storage	11	13.9
Personal hygiene	6	7.6
Contaminated food	25	31.6
Bacteria and microorganisms	13	16.5
No food control	1	1.3
Total	79	100.0
Comments about meals		
Clean	44	86.3
Unclean	7	13.7
Total	51	100.0
Comments about serving window		
Clean	21	46.7
Unclean	24	53.3
Total	45	100.0

supplied by HiMedia India. All media were prepared as per manufacturer's instructions and sterilized at 121°C for 15 min.

Swabbing

By using sterilized cleansers, swabs were collected from the target surfaces and hands of students, teachers and food handlers serving foods. Length and width of the surfaces were measured by using scale. Test tubes were transported to laboratory in a cooler and immediately analyzed upon arrival.

Aerobic plate count

Aerobic plate counts (APC), yeast and mold count, and *S. aureus* determination were carried out according to the method outlined in (Yousef and Carlstrom, 2003). Colonies developed in the petri dish were counted by using colony counter and expressed as CFU/cm^2. *Escherichia coli* was investigated by the most probable number (MPN) technique. Tubes showing gas production in EC broth were streaked on Eosin Methylene blue (EMB) agar to confirm the presence of *E. coli*, which appears bluish with greenish metallic sheen on EMB plates.

RESULTS AND DISCUSSION

Description of the study population

One hundred and forty-seven respondents participated in the study. The respondents lived in the city of Al Ain in the Emirate of Abu Dhabi and their age groups are shown in Figure 1. As shown in Figure 1, the majority of the population under study is between 16 and 19 years of age representing 48%. The second largest group falls between 12 and 15 years while 11% of them fall in the range of 20 to 25 years and only 8% of them are above 26 years of age.

Figure 2 shows educational profile of the study group. From the figure, it can be seen that most of the participants are secondary school students consisting 52% of the population. The primary school students represent 31% of the population while the remaining 17% consisted bachelors, diploma holders and postgraduate qualification holders representing teachers and workers.

Observation of food safety behavior in schools

Eight schools were randomly selected and approached. After the approval of administration, three female observers who were graduating students from the Department of Food Science, College of Food and Agriculture, United Arab Emirates University visited the schools for evaluating food safety behavior of students and teachers. The observers also inspected general

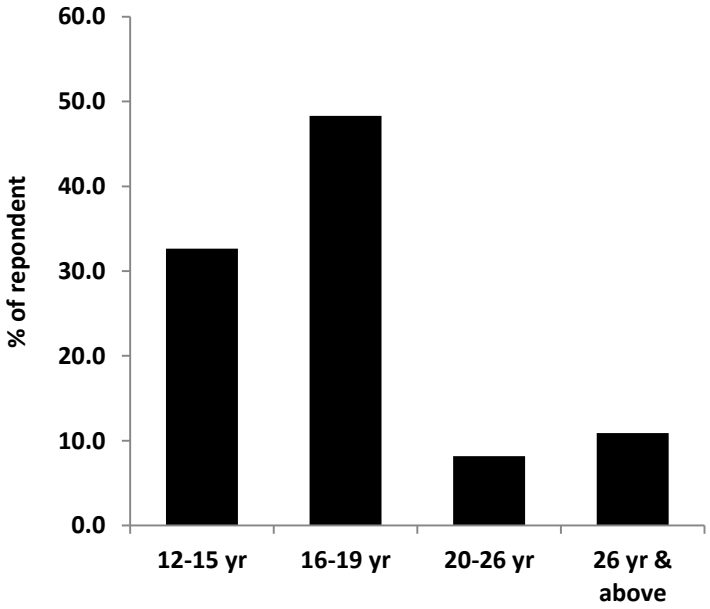

Figure 1. Age brackets of study group in percentage.

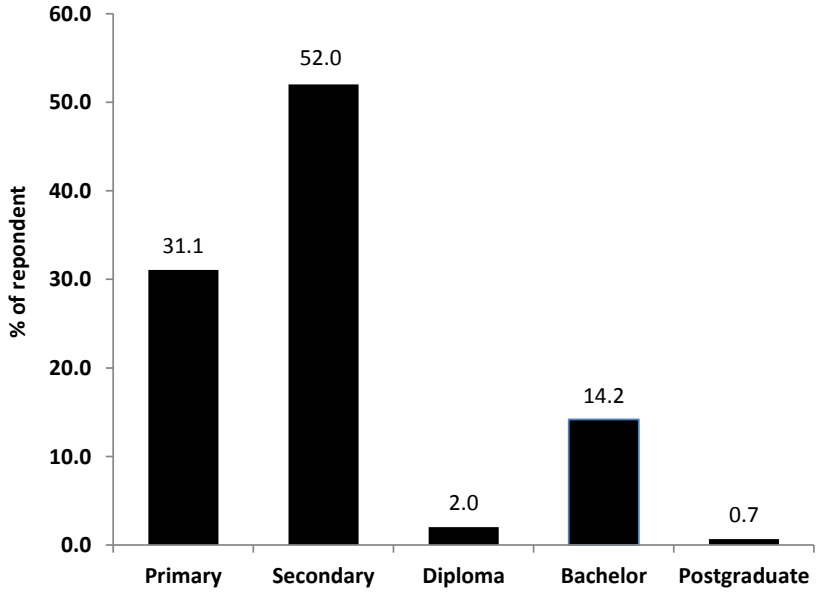

Figure 2. Educational profile of study group in percentage.

conditions of premises and facilities used to serve food from food safety point of view. Information recorded is presented in Table 1.

As shown in Table 1, refrigeratable foods were held at 5°C or below only in four schools (50%). With respect to cleanliness of refrigerators, six out of eight schools visited had unacceptable level of dirt in their refrigerators. Failure to keep food at the appropriate temperature and the inability to keep the refrigerators clean suggest that participants have limited knowledge about the relationship between temperature and microbial proliferation and cross contamination. This observation is in agreement with what have been reported by Ovca et al. (2014). In a study to evaluate food safety awareness, knowledge and practices among students in Slovenia, the researchers reported that 57.2% of respondents do not pay adequate attention to the effect of temperature on microbial growth. When prepared food is not consumed

immediately, considerable number of respondents in the above-mentioned study (48.8%) reported that they do not keep the leftover food in refrigerator. In the event that the leftover food is to be re-heated in the kitchen, the process is far from ideal and most of them do not do it to the recommended temperature (Ovca et al., 2014). In a previous study conducted by JevŠnik et al. (2008) to evaluate consumer awareness of food safety, it was found that 43.7% of respondents do not know the temperature of their home refrigerator.

From the data shown in Table 1, it is clear that four out of the eight schools selected do not keep refrigeratable food at right temperature. Although they have refrigerators, six schools do not keep refrigerators clean. With regard to availability of storage facilities, it was observed that five out of eight schools visited were found to be storing foods in proper conditions. Remaining three schools did not keep foods properly in store. With respect to distance of food from the floor in store, Table 1 shows that in four schools, food is kept at least 6 inches above the ground level. However, there are four schools which do not apply this practice.

For personal hygiene, Table 1 shows that 62.5% of the people observed wash their hands after using the toilet. However, there are 37.5% who do not adhere to this important practice. Even those who are doing it need to be trained on how to do it properly. For the evaluation of cleanliness of workers, results showed that 62.5% of them were not only wearing unclean uniform but also were not changing gloves and utensils when needed. This observation is relatively lower than what had been reported by Santana et al. (2009) who found that 86.7% of the workers in the schools who participated in their study failed to have proper personal hygiene practices and did not use hair restraints or proper uniforms.

With respect to location and cafeterias-design (Table 1), four out of the eight schools visited had suitable location area which was free from unsanitary vicinity such as trash collection sites or exposed to roaming animal, pets and rodents. The other four schools were without proper location and exposed to various physical hazards. Similarly, the fixtures such as doors, windows and surfaces of working area in the cafeteria were not suitable in many schools. In a similar study conducted by Santana et al. (2009) in Brazil, the researchers found that the main food safety related problems of the schools were improper location, improper ventilation, free access by people who do not work in the cafeteria and areas of food contact surfaces not properly cleaned. The researchers also observed that poor lighting and unprotected window screen against insects were part of the identified problems.

In the schools visited, foods are served to students in dishes. Most of the time these dishes are not adequately clean to ensure that cross contamination is avoided. As an example, Table 1 showed that in four out of the eight schools visited, dishes were washed and dried in a proper way; however, there were four schools in which this activity was not a routine. Regarding the appropriateness of material used for making contact surfaces, results showed that 5 out of eight 75% of school do not have smooth benches that can be easily washed and maintained properly. For pest control system, results indicated that the pest control system adopted by these schools is not robust enough to eliminate the potential source of pest invasion Table 1. In five schools out of the eight, evidence of the presence of pest is vivid. The proximity of some serving site of the schools to waste collection is also a real concern.

Assessment of food safety knowledge of students, teachers and food handlers in schools

In order to have a clear understanding about food safety knowledge and practice of the population under investigation, the present study investigated where these people eat their school meals during the break time. Results have shown that the majority of the respondents 60.3% eat their meal in school playground (Table 2). The school playground is an open place where students run and play during their break. The physical activity and big number of students moving around may expose the area to more dust and serve as a potential source of contamination. Therefore; from food safety point of view, the school playground may not be an ideal place to eat meal. The fact that majority of students eat their meal in an open place suggesting limited knowledge of food safety. In a study conducted by Ovca et al. (2014) in Slovenia to evaluate food safety awareness, knowledge and practice among students, it was found that only 65.8% of the students were able to give correct answers to the questions asked. These questions fell within the range of self-reported knowledge of respondents in this study. In another study conducted by Tan et al. (2013) to assess hand hygiene knowledge, attitudes and practices among food handlers at primary schools in Hulu Langat district, Selangor (Malaysia), the researchers reported 85.4% of the respondents under study have good hand hygiene knowledge, which is a critical part in food safety. A population of 94% was reported for cross contamination, followed by personal hygiene with a population of 79.9%. For the frequency of occurrence of food poisoning in this study, 73% reported that they never had suffered from food poisoning while 26.8% said they had suffered from the problem before and this percentage was twofold of what had been reported by Ovca et al. (2014).

With respect to source of information about knowledge of food safety, the majority of the respondents (80%) reported that they read article, magazine or books to enrich their food safety knowledge, which is a good practice.

For the assessment of hand washing practices, results

are presented in Table 3. A population of 96% of the respondents said they washed their hands after using a washroom. This value is much higher than what has been observed by the team of visitors who conducted this study 62.5% (Table 1). To find out whether water and soap were used to wash hands, Table 3 showed that 84% of the respondents confirmed that they used water and soap during hand washing. This value is comparable to what has been reported by Hassan and Dimassi (2014). In an assessment of Food safety and handling knowledge and practices of Lebanese University students, these researchers reported that 86.7% of the study population mentioned they wash their hands with water and soap. The proportion that uses only water to wash hands was 4.5% relatively lower than what has been found in this study 14.8%, Table 3.

