*International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-4, Issue-6, June 2018*
*ISSN: 2395-3470*
*www.ijseas.com*

# Biocontrol traits of *Bacillus* strains isolated from *Osyris lanceolata* rhizosphere in the Northern Burundi

Venant NIHORIMBERE[1] & Marc ONGENA[2]

[1]University of Burundi, Faculty of Agronomy and Bio-Engineering, Department of Food Science and Technology, 02 avenue de l'UNESCO, P.O. Box 2940 Bujumbura-Burundi
[2]University of Liège, Gembloux Agro Bio Techn, Microbial and Plant Interactions, 02 Passage des déportés, 5030 Gembloux

**Abstract**
In the Northern provinces of Burundi such as Kirundo and Muyinga, natural climate conditions favor the wild plant development named *Osyris lancelata* which is greatly known for its medicinal properties and has since decades attracted significant attention as potential perfumery oils extraction. However, this plant development and production is hampered by fungi pathogens dominating in that area resulting in economic losses. In this study, we wanted to evaluate the potential of *Bacillus* spp. as biocontrol agent to fight some fungal pathogens infesting *Osyris* species. To the authors' knowledge, this report is the first to characterize biocontrol traits of *Bacillus* species cohabiting the rhizosphere of *Osyris lanceolata.* A total of twenty six *Bacillus* strains were isolated; and four of which (15%) were screened for their important antifungal activity against *Fusarium oxysporium, Botrytis cinerea, Aspergillus niger, Cladosporium cucumerinium* and *Alternaria alternata*. The screened isolates were based on *16S r-RNA* and *gyrase-A* (*gyr-A*) genes analyses, and were closely related to *B. amyloliquefaciens, B. velezensis*, *B. subtilis sub sp spizezenii and Panaebacillus polymyxa* species. The strain S499 has been selected as the model *Bacillus* strain based on its high *in vitro* antagonistic activity correlating with a huge potential to secrete fungitoxic cyclic lipopeptides. Most of the isolates produced in opt-medium cyclic lipopeptides such as *surfactins* and *iturins*. The *B. amyloliquefaciens* (9SRTS) and the model type strain (S499) produced *Iturin A* and the *Bacillus velezensis* (26SRTS) and the commercialized strains (FZB42) produced Bacillomycin D. The production of fengycins was detected only in these two isolates. However, the *Paenebacillus polymyxa (18SRTS)* didn't produce any type of lipopeptides. All the tested *Bacillus* isolates produced cellulase but the protease activity was observed only in the *B. amyloliquefaciens* species (*9SRTS)* and (*FZB42)*. The *Bacillus* strains synthesized 6 to 52 µg/ml of indol-3 acetic acid and siderophores with more than 10 mm wide orange zones on chrom azurol S. These results suggested that the *Bacillus* strains isolated in this work have strong effects against fungi tested *in vitro* and may be used further as bioinoculants to improve crop growth and health.

**Keywords**: *Bacillus*; *Osyris lanceolata;* rhizosphere; antifungal activity; biocontrol traits

## 1. Introduction

*Osyris lanceolata* (East African Sandalwood) is an evergreen shrub to small tree (1-6 m) in the family *Santalacea*. This species is mainly a dryland species with spatial distribution in Southern Africa and Eastern African countries such as Kenya, Tanzania and Burundi. In Burundi, the species has been recorded in the Northern provinces like Kirundo and Muyinga. It occurs as isolated individuals, in close association with other woody species, and does not occur communally in large numbers. Traditional healers have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms [1].

Locally, the wood is used to smoke milk containers to obtain an excellent aroma. At industrial level, the wood and the bark oils are reportedly used to extract highly valued perfumes and other cosmetics [2]. It is also used as an ingredient for quality lotions and rare soaps. The plant is highly valued by the local communities for its medicinal properties, providing income generation [3]. For instance, it is reported to threat gastroenteritis, stomach aches, diarrhea, ulcers, snakebites [2]. The species has been of little importance, until recently when it was captured in the limelight due to its overexploitation to meet the international demand for its perfumery and medicinal products in the treatment of hepatitis. Since then, demands for this plant medical products are increasing due to growing recognition of their benefic effects of being non-toxic, having no side effects for health and their subsequent affordable prices [4]. Nevertheless, this plant is getting threatened by some diseases especially of fungi origins, impacting the economic income generated by that plant. Many diseases symptoms are caused by different fungi and may be attributed to