When the respondents were asked to what extent, they have agreed with the statement that meals should not be left more than four hours at room temperature, 74.6% of them agreed (Table 4). However, in reality, the percentage reported by Abushelaibi et al. (2015) is much lower than that. In an evaluation of the effect of person-in-charge training program which was conducted by the Dubai Municipality, the researchers found that when a similar question was asked to food handlers, only 35.4% of them were able to give correct answer. For proper keep up of food establishment, 95.2% of the people who answered the questionnaire of this study said they hate insects and cockroaches in particular in food serving areas. When asked where they keep food, only 42% of those who answered the questionnaire said they keep food in refrigerator. The remaining 58% leave their food on the counter in the cafeteria at room temperature. Considering the high temperature in the United Arab Emirates which sometimes reaches 48°C, this practice could be very dangerous resulting in disease causing microbial proliferation. With respect to food handling practices, the self-reported hand hygiene finding is shown in Table 4. As it can be seen, only 16.7% of food handlers wear gloves while handling foods. This implies that more than 80% of them handle food with bare hands. Handling foods without proper gloves could be an important means of transmitting foodborne illness especially if hand hygiene practices are inadequate. In a similar study conducted by Tan et al. (2013) in Malaysia, it was reported that more than 90% of the participants wore gloves when touching ready to eat foods. This percentage is much higher than what has been found in our study. Therefore, the need to educate people on hand hygiene knowledge and practices in our schools is evident.

Like any other business, food sale is not same every day. Sometimes food prepared is not sold the same day and is stored until used. Therefore, proper actions are needed to be taken to ensure food being sold to consumers is fit for consumption. In order to have an idea about how food handlers manage the leftover food, the food sellers were asked what action they usually take when food is not all sold. The answers received showed that 65.3% of sellers throw away the remaining food while 26.5% of them give it free to students. Only 8.2% reported they store it for the next day (Table 4).

Regarding the cause of food poisoning, the study investigated the understanding of school children and teachers about the major causes. The results are stipulated in Table 4. Here, it can be seen that people interviewed had different understanding about the causes of food poisoning. Some of them said exposing food to the external environment for long time is one of the problems associated with food poisoning. Others reported that inadequate cooking time or temperature is an issue. However, the majority of them 48.1% believed that contaminations and microbial agents like bacteria are the main causes. For the general comments on the meals that were being sold at the school, 86.3% of the people surveyed felt that meals were clean while 13.7% believed the meals were not clean enough. As far as the cleanliness of the serving windows are concerned, 46.7% thought the windows through which foods were served to students and teachers were clean while 53.3% held opposite view.

Microbiological assessments of personal hygiene of student, teachers and workers

In each of the eight schools visited, swabbing technique was conducted on hands of students, teachers and workers in addition to surfaces of serving windows and tables to evaluate the level of hygiene on these frequent potential sources of contamination. Results of microbiological analysis for aerobic plate count, yeast and mold, *E. coli* and *S. aureus* are shown in Table 5.

In all the schools, the average aerobic plate counts were relatively higher in workers hands 7.6×10^3 CFU/cm^2 than students and teachers with counts 10.6×10^2 and 6.1×10^2 CFU/cm^2, respectively. These aerobic plate counts were found to be much lower than those reported by Santana et al. (2009). In a similar study conducted in Brazilian public schools, researchers found that in some schools, values as high as 6 log cycles were found in the hands of food handlers. However, no contamination with *Staphylococci* coagulase-positive or thermo tolerant coliforms was observed. Aerobic plate count higher that 3 log cycle is considered as poor sanitary condition which means there are potential risks of the presence of pathogenic microorganisms. Close examination of results showed that in all the 40 swab samples taken, the bacterial loads were relatively high. In fact a microbial load as high as 7.5×10^3 CFU/cm^2 was found on the hand of one of the workers. For serving windows and surfaces of serving tables, the aerobic plate counts were 2.8×10^3 and 5.8×10^2 CFU/cm^2, respectively. JevŠnik et al. (2013) assessed bacteriological test of kitchen surfaces and participants' hands in Slovenia and reported high aerobic

Table 5. Results of bacteriological analysis of swabs for students, teachers and workers hands.

Swab (n= 8)	Aerobic plate count			Yeast and mold		
	No. of +ve	Lowest CFU/cm^2	Highest CFU/cm^2	No of +ve	Lowest CFU/cm^2	Highest CFU/cm^2
Student hands	8	<10	10.6x10^2	2	1.5	1.7
Teachers hands	8	<10	6.1x10^2			
Workers hands	8	<10	7.6x10^3	2	2	16
Service window	8	<10	2.8x10^3			
Service table	8	<10	5.8x10^2	1	1	2

Swab (n=8)	*E. coli*			*Staphylococcus*		
	No. of +ve	Lowest CFU/cm^2	Highest CFU/cm^2	No. of +ve	Lowest CFU/cm^2	Highest CFU/cm^2
Student hands	1	1		2	1	2
Teacher hands	1	1				
Worker hands	1	2		1	2	
Services windows						
Services table				1	3	

Colony Forming Unit (CFU).

plate count on hands, kitchen counters and plastic dishes. For yeast and mold, swab samples from five schools showed presence of yeast and mold. The highest yeast and mold count of 16 CFU/cm^2 was found on the hands of a worker. This finding is similar to study conducted by JevŠnik et al. (2013) where they found considerable colonies of yeast and mold on knives for chopping meat.

On comparing, the microbiological load observed on the hands of students, teachers and workers in this study with other studies (Santana et al., 2009; JevŠnik et al., 2013) the loads reported here are not that high. However, the presence of *E. coli* and *S. aureus* on hands of students, food handlers and even a teacher is worrying. Coliform bacteria are important microbiological sanitary indicators which suggest there is a need for hygiene in processing and handling of foods. The presence of coliforms and *E. coli* may indicate fecal contamination because of lack of hygiene after using the toilet. Contaminated hands play a key role in carrying fecal flora from one host to another (Curtis and Cairncross, 2003). The hygiene and sanitary microbiological indicators can differ from one country to another. In the United Arab Emirates, the maximum limit is not known to the authors however, in places such as Slovenia, the law on specific measures of food poisoning and their prevention (Slovenian Official Gazette No. 24/1981) set the maximum number for aerobic plate count to 200 CFU per 20 cm^2. This number is much lower than what is reported in this study.

Conclusion

From the preliminary findings in the first part of the study, it can be noted that 37.5% of respondents do not wash their hands properly after using toilet. This revelation showed that proper personal hygiene is not adequately maintained in the school visited. For the evaluation of cleanliness of workers, it was found that 62% of those who serve food were not only wearing unclean uniform but also were not changing gloves as recommended. This finding also is another important setback that needs attention. With respect to cleanliness of refrigerators, the majority of the schools 75% do not keep their refrigerators clean enough. For the second part, results showed that 60.3% of respondents eat their meal in school playground. Utilization of playground for eating and physical activity simultaneously may expose the area to more dust, which serves as a potential source of contamination. For the appreciation of the importance of food safety, it was found that 87% of those who participated in the study appreciated the importance of food safety in maintaining good health, which means they are prepared to actively participate in future food safety intervention programs. In the third part of the study, the microbiological evaluation of surfaces and hands of students and food handlers revealed that the general microbiological conditions although were not unusual but the presence of *E. coli* and *S. aureus* on hands of students, food handlers and even a teacher is worrying. It is worrying because it can expose students to bacterial food poisoning such as salmonella and staphylococcus, which result into vomiting and severe abdominal pain. Therefore, it can be concluded that there is a need of food safety intervention in the schools. The information gathers from this study suggests that, personal hygiene and keeping food at proper temperature should be the points of focus in any future food safety intervention activity.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This work was part of senior student projects from Department of Food Science, College of Food and Agriculture, United Arab University. The authors are grateful to all the department members who supported the student during conducting this valuable part in their study plan specially, Mr. Abdulkadar Wasesa and Mr. Ismail Abdul Haleem.

REFERENCES

Aziz SAA, Dahan HM (2013). Food Handlers' Attitudes towards Safe Food Handling in School Canteens. Proc. Soc. Behav. Sci. 105:220-228.

Abushelaibi MA, Jobe B, Afifi HS, Mostafa B, Murad AA, Mohammed KA (2015). Evaluation of the effect of Person-in-charge (PIC) program on knowledge and practice change of food handlers in Dubai. Food Control 50:382-392.

Curtis V, Cairncross S (2003). Effect of washing hands with soap on diarrhea risk in the community: A systematic review. Lancet Infect. Dis. 3:275.

Gibson LL, Rose JB, Haas CN, Gerba CP, Rusin PA (2002). Quantitative assessment of risk reduction from hand washing with antibacterial soaps. J. Appl. Microbiol. 92(1):136-143.

Green LR, Selman CA, Radke V, Ripley D, Mack LC, Reimann DW (2006). Food worker hand washing practices: An observation study. J. Food Prot. 769:661-666.

Hassan HF, Dimassi H (2014). Food safety and handling knowledge and practices of Lebanese university students. Food Control 40:127-133.

JevŠnik M, Hlebec V, Raspor P (2008). Consumers' awareness of food safety from shopping to eating. Food Control 19(8):737-745.

JevŠnik M, Ovca A, Bauer M, Fink R, Oder M, SevŠek F (2013). Food safety knowledge and practices among elderly in Slovenia. Food Control 32(2):284-290.

Marzano MA, Balzaretti CM (2013). Protecting child health by preventing school-related foodborne illnesses: Microbiological risk assessment of hygiene practices, drinking water and ready-to-eat foods in Italian kindergartens and schools. Food Control 34:560-567.

Nicholas DAMD, Mackinnon L, Rowe SM, Bean NH, Griffin PM, Mead PS (2002). Foodborne disease outbreaks in United States schools. Pediatr. Infect. Dis. J. 21:623-628.

Ovca A, JevŠnik M, Raspor P (2014). Food safety awareness, knowledge and practices among students in Slovenia. Food Control 42:144-151.

Paulson DS (2000). Hand-washing, gloving and disease transmission by the food preparer. Dairy Food Environ. Sanit. 20:838-845.

Robert Koch Institute (2012). Outbreak of acute vomiting with diarrhea in children and youths over, cause identified. Germany: Federal Ministry of Health Available from http://www.rki.de/EN/Home/Outbreak_AV.html.

Su HP, Chiu SI, Tsai JL, Lee CL, Pan TM (2005). Bacterial food-borne illness outbreaks in Northern Taiwan, 1995-2001. J. Infect. Chemother. 11(3):146-151.

Santana NG, Almeida RCC, Ferreira JS, Almeida PF (2009). Microbiological quality and safety of meals served to children and adoption of good manufacturing practices in public school catering in Brazil. Food Control 20:255-261.

Slovenian Official Gazette (1981). Law on specific measures in food poisoning and their prevention. 24:1701-1706.

Sneed J, Strohbehn C, Gilmore SA, Mendonca A (2004). Microbiological evaluation of foodservice contact surfaces in Iowa assisted-living facilities. J. Am. Diet Assoc. 104(11):1722-1724.

Soares LS, Almeida RCC, Cerqueira ES, Carvalho JS, Nunes IL (2012). Knowledge, attitudes and practices in food safety and the presence of coagulase-positive staphylococci on hands of food handlers in the schools of Camaçari, Brazil. Food Control 27:206-213.

Staskel MD, Briley ME, Field LE, Barth SS (2007). Microbial Evaluation of Foodservice Surfaces in Texas Child-Care Centers. J. Am. Diet Assoc. 107(5):854-859.

Tan SL, Bakar FA, Karim MSA, Lee HY, Mahyudin N A (2013). Hand hygiene knowledge, attitudes and practices among food handlers at primary schools in Hulu Langat district, Selangor (Malaysia). Food Control 34(2):428-435.

WHO-World Health Organization (2007). Food safety and food-borne illness. Fact sheet No. 23. Available at http://wwwwho.int/mediacentre/factsheets/fs237/en/ (Reviewed March).

Yousef A, Carlstrom C (2003). Food Microbiology: A laboratory manual. Hoboken, New Jersey. USA: John Wiley & Sons, Inc.

academicJournals

Vol. 10(15), pp. 521-527, 21 April, 2016
DOI: 10.5897/AJMR2016.7958
Article Number: D820D6E58168
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro activities of *Trichoderma* species against *Phytophthora parasitica* and *Fusarium oxysporum*

E. Osorio-Hernández[1]*, J. Hernández-Morales[2], V. Conde-Martínez[2], A. C. Michel-Aceves[3], J. A. Lopez-Santillan[1] and J. A. Torres-Castillo[4]

[1]Facultad de Ingeniería y Ciencias de la Universidad Autónoma de Tamaulipas, Cd. Vitoria, 87000, Tamaulipas, México.
[2]Colegio de Postgraduados, Montecillo, 56230, Estado de México, México.
[3]Centro de Estudios Profesionales del Colegio Superior Agropecuario del Estado de Guerrero, Colonia Centro. Iguala, 40000, Guerrero, México.
[4]Universidad Autónoma de Tamaulipas-Instituto de Ecología Aplicada, Cd. Vitoria, 87029 Tamaulipas, México.

Received 9 February, 2016; Accepted 8 April, 2016

***Trichoderma* species usually produce chitinases and glucanases with inhibitory effects on several phytopathogens. In this work, the effects of such enzymes activities were evaluated on *Phytophthora parasitica* and *Fusarium oxysporum*. Enriched enzyme extracts were obtained from *Trichoderma asperellum, Trichoderma virens, Trichoderma gamsii* and *Trichoderma logibrachiatum*, which were grown on supplemented media with chitin or lamminarin. Chitinase and glucanase activity were confirmed by spectrophotometric methods. *T. longibrachiatum* showed the highest level of total and specific activity for both hydrolytic enzymes. Filtrated media were submitted to antagonism assays and results showed that chitinases from *T. longibrachiatum* inhibited 15% of *P. parasitica* and 45% of *F. oxysporum* mycelial growth;on the other hand, no inhibition was detected to *P. parasitica* with any other media. Glucanase activity from *T. longibrachiatum* showed 40% of mycelial growth inhibition on *F. oxysporum* and no other species showed inhibitory effects.**

Key words: Antibiotic, biological control, enzymes, phytopathogens.

INTRODUCTION

Phytophthora parasitica and *Fusarium oxysporum* infect a wide range of host plants and cause significant damage on important crops around the world (Leslie and Summerell, 2006; Martin et al., 2014). To date, most of the phytosanitary management has been done through chemical control strategies; nevertheless, some strains of *P. parasitica* and *F. oxysporum* have developed resistance to several fungicides (Lucas et al., 2008). Due to this, searching for alternative biological strategies to control plant diseases has been growing in recent years

*Corresponding author. E-mail: eosorio@uat.edu.mx, osorio@hotmail.com.

(Anitha and Rabeeth, 2010). Amongst these, the use of *Trichoderma*, has received huge attention, because of its adaptability and diverse action mechanisms (mycoparasitism, antibiosis, competence and induction of endogenous plant defense) (Vinale et al., 2014). *Trichoderma* species are widely used in conventional agriculture and for biopesticide industry as biological agents or as enzymes sources. *Trichoderma* spp. produce several enzymes involved in their biological processes, including chitinases, glucanases, cellulases, proteases and amylases (Gajera et al., 2012; González et al., 2012); among these, chintinases and β-glucanases are the more relevant in the mycoparasitism. Chitinase 46-KDa (CHIT 46) affects the growth and cell wall of *Sclerotium rolfsii* and *Rhizoctonia solani* (Lima et al., 1999); β-1,6 glucanase, chitinase and protease produced by *Trichoderma harzianum* and *Trichoderma viride* also showed the ability to degrade cell walls of *S. rolfsii* (Parmar et al., 2015); furthermore, an extracellular alkaline-protease produced by *T. harzianum* of 18.8 kDa and active at pH 7 and 8 hydrolyzed cell-wall proteins from *Crinipellis perniciosa*, a pathogen with high resistance to antagonist microorganisms. *P. parasitica* and *F. oxysporum* also are sensible to lytic enzymes, as demonstrated by Budi et al. (2000), where the confrontation of several hydrolytic enzymes (chitinases, cellulases, proteases and pectinases) from *Paenibacillus* species promoted cell-wall and cell disorganization in some phytopathogens. The ability of *Trichoderma* spp. to parasite fungal phytopathogens is well known, and it is associated with formation of rolling, hooks and apresoria used to penetrate cell walls combined with enzymes, including chitinases and glucanases (Zeilinger and Omann, 2007). These enzymes attack cell walls structures formed almost exclusively of polysaccharides as chitin and glucanes (Küçük and Kivanc, 2004). Based on this, the main goal of this work was the quantification of chitinase and glucanase activities produced by four *Trichoderma* spp. and the evaluation of the inhibitory effect on the mycelial growth of *P. parasitica* and *F. oxysporum*.

MATERIALS AND METHODS

Biological material

P. parasitica and *F. oxysporum* were isolated from Roselle plants (*Hibiscus sabdariffa L.*) with specific symptoms in the Laboratory of Biochemistry from the Colegio de Postgraduados, Campus Montecillo, Estado de Mexico, Mexico. *P. parasitica* was identified based on taxonomic keys from Erwin and Ribeiro (1996) and *F. oxysporum* was identified through taxonomic keys of Leslie and Summerell (2006).

To evaluate several *Trichoderma* species, *Trichoderma virens* (T6), *Trichoderma asperellum* (T9), *Trichoderma gamsii* (T13) and *Trichoderma longibrachiatum* (T19) were used. *Trichoderma* spp. were obtained from strain collection at the Instituto of Fitosanidad, Colegio de Postgraduados, Campus Montecillo, Estado de Mexico, Mexico. Species were identified with morphology and ITS sequencing by Esparza-Luna (2009) (unpublished data).

Chitinases and glucanases production

Trichoderma spp. were grown in liquid media to produce chitinases and glucanases according to (Almeida et al., 2007). Erlemeyer flasks were filled with 200 ml of culture media (1 L was prepared by mixing 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4/H_2O$; 2 mg of $FeCl_3$, 150 ml of V8 commercial juice, 10 g de PVP and distilled water to 1 L). Salts for media preparation were purchased from J. T. Baker (PA, USA), PVP was purchased from Sigma-Aldrich, MO, USA and the commercial juice was obtained from JUMEX (MO, Mexico). Two culture media were prepared by addition of 1% of chitin from shrimp shells (Sigma-Aldrich, MO, USA) and 0.1% of laminarin from *Laminaria digitata* (Sigma-Aldrich, MO, USA) to induce chitinases and glucanases production, respectively. The final pH of media was 6 and then was autoclaved at 121°C for 20 min. Separately, each species was inoculated in a flask with the addition of 1 ml of 1×10^6 conidia/ml and incubated at 25°C under 12h:12 h (L:D) in continuous stirring (Thermo Scientific MaxQ 4000 Incubated Shake, MA, USA) for 5 days for chitinase and eight days for glucanase production. After incubation, media were filtered through paper Whatman No. 1 (Maidstone, UK) and centrifuged at 5,000 rpm for 15 min, supernatant was adjusted to 20 ml and used as source for protein quantification, chitinases and glucanases assays and for the inhibition assays (sterilized through a 0.22 µm filter (Millex® Millipore, MA, USA)).

Chitinase activity detection

Five tubes were marked for the experiment, in the first, 1 ml of water was added as a reaction blank, in the remaining tubes 1 ml of filtered media was added and then 1 ml of substrate solution (3.8 mg/ml of chitin in Citrate McIlvaine buffer at pH 5 (prepared with 49 ml of 0.1 M solution of citric acid plus 51 ml of 0.2 M solution of sodium phosphate)) was incorporated. One tube was heated at 95°C in 10 min, after this, heated tube and remaining tubes were incubated at 37°C for 24 h. After incubation, 500 µl of each tube were placed in new tubes and heated at 95°C for 10 min and followed by addition of 100 µl of 0.8 M potassium tetraborate and then tubes were heated again during 5 min. Tubes were incubated at 37°C during 20 min and 3 ml of *p*-dimethylamino benzaldehyde solution (Sigma-Aldrich, MO, USA) (1 g was dissolved in 100 ml of 1.25% HCl - 8.75% aqueous solution) were added. Absorbance was recorded at 544 nm (spectrophotometer JENWAY 6305, OSA, UK). All reactions were done with four replicates and absorbance was compared with a standard curve, prepared with 0, 25, 50, 75, 100, 125, 150, 175 and 200 µg/ml of N-acetylglucosamine in McIlvaine buffer. Chitinase activity was related to release of N-acetylglucosamine by comparison of the recorded absorbance from the initial heated tube minus average absorbance of the remaining tubes. Total chitinase activity (TA) and specific chitinase activity (SA) were calculated according to González et al. (2012) and expressed as µmol of N-acetylglucosamine/h and SA was expressed as µmol of N-acetylglucosamine/µg of protein/h. Protein quantification was according to Bradford (BIORAD, CA, USA) (Bradford et al., 1976).

Glucanase activity detection

Glucanase production was determined according to four filtered *Trichoderma* spp. media. Five tubes were marked as “X” and five as

"Y". One milliliter of 10 mg/ml lamminarin dissolved in 0.1 M citrate buffer pH 5 (0.1 M $C_6H_5Na_3O_7$ and 0.1 M citric acid, in 21:29 ratio) was added in each tube. Then, 250 µl of filtered media were added only in tubes marked as "X" before incubation (4 h at 40°C in a warm water bath). After incubation, 250 µl of filtered media were added in tubes marked as "Y" and then 2 ml of copper reactive (CR) was added to all tubes and immediately heated by immersion in boiling water for 10 min followed by addition of 2 ml of arsenomolybdate. Final volume was adjusted at of 25 ml with water. Solution was stirred in 1 min and finally absorbance was recorded at 595 nm (spectrophotometer JENWAY 6305). Calibration blank was prepared with water (Bruce et al., 1995).

CR solution was prepared previously and consisted of Cu I solution (16.1 g $KNaC_4H_4O_6.4H_2O$, 24 g of Na_2CO_3, 16 g of $NaHCO_3$, 80.44 g of Na_2SO_4 dissolved in distilled water to a final volume of 800 ml) and Cu II solution (4 g of $CuSO_4.5H_2O$, 36 g of Na_2SO_4 dissolved in a final volume of 200 ml), both solutions were mixed before using. The arsenomolybdate solution was prepared by mixing 25 g of $NH_4Mo_7O_{24}.4H_2O$ in 450 ml of water and 21 ml of concentrated H_2SO_4 and 3 g of $Na_2HAs_5O_4.7H_2O$ in 25 ml of water incubated at 37°C for two days. Salts for solutions preparation were purchased from J. T. Baker (PA, USA). Standard curve was made with 25, 50, 75, 100, 125 and 150 µg glucose/ml. Absorbance differences between X and Y were used to detect glucanase activity, detection was done with four replicates. Glucanase activity was calculated according to González et al. (2012), where total glucanase activity was expressed as µmol glucose/h, and glucanase specific activity was expressed as µmol glucose/h/µg of protein.