*Alternaria alternata*, *Alternaria porri*, *Botrytis cinerea*, *Drechslera (Cochliobolus) hawaiiensis*, *Fusarium avena- ceum*, *Fusarium culmorum* and *Sclerotinia sclerotiorum* [5-6]. The application of agrochemicals is necessary to protect this species but these products have several negative side effects [7]. The plant growth-promoting bacteria (PGPB) can be used as an alternative way to the use of such xenobiotic compounds [8]. It has been noted by many researchers that *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Azospirillum*, *Klebsiella*, and *Enterobacter* isolated from the rhizosphere of various crops, showed synergistic effects on plant growth [9]. PGPB can affect plants directly or indirectly [10]. The indirect mechanisms consist of the lessening or prevention of one or more phytopathogenic organisms deleterious effects [11-12], including several mechanisms as, i) antibiotic production, ii) depletion of iron from the rhizosphere, iii) synthesis of antifungal metabolites, iv) production of fungal cell wall lysing enzymes, v) competition for sites on roots and vi) induced systemic resistance. The direct promotion of plant growth, for the most part, of PGPR, entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients like nitrogen (N) or phosphorus (P) from the environment [13] or the production of phytohormones which stimulate plant growth [14]. It has been reported that the PGPR of apple showed the growth-promoting effect with possible involvement of the plant growth regulators indole-3-acetic acid (IAA) and cytokinin [15]. The *Bacillus* species offer over the other genera several advantages because of their capacity to produce spores in unfavorable environmental conditions which facilitates the conversion of spore suspensions to powder formulations without killing bacteria [16]. Thus, a number of *Bacillus* and *Paenibacillus* spp. have been developed commercially as biological fungicides, insecticides and nematicides or generic plant growth promoters, and their use in agriculture has recently been reviewed [17-18].

This study aimed to answer to the question: Is there a *Bacillus* spp. in the rhizosphere of *Osyris* species that could fight some fungi devasting that plant. To the authors' knowledge, this work is the first to characterize *Bacillus* biocontrol isolates from a rhizosphere of *Osyris lanceolata*. The main objectives were to: (I) isolate the predominant antifungal *Bacillus* species; (II) evaluate their *in vitro* biocontrol traits in order to use them further as bio-inoculants strains.

## 2. Material and methods

### *2.1. Isolation of aerobic endospore-forming Bacillus strains*

The Sample soil was collected from the rhizosphere of *Osyris lanceolata* where one gram of soil sample was mixed with nine ml of 0,9 % of sodium chloride (NaCl). Serial decimal dilutions were prepared and pasteurized (12 min, 80 °C) to eliminate non-sporulated bacterial forms. The *Bacillus* strains were isolated by plating the treated decimal dilutions on a rich LB agar medium [19]. After 24h of incubation at 30 °C, the grown bacteria were purified and stored by freezing at -80 °C.

### *2.2 Antagonism test*

The antifungal activity was investigated on PDA Petri dishes by the dual culture technique. The bacteria were inoculated on the edge of the plates and the mycelial plugs (5 mm) of *Fusarium oxysporium, Botrytis cinerea, Aspergillus niger, Cladosporium cucumerinium* and *Alternaria alternata* were deposited in the center. Plates were incubated at room temperature during 2 to 6 days, depending on the tested fungi growth rate. Mycelia growth inhibition was calculated as the reduction percentage of mycelia expansion compared with control plates without bacteria [20]. Mean values and standard deviations were calculated from three repetitions used for each fungal strain.

### *2.3 Spore yields determination*

*Bacilli* isolates were grown at 30 °C for 72 h in agitated flasks (180 rpm) in a liquid medium (named Opt medium) as described by Jacques *et al.* (1999). Bacterial suspensions obtained at the end of culture were treated by heating them at 80 °C for 12 min followed by immediate cooling to room temperature in cooled water. After sequential dilution, 100 µl of samples were spread on LB plates. These plates were incubated at 37 °C for 24 h, and the spores yield were determined by counting colony-forming unit (CFU) per milliliter or spores per milliliter. All the experiments were performed as three replicates.

### *2.4 Bacillus strains identification*

A total DNA was extracted from *Bacillus* liquid cultures by the wizard Genomic DNA purification kit (Promega), using the manufacturer's instructions. The primers used for the PCR amplification were the universal primers 16SP0 (GAA GAG TTT GAT CCT GGC TCAG) and 16SP6 (CTA CGG CTA CCT TGTTAC GA) for *16S r-RNA gene* (22) and *gyr-A.f* (CAG TCA GGA AAT GCG TAC GTC CTT) and *gyr-A.r* (CAA GGT AAT GCT CCA GGC ATT GCT) for *gyr-A* gene [23]. The purification of the PCR products was achieved using the GFX PCR DNA and Gel Band Purification Kit. The amplified genes were sequencing using the same primers sited above and the obtained sequences were corrected by the Bio-edit program. To identify the *Bacillus* isolates, the

resulted sequences were compared to those previously published in Genbank using the BLASTN program.