Chitinase and glucanase activity against fungal phytopathogens

Aqueous fraction of *Trichoderma* spp. cultures with chitinase or glucanase activity were sterilized through 0.22 µm filter (Millex®, Millipore) and mixed separately with warm PDA (Bioxon, Estado de Mexico, Mexico) (ratio 1:1) before filling Petri dishes (8 cm). In each plate, a 5 mm-piece of active mycelium of *P. parasitica* (5 days) or *F. oxysporum* (7 days) was placed and incubated at 25°C. Controls were established using PDA with distilled water (ratio1:1). Radial growth in cm was recorded every two days until the media were completely covered by mycelium (five days for *F. oxysporum* and seven days for *P. parasitica*). To estimate conidial production of *F. oxysporum*, 10 pieces 5 mm-diameter were stirred in 10 ml of distilled water to obtain conidial suspensions which were quantified in a Neubauer chamber (Wertheim, GE). Results from three dishes were recorded and expressed as number of conidia in 1 cm of PDA. Number of colony-forming units (CFU) was evaluated by inoculation of conidia in PDA and counted 48 h later.

Experimental design and data analyses

A completely randomized design was used with 9 treatments (4 glucanase activities and 4 chitinase activities and a control) with 5 replicates for two pathogens (*P. parasitica* and *F. oxysporum*). Response variables were mycelial radial growth, the inhibition percentage (calculated with the formula: [I (%) = (1 – T /C) × 100]; where, I= Inhibition percentage of pathogen growth by antagonists, C=radial growth in control, T=radial growth in the treatments), number of conidia and number of CFU. Analyses of variance (ANOVA) was used to detect differences between treatments and comparison means were done by Tukey test (P≤0.05) with SAS program version 9 (2002).

RESULTS AND DISCUSSION

Chitinases and glucanases quantitation

The four *Trichoderma* spp. produced chitinases and glucanases at different levels; in general, chitinase TA (Total chitinase activity) values were between rates of 0.107 and 0.228 µmol of N-acetylglucosamine/h^{-1} and SA (Specific Chitinase activity) rates were from 0.102 to 0.255 µmol h^{-1} μg^{-1} de N-acetylglucosamine/µg of protein. Statistical analyses detect differences (P≤0.0001) between species in TA and AE when chitinase activity was analyzed (Table 1). Total and specific activities were found higher in *T. longibrachiatum*, but this kind of activity was wide spread in all species, which seems typical in *Trichoderma* as reported for strains of *T. harzianum* (Michel-Aceves et al., 2005); however, differences in activities levels between species was evident and coincide with results from González et al. (2012) and Lee et al. (2012), who related such differences to endogenous characteristics, media composition and to culture age; such differences are considered useful by several authors to select *Trichoderma* spp. for biological control. Several studies on *Trichoderma* spp. have demonstrated the secretion of hydrolytic enzymes, including chitinases and glucanases, which have the ability to breakdown cell wall and penetrate the mycelium of other species (González et al., 2011).

It is well known that fungal β-glucanases have roles in glucan mobilization in the cell wall and also during nutrient depletion (Parmar et al., 2015), callose degradation, pathogenesis mechanism of antagonist fungi and nutrition of mycoparasites, for example, β-1,3-glucanases was associated with the lysis and wall degradation of *Sclerotium* spp. Glucanase activities obtained from four *Trichoderma* spp. fluctuated from 0.22 to 0.47 µmol of released glucose, while specific activity varied from 1.24 to 2.78 (Table 2). *T. longibrachiatum* showed the highest activity (0.47 µmol); however, *T. gamsii* and *T. virens* showed the higher specific activity, supporting that production depends on the species, which is similar to that reported by Parmar et al. (2015). Glucanase activity has been reported in several species of *Trichoderma* by Michel-Aceves et al. (2005), where enzyme production was *T. koningii* (1747.2 µmol), *T. longibrachiatum* (1400 µmol), *T. virens* (1045 µmol) and *T. harziamun* (1232 µmol), being higher than levels detected in this work, probably due to the culture conditions or genetic background of each species, similar to results reported by González et al. (2011), who indicated that the β-1,3 glucanase specific activity of *Trichoderma* spp., showed variations associated with variation in the substrate carbon source. On the other hand, Bruce et al. (1995) reported variations of total activity from 50 to 241 µmol of released glucose; nevertheless, specific activity fluctuated between 0.9 and

Table 1. Chitinase activity in *Trichoderma* species cultured in medium supplemented with 1% chitin.

Species	Total chitinase activity (µmol of released N-acetylglucosamine h^{-1})	Specific chitinase activity (µmol of released-acetylglucosamine h^{-1} $µg^{-1}$ of protein)
T. gamsii	0.151[ab]	0.102[b]
T. virens	0.107[b]	0.179[ab]
T. longibrachiatum	0.228[a]	0.255[a]
T. asperellum	0.164[ab]	0.165[b]

[z]Treatments with the same letter are statistically similar between them (Tukey, P ≤0.05).

Table 2. Glucanase activity in *Trichoderma* species cultured in medium supplemented with 0.1% laminarin.

Species	Total activity (µmol h^{-1} de glucose)	Specific activity (glucose µmol h^{-1} $µg^{-1}$ de protein)
T. gamsii	0.35[abz]	2.78[a]
T. virens	0.28[ab]	2.44[a]
T. longibrachiatum	0.47[a]	1.24[b]
T. asperellum	0.22[b]	1.26[b]

[z]Treatments with the same letter are statistically similar between them(Tukey, P ≤0.05).

Table 3. Effect of chitinase activity from *Trichoderma* species on mycelial growth and reproductive potential of *P. parasitica* (Pp) and *F. oxysporum* (Fo).

Species	Growth of *Pp* (cm)	Growth of *Fo (cm)*	Number of Conidia *Fo*	Number of CFU of *Fo*
T. virens	7.9[az]	7.8[a]	11.3[a]	153[a]
T. gamsii	7.7[a]	8.0[a]	5.6[a]	56[c]
T. asperellum	7.5[a]	7.8[a]	6.6[a]	138[ab]
T. longibrachiatum	6.7[b]	4.4[c]	6.3[a]	89[bc]
Control (Water)	3.4[c]	5.9[b]	2.0[a]	41[c]

[z]Treatments with the same letter are statistically similar between them (Tukey, P ≤0.05). CFU (Colony-forming units).

4.44 µmol h^{-1} $µg^{-1}$ of glucose/mg protein and was in the range of results reported here, which is indicative that enzyme production is also influenced by culture media composition and the *Trichoderma* spp. evaluated.

Evaluation of chitinase and glucanase activity against *P. parasitica* and *F. oxysporum*

Production of enzymes by *Trichoderma* as part of their mycoparasitic process, where chitinases and glucanases are the most relevant enzymes has been reported (Küçük and Kivanç, 2008; Parmar et al., 2015). Gajera et al. (2012) and Parmar et al. (2015) indicated that chitinases and glucanases produce the lysis of fungal cell walls in some phytopathogens. Based on this, chitinase effects on *P. parasitica* mycelium growth was different between the *T. gamsii, T. asperellum* and *T. longibrachiatum* (Tables 3 and 4). On the other hand, enzyme activity obtained from *T. asperellum, T. virens* and *T. gamsii* induced abundant mycelial growth of *P. parasitica*, which could be attributed to enzyme inhibitors possibly produced.

Antagonistic activity could be also influenced by specific recognition at the molecular level, which determines the success or failure for the control; for example, the glucanase AGN13.1 recognized the cell wall of *Aspergillus niger, Botrytis cinerea, Colletotrichun acutatum, F. oxysporum, Penicillium aurantiogriseum* and *R. solani* but did not affect other evaluated fungi (Ait-Lahsen et al., 2001). This suggests that no evident inhibitory effects observed here by the four *Trichoderma*

Table 4. Effect of glucanase activity from *Trichoderma* spp., on mycelial growth and reproductive potential of *P. parasitica* (*Pp*) and *F. oxysporum* (*Fo*).

Species	Growth of *Pp* (cm)	Growth of *Fo* (cm)	Number of conidia in *Fo*	CFUof *Fo*
T. virens	7.7[az]	8.0[a]	8.3[a]	87[bc]
T. gamsii	7.3[b]	8.0[a]	8.3[a]	124[ab]
T. asperellum	7.2[b]	8.0[a]	9.6[a]	149[a]
T. longibrachiatum	7.2[b]	4.8[c]	3.3[a]	97[abc]
Control	3.4[c]	5.8[b]	2.0[a]	41[c]

[z]Treatments with the same letter are statistically similar between them(Tukey, P ≤0.05).

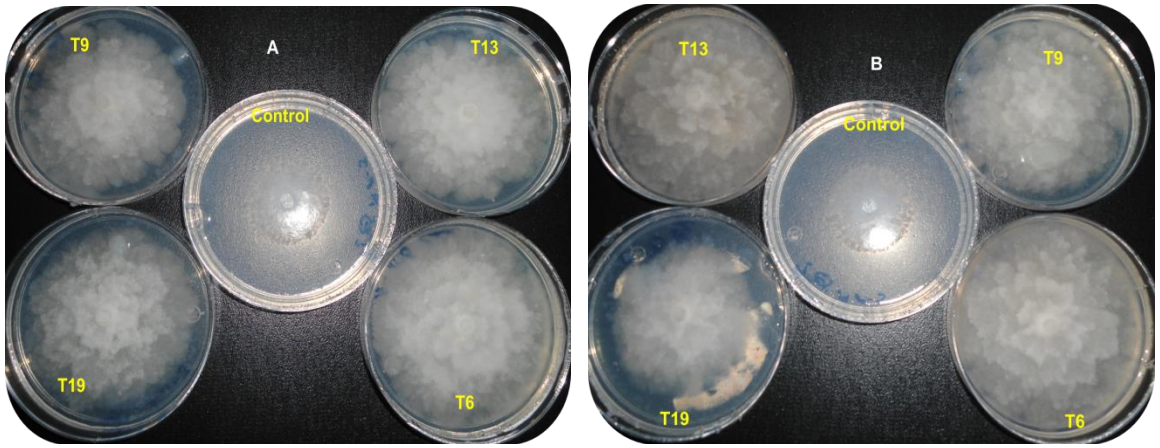

Figure 1. Chitinases (A) and glucanases (B) activity from *T. asperellum* (T9*)*, *T. virens* (T6*)*, *T. gamsii* (T13*)*, *T. longibrachiatum* (T19*)* and Control on *P. parasitica* growth.

spp. could be related to absence of specific recognition on *P. parasitica* and *F. oxysporum*.

Some reports indicated that *P. parasitica* could induce glucanase production to defend itself against antagonists, which in addition to the possible enzyme inhibitor production could be part of factors that influenced the null inhibitory effects; however, this remains for confirmation. Chitinase and glucanase antibiotic effect on mycelial growth of *F. oxysporum* showed significant differences between treatments (P≤0.0001) (Figure 1). *T. longibrachiatum* showed the highest inhibitory effect with a reduction of 40% (4.4 cm) when chitinase activity was evaluated and 45% (4.8 cm) when glucanase were used (Table 3). Michel-Aceves et al. (2005) indicated that glucanases and chitinases produced by *T. koningii* and *T. harzianum* presented variations in their accumulation; nevertheless, also inhibited growth of *F. oxysporum*. Nevertheless, Gajera et al. (2012) and Parmar et al. (2015) reported that a significant positive correlation between percentage growth inhibition (*Macrophomina phaseolina* and *S. rolfsii)* of test fungus and lytic enzymes (chitinase, β-1,3-glucanase and protease) in the culture medium of antagonist treatment established a relationship to inhibit growth of fungal pathogen by increasing the levels of these enzymes.

Similarly to *P. parasitica* results, it was also observed an abundant growth of *F. oxysporum* when challenged with enzymes activities of *T. asperellum, T. virens* and *T. gamsii* (Figure 2); however, *F. oxysporum* inoculated in media containing enzyme activities did not produce violet pigments, typical of this species, which accumulation was observed in control media, indicating that extracts did not affect the growth rate but could have effects at other levels. Such inhibition of pigment accumulation could diminish pathogenic potential, because such pigments usually are associated with pathogenic roles and are important in the interaction phytopathogen-plant (Medentsev et al., 2005). Nelson et al. (1993) reported that *F. oxysporum* produce secondary metabolites like micotoxins, trichothecenes, pigments, antibiotics and phytotoxins that could have influence on the null inhibition of *F. oxysporum* growth. Number of conidia and CFU were different (P≤0.0001) between treatments and it demonstrated a relation with the higher mycelium growth

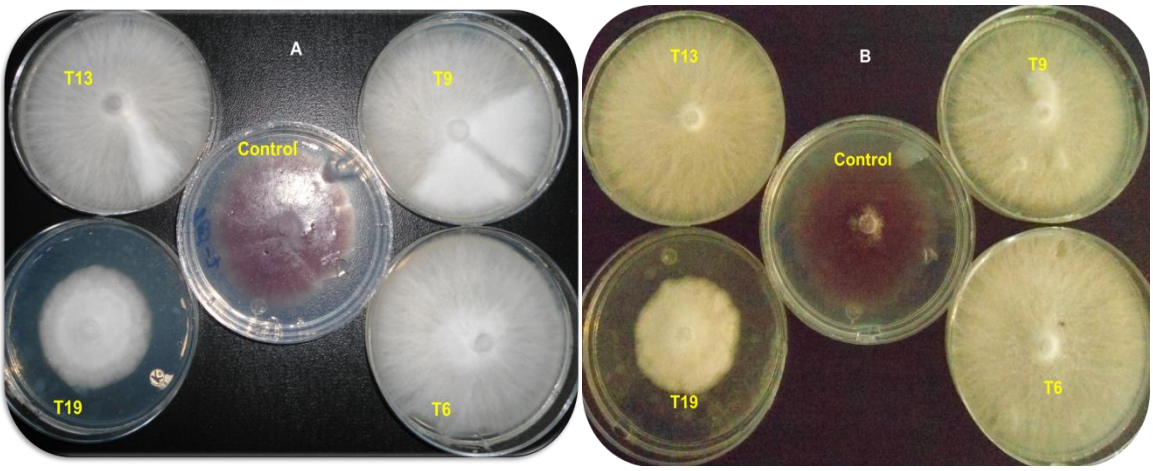

Figure 2. Chitin (A) and glucanase (B) activity of *T. asperellum* (T9), *T. virens* (T6*)*, *T. gamsii* (T13*)*, *T. longibrachiatum* (T19*)* and control on *F.oxysporum* growth.

that presented the higher number of conidia and CFU, demonstrating that an important parameter for reproduction as the conidial viability was not affected, which contrasted with report of Michel-Aceves et al. (2005) who found a 95% reduction in conidial production and around 70% of loss of viability when *T. harzianum* and *T. koningii* were used, rising the arguments to consider that physiological conditions, genetic background and nutritional availability are directly related with antagonistic effects of each *Trichoderma* spp., which should be considered as part of the production management and for control strategies that guarantees successfully plant protection.

Conclusions

All the *Trichoderma* spp. produced hydrolytic activities depending on the carbon source and activity levels were independent of the species. *T. longibrachaitum* was the species with higher total and specific enzyme activities of both activities; it also showed antagonistic effect on both phytopathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors wish to thank Colegio de Postgraduados and Consejo Nacional de Ciencia y Tecnologia (CONACYT) for the providing scholarship to the first author (Scholarship number: 50928) and to the grant SAGARPA-163972.”

REFERENCES

Ait-Lahsen H, Soler A, Rey MJ, de la Cruz J, Monte E, Llobell A (2001). An antifungal Exo-α 1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. Appl. Environ. Microbiol. 67(12):5833-5839.

Almeida FB, Cerqueira FM, Silva RN, Ulhoa CJ, Lima AL (2007). Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: Evaluation of coiling and hydrolytic enzyme production. Biotechnol. Lett. 29:1189-1193.

Anitha A, Rabeeth M (2010). Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus*. Afr. J. Plant Sci. 4:061-066.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bruce A, Srinivasan U, Staines HJ, Highley TL (1995). Chitinase and laminarinase production in liquid culture by *Trichoderma* spp. and their role in biocontrol of wood decay fungi. Int. Biodeter. Biodegrad. 23(4):337-353.

Budi SW, van Tuinen D, Arnould C, Dumas-Gaudot E, Gianinazzi-Pearson V, Gianinazzi S (2000). Hydrolytic enzyme activity of *Paenibacillus* sp. Strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. Appl. Soil. Ecol. 15:191-199.

Phytophthora diseases worldwide. American Phytopathological Society (APS Press).

Esparza-Luna, LL (2009). *In vitro* effectivity natives strains of *Trichoderma* spp. In isolates of *Phytophythora parasitica* D. that was obtained of plants of roselle. Master Thesis, Colegio de Postgraduados, campus Montecillo.

Gajera HP, Bambharolia RP, Patel SV, Khatrani TJ, Goalkiya BA (2012). Antagonism of *Trichoderma* spp. against *Macrophomina phaseolina:* Evaluation of Coiling and Cell Wall Degrading Enzymatic Activities. J. Plant Pathol. Microb. 3:149.

González I, Infante D, Martínez B, Arias Y, González N, Miranda I, Peteira B (2012). Induction of chitinases and glucanases in *Trichoderma* spp. strains intended for biological control. Biotecnología Aplicada 29(1):7-11.

González I, Infante D, Peteira B, Martínez B, Arias Y, González N, Miranda I (2011). Biochemical characterization of promising isolates of *Trichoderma* spp. as biological control agents. II. Expression of glucanase activity. Rev. Protección Veg. 26:23-29.

Küçük C, Kivanc M (2004). *In vitro* antifungal activity of strains of *Trichoderma harzianum*. Turk. J. Biol. 28: 111-115.

Lee CK, Darah I, Ibrahim CO (2012). Production and optimization of cellulase enzyme using *Aspergillus niger* USM AI 1 and comparison with *Trichoderma reesei* via solid state fermentation system. Biotechnol. Res. Int. pp. 1-6.

Leslie JF, Summerell BA (2006). The Laboratory *Fusarium*. Manual. Blackwell Publishing. Ames, Iowa, USA. 2:10.

Lima L, De Marco JL, Ulhoa CJ, Felix CR (1999). Synthesis of *Trichoderma* chitinase which affects the *Sclerotium rolfsii* and *Rhizoctonia solani* cell walls. Folia Microbiol. 44:45-49.

Lucas AJ, Bower LA, Coffey MD (2008). Fungicide resistance in soil-borne *Phytophthora* species. EPPO Bull. 1365-2338.

Martin NF, Blair J, Coffey E, Michael DA (2014). Combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora*. Fung. Genet. Biol. 66:19-32.

Medentsev AG, Arinbasarova AY, Akimenko VK (2005). Biosynthesis of naphthoquinone pigments by fungi of the genus *Fusarium*. Appl. Biochem. Microbiol. 41(5):573-577.

Michel-Aceves AC, Otero-Sánchez MA, Rebolledo-Domínguez O, Lezama-Gutiérrez R, Ochoa-Moreno ME (2005). Production and antagonistic effect of chitinases and glucanases from *Trichoderma* spp. in the inhibition of *Fusarium oxysporum* and *Fusarium subglutinans in vitro*. Revista Chapingo Serie Horticultura 11:273-278.

Nelson PE, Desjardins AE, Plattner RD (1993). Fumonisins, mycotoxins produced by *Fusarium* species: Biology, chemistry, and significance. Ann. Rev. Phytopathol. 31:233-252.

Parmar HJ, Bodar NP, Lakhani HN, Patel SV, Umrania VV, Hassan MM (2015). Production of lytic enzymes by *Trichoderma* strains during *in vitro* antagonism with *Sclerotium rolfsii*, the causal agent of stem rot of groundnut. Afr. J. Microbiol. Res. 9(6):365-372.

SAS OnlineDoc® 9 Copyright © (2002). By SAS Institute Inc. Cary NC USA.

Vinale F, Sivasithamparam K, Ghisalberti EL, Woo SL, Nigro M, Marra R, Lombardi N, Pascale A, Ruocco M, Lanzuise S, Manganiello G, Lorito M (2014). *Trichoderma* Secondary Metabolites Active on Plants and Fungal Pathogens. Open Mycol. J. 8:127-139.

Zeilinger S, Omann M (2007). *Trichoderma* biocontrol: Signal transduction pathways involved in host sensing and mycoparasitism. Gene. Regul. Syst. Biol. 1:227-234.

academicJournals

Vol. 10(15), pp. 528-534, 21 April, 2016
DOI: 10.5897/AJMR2015.7784
Article Number: DAECED558170
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Biocontrol of *Salmonella* Typhimurium growth in tomato surface by bacteriophage P22

Osvaldo López-Cuevas[1], Nohelia Castro-del Campo[1], Karina Ramirez[2] and Cristóbal Chaidez[1]*

[1]Laboratorio Nacional para la Investigación en Inocuidad Alimentaria, Centro de Investigación en Alimentación y Desarrollo A. C. Carretera a El dorado Km. 5.5, Campo El Diez, Culiacán, Sinaloa 80110, Mexico.
[2]División de Estudios de Posgrado e Investigación, Instituto Tecnológico de Culiacán. Calle Juan de Dios Bátiz, No. 310 pte. Colonia Guadalupe, Culiacán, Sinaloa 80220, México.

Received 30 September, 2015, Accepted 18 March, 2016

The aim of this research was to evaluate the effect of bacteriophage P22 on *Salmonella* Typhimurium in artificially inoculated tomato surface, under simulating condition of storage (10°C) and marketing (20°C) during seven days. First, we demonstrated the *in vitro* ability of phage P22 to reduce host population density to undetectable levels two hours after inoculation; however, *S.* Typhimurium was able to regrow up to 5.5 Log_{10}CFU/ml after 24 h. *S.* Typhimurium-inoculated tomatoes stored at 10°C and exposed to P22 exhibited a mean bacterial reduction of 3.02 Log_{10}CFU/tomato, while those stored at 20°C showed a mean reduction of 0.7 Log_{10}CFU/tomato at day seven. Although phage P22 was able to reduce *S.* Typhimurium on tomato surface, the *in vitro* results indicate that a larger initial concentration of phages is required in order to ensure sustained inactivation of *S.* Typhimurium.

Key words: Biocontrol, bacteriophages, *S.* Typhimurium, tomato.

INTRODUCTION

The number of foodborne illness outbreaks linked to fresh produce has increased in the last years (Beuchat, 1996; Lynch et al., 2009). Pathogens most commonly associated with the consumption of fresh produce are viruses, bacteria and protozoa (DeWaal et al., 2007). These microorganisms cause approximately 76 million new cases of food-related illness in the United States each year, resulting in 325,000 hospitalizations and 5,000 deaths, while the economic cost generated by these events is around 152 billion dollars (Scharff, 2010). Consumption of tomatoes and peppers has been recently responsible of foodborne outbreaks caused by *Salmonella* serovars, which have involved from sporadic cases to multistate outbreaks, implicating severe problems in public health (Hedberg et al., 1999; Behravesh et al., 2011). Fresh produce contamination can occur from the field to the table; according to Lynch et al. (2009) the probability of contamination increases in specific production steps, including field work, packinghouse, and also during final processing in the

*Corresponding author. E-mail: chaqui@ciad.mx. Tel: (52) 667 7605536. Fax: (52) 667 7605537.

kitchen. *Salmonella* is the main bacteria responsible for fresh produce outbreaks (Hanning et al., 2009; Fatica and Schneider, 2011), being the leading cause of hospitalization and deaths with a 35 and 28%, respectively (González and Rojas, 2005). Outbreaks of *Salmonella* Saint Paul occurred in 2008 and 2013 in the United States sickened 1,442 people in 43 states, and 81 people in 18 states, respectively. These outbreaks were associated (as suspects) with the consumption of jalapeño pepper and cucumber (CDC, 2008, 2013a). The occurrence of outbreaks evidences the failure of physical and chemical interventions methods (Parish et al., 2003).