### *2.5 Detection of enzymatic activities chitinase, protease and cellulase*

Enzymatic activities were assessed in a qualitative way through a halo formation on solid media containing colloidal chitin, milk powder and carboxymethyl cellulose substrates to reveal chitinase, protease and cellulase activities [24].

### *2.6 Lipopeptides production*

The *Bacillus* strains were grown in agitated flasks (180 rpm) containing the opt medium at 30 °C for 72 h. Cultures were centrifuged at 15,000 g for 20 min. The supernatant samples were loaded on C18 solid-phase extraction cartridges (900 mg, Alltech) and lipopeptides were desorbed with 100 % ACN. The resulting samples were analyzed by reverse phase HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyzer) on a X-terra MS (Waters) 150*2.1 mm, 3.5 µm column as previously described by Nihorimbere *et al.* (2012) [25]. In this work, a single elution gradient allowing the simultaneous measurement of all three lipopeptides families was used. The water acidified with 0.1 % formic acid (A) and acetonitril (ACN) acidified with 0.1 % formic acid (B) were used as a mobile phase. The flow rate was maintained at 0.5 mL $min^{-1}$ and the column temperature at 40°C, with a gradient of 35min (43 % - 80 %, vol/vol ACN in 18 min; 100 %, vol/vol ACN for 9 min, and 43 %, vol/vol ACN in 8 min). Compounds were identified on the basis of their retention times compared to purified standards. The identity of each homologue was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization conditions in the MS as source temp., 130 °C; desolvation temp., 250 °C; nitrogen flow, 500 l/h; cone voltage, 70 V. The positive ion mode was used for analysis of all three families because a higher signal/background ration was obtained compared to negative ion recording.

### *2.7Production of Indole 3 acetic acid (IAA)*

Indol acetic acid production was assayed calorimetrically by using the Salkowski reagent (0.01 M $FeCl_3$ in 36% $H_2SO_4$) as described by [26]. TGE (composition per liter: triptone 5 g, glucose 10 g, beef extract 3g ) supplemented with 5 mM of L-tryptophan was inoculated with the *Bacillus* isolates, incubated at 30 ºC and 160 rpm for 4 days. 300 µl of the Salkowski reagent was added to 100 µl of cultures in a microplate. After 15 minutes in the dark, color reaction intensity was estimated by measuring absorbance at 535 nm. The TGE containing L-tryptophan mixed with the salkowski reagent was used as a blank. The IAA concentration was determined with a standard curve. The test was achieved in duplicate.

### *2.8 Production of siderophores*

The *Bacillus* isolates were streaked on azurol S medium (CAS-medium) as described by Husen (2003) [27] and the siderophore production was indicated by the formation of yellow-orange halos around the colonies after incubation. This test was achieved in three replicates.

## 3. Results

### *3.1. Isolation, identification and spore yields of Bacillus isolates*

Twenty six *Bacillus* strains were isolated from the rhizosphere of *Osyris lancelata*. Four isolates were screened for their ability to inhibit growth of some phytopathogenic fungi such as *Fusarium oxysporium, Botrytis cinerea, Aspergillus niger, Cladosporium cucumerinium*and *and Alternaria alternata* (Fig. 1).

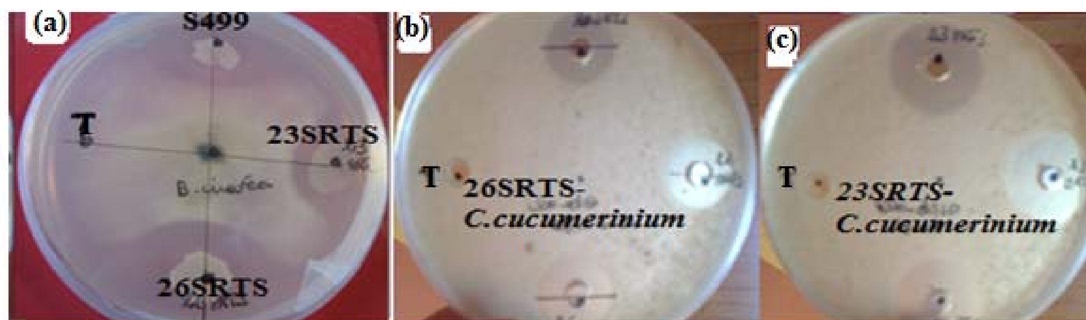


**Fig. 1** *In vitro* growth inhibition of the phytopathogens; *Botrytis cinerea* (a) and *Cladosporium cucumerinium* (b and c), developed by the *Bacillus* isolates and their supernatants, in comparison with the commercialized type strain (S499). The bacteria were isolated from the rhizosphere of *Osyris lanceolata*. The antagonism test was carried on potato dextrose agar (PDA), by the dual culture technique. The