In this regard, the use of bacteriophages as biocontrol, individually or combined with chemical products, has been proposed (Xu, 1999). Previous reports have indicated the efficacy of bacteriophages to eliminate *Salmonella* from meat, fresh produce and processed foods. Leverentz et al. (2001) applied a mixture of 4 lytic bacteriophages (SCPLX-1) specific for *Salmonella* Enteritidis on pre-cut melons and apples stored at temperatures of 5, 10 and 20°C in a period of 168 h achieving bacterial reductions of 3.5 Log_{10}CFU/melon at 5 and 10°C, and 2.5 Log_{10}CFU/melon at 20°C. Another study showed the effect of bacteriophages over *S.* Enteritidis suppression in Cheddar cheese produced from raw and pasteurized milk and stored at 8°C; *S.* Enteritidis did not survive in pasteurized milk cheese after 89 days in the presence of the phage. However, *S*. Enteritidis was able to survive around 50 CFU/g in raw milk cheese containing phage even after 99 days of storage (Modi et al., 2001). Waseh et al. (2010) orally administered P22 phage tailspike protein in chicken infected with *Salmonella* and found a reduction in bacterial colonization. These findings coincide with the ability of bacteriophages to reduce bacterial populations from foods.

Considering that *S. enterica* is a major foodborne pathogen generally involved in fresh produce-borne outbreaks (CDC, 2013b) and that tomatoes have been implicated in recent illness cases (CDC, 2013c), in this study we determined the efficacy of bacteriophage P22 to reduce *S. enterica* subsp. *enterica* serovar Typhimurium on tomato surface in order to demonstrate the feasibility of successful biocontrol against *Salmonella*.

MATERIALS AND METHODS

Bacterium inoculum

Host strain: *Salmonella* Typhimurium LT2 (ATCC® 19585™) inoculum was obtained by the addition of a loop of the bacterium to 6 L of trypticase soy broth (TSB, Bioxon, México) and incubated for 24 h at 37°C. Bacteria were concentrated to 500 ml using an ultra-filtration system (Hernandez-Morga et al., 2009). The bacterial concentrate was then centrifuged at 13,800×*g* for 10 min at 4°C (Thermo IEC Multi RF, 8466, USA). The pellet was washed two times with 100 ml of sterile monobasic phosphate buffered solution (PBS) [0.01 M, pH 7.2, Sigma-Aldrich, Inc. Saint Louis, MO, USA] and resuspended in 6 L of PBS. The cell suspension was adjusted to a final OD_{600} of 1 (Eppendorf, Hamburg, Germany), according to Leverentz et al. (2001). The bacterial inoculum was quantified using the spread plate technique to obtain a final concentration of viable bacteria of 10^9 CFU/ml (APHA, 1998).

Bacteriophage P22 propagation

Bacteriophage P22 was kindly provided by Dr. Charles P. Gerba (University of Arizona). In order to propagate the bacteriophage, five millilitre of the P22 stock and 50 ml of *S.* Typhimurium (10^9 CFU/ml) were added to two litters of sterile PBS, pH 7.2 (Whichard et al., 2003), and incubated in a water bath overnight at 37°C and 110 rpm (Shak R Bath 3580 R, Lab-Line Instruments, Inc, USA). The mixture was then concentrated to 500 ml with an ultra-filtration system adapted from Hill et al. (2005) and Hernández-Morga et al. (2009), followed by centrifugation at 13,800×*g* for 10 min at 4°C. The supernatant was filtered through sterile cellulose acetate 0.22 µm membrane filters (Corning®, NY, USA). The bacteriophage titer was determined by the soft agar overlay technique (APHA, 1998). The final bacteriophage concentration was 10^{11} PFU/ml.

In vitro assay for bacterial biocontrol

In order to determine the ability of bacteriophage P22 to lyse *S.* Typhimurium (ATCC® 19585™), an experiment in liquid culture at 37°C for 48 h was performed. One colony of *S.* Typhimurium was grown in 10 ml of TSB at 37°C overnight. Then, aliquots of 1 ml were individually transferred to six flasks, each containing 49 ml of pre-warmed TSB and incubated at 37°C until the OD_{600} was 0.5 (ca. 1.5×10^8 CFU/ml). The bacteriophage P22 was individually inoculated into the bacterial cultures at a theoretical MOI of 100 and 1000 (in duplicated); additionally, two flasks were not inoculated (Control). Bacterial titer was measured in Hektoen agar plates at 2, 4, 6, 8, 12, 24 and 48 h after bacteriophage inoculation. The experiment was performed in duplicate.

Tomato preparation

Roma saladette tomatoes under physiological maturity were obtained from the greenhouse of the Centro de Investigación en Alimentación y Desarrollo, Culiacán Station. Homogeneous size and free of mechanical damage tomatoes were selected and disinfected by immersion into a sodium hypochlorite solution (Fermont, México) adjusted at 300 mg/L for 10 min. Then tomatoes were immersed in a 5% sodium thiosulfate solution (Fermont, México) for 10 min, and finally rinsed with sterile purified water and stored at 4°C for 12 h prior use. Tomatoes were evaluated for the absence of microbial contaminants after the disinfection procedure.

Biocontrol of *S.* Typhimurium on tomato surface

For this purpose, the immersion method was performed as previously described by Chaidez et al. (2007) with some modifications. Briefly, 72 tomatoes were divided into three groups: the first group consisted of 24 *S.* Typhimurium -inoculated tomatoes sprayed with bacteriophage P22; the second group had 24 *S.* Typhimurium -inoculated tomatoes sprayed with sterile PBS; and the third group had 24 tomatoes sprayed with P22 alone (control). For the first two groups, tomatoes were immersed for 15 min in 6 L of bacterial suspension (10^9 CFU/ml) with constant agitation, whereas the last group (control) was not inoculated with anything. Then, all of tomatoes were dried for 1 h in two parallel sterile glass rods located in a biosafety cabinet and stored in sterilized bags (Ziploc®, SC Johnson USA) at 4°C until use (Lang et al., 2004).

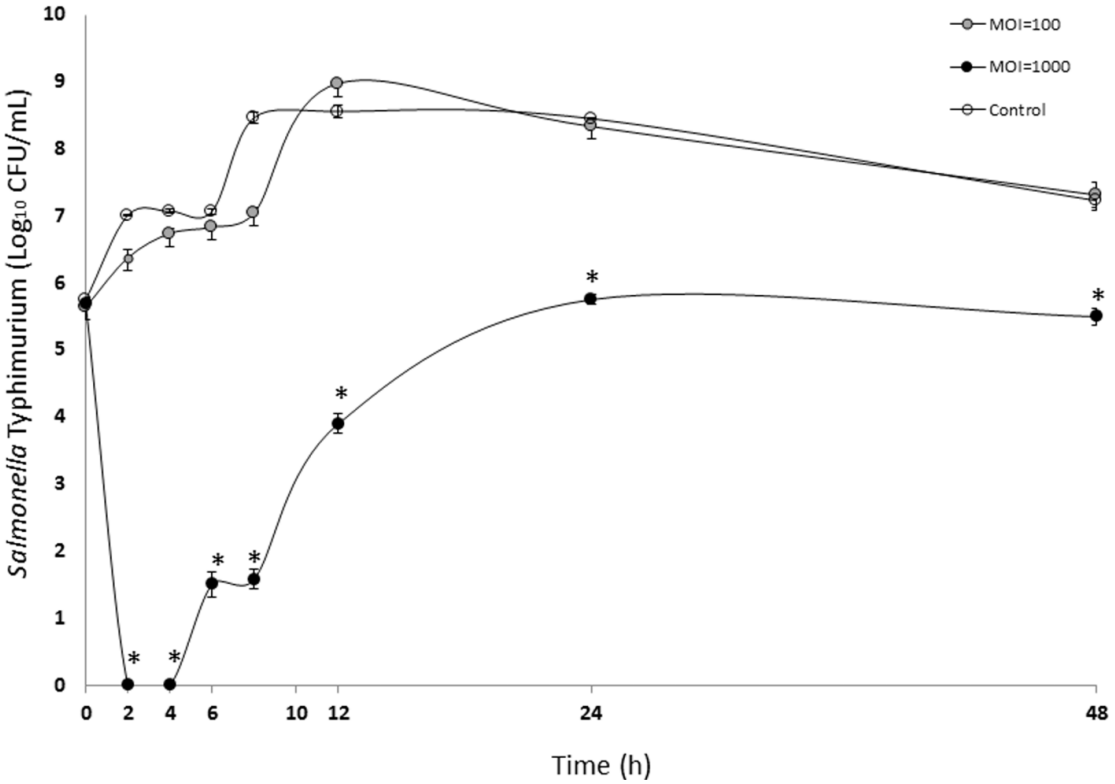

Figure 1. *In vitro Salmonella* infection by bacteriophage P22. Bacteria were infected with phage P22 at MOI of 100 and 1, 000 at 37°C for 48 h. Data represents mean Log_{10}CFU/ml ±SE. Significant differences (* *P<0.005*) compared with the non-infected control group are indicated.

Bacteriophage P22 (10^{11} PFU/ml) was sprayed onto *S.* Typhimurium -inoculated and control tomatoes for 45 s from a distance of 45 cm with a manual sprayer (SPRAY-33, Truper®, México) to simulate packinghouse spray disinfecting conditions. Following bacteria and bacteriophage inoculation, one set of each group was stored at 10°C and another set was stored at 20°C. Tomatoes were analyzed every 24 h for 7 days. Two independent experiments were performed for each temperature with 3 replicates per experiment.

Recovery and enumeration of *S.* Typhimurium and bacteriophage P22

Treated tomatoes (n=3), as described above, were randomly chosen and evaluated every 24 h for 7 d for bacteria and phage recovery. Each tomato was placed in a sterile plastic bag containing 100 ml of PBS and manually rinsed by rubbing for 1 min to remove *S.* Typhimurium and bacteriophage from the surface (Hirotani et al., 2002). Samples were serially diluted and plated in duplicate on XLD agar (Bioxon, México). Plates were incubated at 37°C and enumerated after 24 h. Bacteriophage concentration was determined using the soft agar overlay as previously described (APHA, 1998). Data was expressed as Log_{10}CFU/tomato and Log_{10}PFU/tomato, for bacterial and viral quantification, respectively.

Statistical analysis

The survival of *S.* Typhimurium on tomato surface was determined by a design of two blocks completely randomized. The persistence of bacteriophage P22 on tomato surface was analyzed with a three complete randomized block design. Efficiency of bacteriophage P22 for *S.* Typhimurium reduction on tomato surface was measured with a three complete randomized block design. Analysis of variance and multiple mean comparisons were conducted through the Tukey test. Differences with P<0.05 were considered significant. Data analysis was performed using MINITAB version 14 (Minitab Inc., State College, PA).

RESULTS

In vitro assay for bacterial biocontrol

The bacteria/bacteriophage challenge in a liquid culture is a passive approach (*in vitro*) to demonstrate the lytic effect of phages against target bacteria. In this case, high multiplicity of infection (MOI) of 100 and 1000 were used to ensure a bacteriophage/bacteria ratio in the system sufficient to infect all target bacteria by at least one phage particle, and induce bacteria lysis in a short period of time. This approach may predict the challenge behavior *in vivo*. Kinetics of *Salmonella* growth is shown in Figure 1. *Salmonella* infected with P22 at the highest MOI ratio of 1,000 showed undetectable levels of bacteria between two and four hours after infection; nonetheless,

measurable bacteria were observed after six hours with a peak value of 5.5 Log_{10}CFU/ml at 48 h after infection (Figure 1, P<0.001). Meanwhile, at the MOI of 100, bacteriophage P22 was unable to significantly reduce *Salmonella* growth, however somewhat delay was observed in bacteria growth between two and four hours after infection (Figure 1, P<=0.005). The bacterial control concentration was 7.48±0.2 Log_{10}CFU/ml after 48 h of incubation. These results indicate that phage P22 is able to lyse *Salmonella* Typhimurium at a higher MOI, however *Salmonella* regrowth at low and high MOI suggests that *Salmonella* may develop Bacteriophage Insensitive Mutants (BIM´s) or a lysogenic cycle due to experimental conditions.

S. Typhimurium survival on tomato surface

To evaluate *Salmonella* survival on tomato surface, tomatoes were inoculated with *S.* Typhimurium and stored at 10 and 20°C for seven days. Tomatoes maintained at 10°C exhibited the lowest *Salmonella* concentrations during storage, with a mean concentration of 6.29 Log_{10}CFU/tomato and maximum/minimum concentrations of 6.5 and 5.83 Log_{10}CFU/tomato, respectively, at day seven. On the other hand, tomatoes stored at 20°C exhibited higher *S.* Typhimurium concentrations than tomatoes stored at 10ºC showing an increasing tendency as the contact time increased (from 7.1 Log_{10} CFU/tomato at time zero to 8.32 Log_{10} CFU/tomato at day seven), with a mean concentration of 7.69 Log_{10}CFU/tomato, which suggest a relationship between temperature and contact time.

Bacteriophage P22 survival on tomato surface

Phage survival was assessed on tomato surface sprayed with P22 alone or in presence of *S.* Typhimurium and stored at 10 and 20°C during the seven days. Both conditions showed decrease on phage survival after the storage period. In tomatoes simultaneously inoculated with *Salmonella* and P22 at 10°C, P22 was reduced up to 7.23 Log_{10} PFU/tomato (3.79 Log_{10} PFU/tomato reduction), as compared to initial phage concentration (11.02 Log_{10}PFU/tomato); on the other hand, tomatoes inoculated only with P22, phage was reduced 4.03 Log_{10} PFU/tomato between day 0 and day seven after inoculation.

High levels of P22 were detected when tomatoes were sprayed with *S.* Typhimurium along with P22 and stored at 20°C. However, a reduction of 3.24 Log_{10}PFU/tomato was observed on day seven with a final concentration of 8.36 Log_{10}PFU/tomato. In contrast, tomatoes inoculated only with P22 showed a reduction of 4.11 Log_{10}PFU/tomato with a final concentration of 6.9 Log_{10}PFU/tomato on day seven. These results show that phage had a reduction in their concentration, regardless of the experimental condition in all cases.

Biocontrol of *S.* Typhimurium on tomato surface

Bacteriophage P22 lytic effect on *S.* Typhimurium inoculated onto tomato surface stored at 10 and 20°C during seven days is shown in Figure 2. Control groups for both temperatures where inoculated only with *Salmonella.* P22 treated-tomatoes stored at 10°C registered a constant decrease of *S.* Typhimurium concentration (Figure 2B). The highest reduction was detected on day seven with 3.02 Log_{10}CFU/tomato, compared to the baseline concentration, while the mean reduction concentration during the seven-day experiment was 1.38 Log_{10}CFU/tomato. P22 treated-tomato and stored at 20°C presented a mean bacterial reduction of 0.7 Log_{10}CFU/tomato seven days after inoculation (Figure 2C). Control groups showed higher *S.* Typhimurium concentrations as compared to tomatoes that received bacteria and P22 (Figure 2B and C).

DISCUSSION

The use of bacteriophages to reduce contamination of *S.* Typhimurium has several advantages over the use of chemical sanitizers. Firstly, bacteriophages are prokaryotic cells specific and, therefore, phages possess no harm to plants and/or animal cells. Secondly, within prokaryotic cells, bacteriophages will only lyse target bacteria, leaving fresh produce microbiota intact (Ackermann and DuBow, 1987; Gill and Abedon, 2003).

However, one of the biggest concerns of using bacteriophages as biocontrol is that they might shift from lytic to lysogenic cycle. During this cycle, phage inserts its nucleic acid into the bacterium and may transfer resistance or virulence genes (if phage contained), resulting in more virulent and resistant bacteria. However, reports have suggested that bacteriophage P22 showed low frequency of lysogenization cycle, which may be attributed to *Salmonella*'s deficiency of adenilatecyclase (*cya* gene) or to the receptive protein (*crp* gene) production (Hong et al., 1971). Furthermore, Steinberg and Gough (1976) demonstrated that *S.* Typhimurium infection by wild type phage P22 causes a lytic response to low multiplicities of infection and a lysogenic response to high multiplicities of infection (MOI >5 more than 90% are lysogenized). Merril et al. (1996) reported the potential use of a mutated variant of P22 as therapeutic agent for bacteremia. In our results, plaques developed by P22 were clear and with 0.5-0.6 mm in diameter (data not shown), which suggests that P22 may have developed lytic cycle in this experimentation (Hong et al., 1971).

The use of high phage MOI has been described to lead

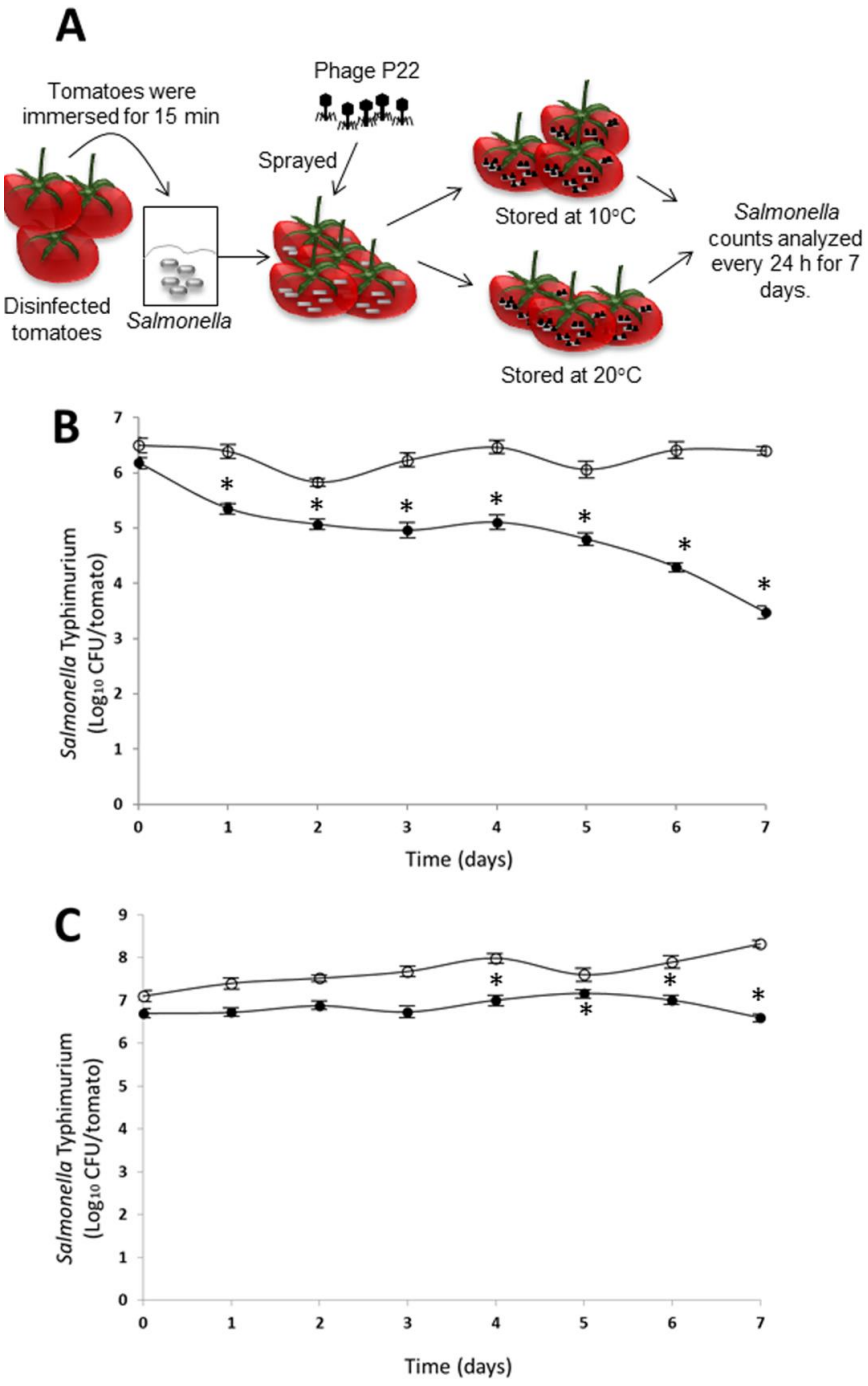

Figure 2. *Salmonella* reduction by bacteriophage P22 on tomato surfaces. (A) Schematic representation illustrating the biocontrol of *S.* Typhimurium on tomato surface. Tomatoes were sprayed with *Salmonella* in the presence of P22 (closed circles) or with *Salmonella* alone (open circles) and stored at 10°C (B) and 20°C (C) during seven days. Data represents mean Log_{10}CFU/tomato ±SE. Significant differences (*P<0.002) compared with the non-infected control group are indicated.

an early bacterial lysis because high virion particles are adsorbed on the bacterial surface and cause the weakened of the cell wall and consequently bacterial lysis, without bacteriophage particles production. Abedon (2011) describes this phenomenon as “Lysis from without (LO)”, which has been demonstrated in various bacteriophages, such as like T4 phages. Although not all phages develop LO, bacterial lysis at high MOI in our

results would be explained by the LO phenomenon.

The *in vitro* infection assay, showed that P22 was able to reduce *Salmonella* to undetectable levels 4 h after phage inoculation; however, after this period the bacterium was able to re-grow, reaching levels close to the control. This effect is most likely associated with the low proportion of P22 in a lysogenic state, which allows *Salmonella* suppression at initial stages; however, lysogenized bacteria could re-grow during long-term incubation (Gama et al., 2013). It is also important to emphasize that enrichment step (rich culture medium and optimal temperature) provide ideal conditions for bacterial growth, whereas *in vivo* conditions might present low nutrient availability or non-permissive temperatures for *Salmonella* growth.

According to our data, *S.* Typhimurium (without P22 treatments) was able to survive in tomato surface at storage temperature of 10°C for at least seven days, showing a marginal reduction of 0.67 Log_{10} CFU/tomato at the end of experimentation. Contrary to what it was observed to tomatoes stored at 20°C, where an increased concentration of *Salmonella* (1.22 Log_{10} CFU/tomato) was observed at seventh day compared to day zero, showing an increasing tendency as increases time; which suggests that *Salmonella* may be growing under these conditions. Other studies also described an increase in bacterial populations when fruits were stored at high temperatures (20 and 25°C) compared to low temperatures (10 and 4°C) where they recorded reductions up to 2 Log_{10} on fresh-cut fresh produce (Leverentz et al., 2001; Castro-del Campo et al., 2004). All microorganisms have an optimal growth temperature at which they exhibit their highest growth rate.

In this sense, the increased concentration of *Salmonella* could be attributed to the mesophilic nature of the bacteria. Zhuang et al. (1995) suggested that viability of *Salmonella* on the surface of tomatoes at 10°C might be a potential survival temperature during transport and storage prior to ripening and consumption; and even more, our results showed that at 20°C (marketing conditions) *Salmonella* was able to grow and increases its concentration, what hypothetically could represent a risk of salmonellosis outbreak if a scenery similar occurs in real conditions; therefore, suitable storage temperature of fruits and vegetables is fundamental to maintain low microbial levels and prevent pathogenic microorganism's growth (Leverentz et al., 2001).

In this investigation, bacteriophage P22 showed a positive lytic effect over *S.* Typhimurium, achieving a maximum reduction of 3.02 Log_{10}CFU/tomato in seven days at 10°C, demonstrating its effectiveness to reduce populations of a pathogenic microorganism, as reported by Sklar and Joerger (2001); Modi et al. (2001); Pao et al. (2004) and López-Cuevas et al. (2012). *Listeria monocytogenes* was reduced in high concentrations (5.7 Log_{10}CFU/ml) in fresh-cut honeydew melon, and higher reductions were achieved through a combination of bacteriophages and bacteriocins (Leverentz et al., 2003). Several studies explore bacteria reductions in fresh-cut produce with phages. For the best of our knowledge, the present study is one of the few studies to be conducted in whole fruit.

This research provides data about the effectiveness of the use of bacteriophages to reduce or eliminate pathogenic microorganisms present on fresh produce. The US-EPA determined the reasonable standard of efficiency of a disinfectant in a fresh produce surface that at least should achieve a 2 Log_{10}CFU reduction of the pathogen population (EPA, 1997). Thus, the use of bacteriophages is a natural alternative, which could benefit both conventional and organic agriculture.

Conclusion

This research data suggests that bacteriophage P22 would provide a protective effect to fresh produce through the production chain as a novel, environmentally safe alternative to control *Salmonella enterica* subsp. *enterica* on fresh produce.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors thank the National Food Safety Laboratory Research personnel (LANIIA-CIAD). We also thank Célida Martinez-Rodríguez for her technical assistance.

REFERENCES

Abedon ST (2011). Lysis from without. Bacteriophage 1(1):46-49.

Ackermann HW, DuBow MS (1987). Practical applications of bacteriophages. In Viruses of prokaryotes: general properties of bacteriophages. CRC Press, Boca Raton, Fla., USA, pp. 143-158.

American Public Health Association [APHA] (1998). Standard methods for the examination of water and wastewater, 20th edition. American Public Health Association, Washington, DCAPHA.

Behravesh CB, Rajal K, Mody MD, Jungk J, Gaul L, Redd JT, Chen S, Cosgrove S, Hedican E, Sweat D, Chávez-Hauser L, Snow SL, Hanson H, Nguyen T, Sodha SV, Boore AL, Russo E, Mikoleit M, Theobald L, Gerner-Smidt P, Hoekstra RM, Angulo FJ, Swerdlow DL, Tauxe RV, Griffin PM, Williams IT (2011). 2008 Outbreak of *Salmonella* Saintpaul Infections Associated with Raw Produce. New. Engl. J. Med. 364(10):918-927.

Beuchat LR (1996). Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59(2):204-216.

Castro-del Campo N, Chaidez QC, Rubio CW, Valdez TJV (2004). Sobrevivencia de *Escherichia coli* y *Staphylococcus aureus* en frutos mínimamente procesados. Rev. Cub. Salud Pública. 30(1):83-86.

CDC (2008). Outbreak of *Salmonella* serotype *Saintpaul* infections associated with multiple raw produce items - United States. Available from http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5734a1.htm.

Accessed 30/10/2014.
CDC (2013a). FDA Investigates Multistate Outbreak of *Salmonella* Saintpaul linked to cucumbers supplied by Daniel Cardenas Izabal and Miracle Greenhouse. Available at: http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm349461.htm. Accessed 13/11/ 2014.
CDC (2013b). Reports of selected *Salmonella* outbreak investigations. Available at. http://www.cdc.gov/salmonella/outbreaks.html. Accessed 13/11/2014.
CDC (2013c). Multistate Outbreak of *Salmonella* Typhimurium Infections Linked to Tomatoes (Final Update). Available at: http://www.cdc.gov/salmonella/typh2006/index.html. Accessed 13/11/2014.
Chaidez QC, López J, Vidales J, Castro-del Campo N (2007). Efficacy of chlorinated and ozonated water in reducing *Salmonella typhimurium* attached to tomato surfaces. Int. J. Environ. Health Res. 17(4): 311-318.
DeWaal CS, Barlow K, Alderton L, Jacobson MF (2007). Outbreak alert closing the gaps in our federal-food safety net. Center for Science in the Public Interest. Available from: http://www.cspinet.org/foodsafety/outbreak_alert.pdf. Accessed 03/12/2014.
Environmental Protection Agency [EPA] (1997). A Set of scientific issues being considered by the agency in connection with the efficacy testing issues concerning public health antimicrobial pesticides. Scientific Advisory Panel September 1997 Meeting Final Report. Available at: http://www.epa.gov/scipoly/sap/1997september/finalsep.htm. Accessed 03/10/2014.
Fatica MK, Schneider KR (2011). *Salmonella* and produce: Survival in the plant environment and implications in food safety. Virulence 2(6):573-579.
Gama JA, Reis AM, Domingues I, Mendes-Soares H, Matos AM, Dionisio F (2013). Temperate Bacterial Viruses as Double-Edged Swords in Bacterial Warfare. PLoS ONE 8(3):e59043.
Gill JJ, Abedon TS (2003). Bacteriophage ecology and plants. APSnet Feature. Available at: http://www.apsnet.org/online/feature/phages/abedon.pdf. Accessed 21/12/2014.
González FM, Rojas HR (2005). Foodborne diseases and PCR: Disease Prevention and diagnosis. Enfermedades transmitidas por Alimentos y PCR: Prevención y diagnóstico. Salud Públ. Méx. 47(5): 388-390.
Hanning IB, Nutt JD, Ricke SC (2009). Salmonellosis Outbreaks in the United States Due to Fresh Produce: Sources and Potential Intervention Measures. Foodborne Pathog. Dis. 6(6):635-648.
Hedberg CW, Angulo FJ, White KE, Langkop CW, Schell WL, Stobierski MG, Schuchat A, Besser JM, Dietrich S, Helsel L, Griffin PM, McFarland JW, Osterholm MT (1999). Outbreaks of salmonellosis associated with eating uncooked tomatoes: Implications for public health. Epidemiol. Infect. 122:385-393
Hernandez-Morga J, Leon-Felix J, Peraza-Garay F, Gil-Salas BG, Chaidez C (2009). Detection and characterization of hepatitis A virus and Norovirus in estuarine water samples using ultrafiltration - RT-PCR integrated methods. J. Appl. Microbiol. 106(5):1579-1590.
Hill VR, Polaczyk AL, Hahn D, Narayanan J, Cromeans TL, Roberts JM, Amburgey JE (2005). Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. Appl. Environ. Microbiol. 71(11):6878-6884.
Hirotani H, Naranjo J, Moroyoqui PG, Gerba CP (2002). Demonstration of Indicator Microorganisms on Surface of Vegetables on the Market in the United States and Mexico. J. Food Sci. 67(5):1847-1850.
Hong J, Smith G, Ames B (1971). Adenosine 3':5'-Cyclic Monophosphate Concentration in the Bacterial Host Regulates the Viral Decision between Lysogeny and Lysis. Proc. Natl. Acad. Sci. USA 68(9):2258-2262.
Lang MM, Harris LJ, Beuchat LR (2004). Evaluation of inoculation method and inoculum drying time for their effects on survival and efficiency of recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated on the surface of tomatoes. J. Food Prot. 67(4):732-741.
Leverentz B, Conway W, Alavidze Z, Janisiewicz W, Fuchs Y, Camp M, Chighladze Y, Sulakvelidze E (2001). Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: A model study. J. Food Prot. 64(8):1116-1121.
Leverentz B, Conway WS, Camp MJ, Janisiewicz WJ, Abuladze T, Yang M, Saftner R, Sulakvelidze A (2003). Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Appl. Environ. Microbiol. 69(8):4519-4526.
López-Cuevas O, Castro-del Campo N, León-Félix J, Valdez-Torres, Chaidez C (2012). Evaluation of bacteriophage Av-08 for simultaneous biocontrol of *Salmonella* Montevideo and *Escherichia coli* O157:H7 in experimentally contaminated chicken skin. J. Food Saf. 32:305-310.
Lynch MF, Tauxe RV, Hedberg CW (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. Epidemiol. Infect. 137:307-315.
Merril C, Biswas B, Carltont R, Jensen N, Creed J, Zullo S, Adhyat S (1996). Long-circulating bacteriophage as antibacterial agents. Proc. Natl. Acad. Sci. 93(8):3188-3192.
Modi R, Hirvi Y, Hil A, Griffiths MW (2001). Effect of phage on the survival of *Salmonella enteritidis* during manufacture and storage of cheddar cheese made from raw and pasteurized milk. J. Food Prot. 64(7):927-33.
Pao S, Randolph SP, Westbrook EW, Shen H (2004). Use of bacteriophages to control *Salmonella* in experimentally contaminated Sprout Seeds. J. Food Sci. 69(5):127-130.
Parish ME, Beuchat LR, Suslow TV, Harris LJ, Garret EH, Farber JN, Busta FF (2003). Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. Comprehens. Rev. Food Sci. Food Saf. 2(1):161-173.
Scharff RL (2010). Health-Related Costs From Foodborne Illness In The United States. For the Produce Safety Project at Georgetown University. Available at: http://publichealth.lacounty.gov/eh/docs/ReportPublication/HlthRelatedCostsFromFoodborneIllinessUS.pdf. Accessed 21/09/2015.
Sklar IB, Joerger RD (2001). Attempts to Utilize Bacteriophage to Combat *Salmonella enterica* serovar Enteritidis Infection in Chickens. J. Food Saf. 21(1):15-29.
Steinberg B, Gough M (1976). Bacteriophage P22 lysogenises efficiently at high multiplicities of infection because *Salmonella typhimurium* DNA synthetic capacity is limited. Nature 263:54-56.
Waseh S, Hanifi-Moghaddam P, Coleman R, Masotti M, Ryan S, Foss M, MacKenzie R, Henry M, Szymanski CM, Tanha J (2010). Orally Administered P22 Phage Tailspike Protein Reduces *Salmonella* Colonization in Chickens: Prospects of a Novel Therapy against Bacterial Infections. PLoS ONE 5(11):13904.
Whichard JM, Sriranganathan N, Pierson FW (2003). Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage felix 01 in liquid culture and on chicken frankfurters. J. Food Prot. 66(2):220-225.
Xu L (1999). Use of ozone to improve the safety of fresh fruit and vegetables. Food Technol. 53(10):58-62.
Zhuang RY, Beuchat LR, Angulo FJ (1995). Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. Appl. Environ. Microbiol. 61(6):2127-2131.

African Journal of Microbiology Research

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals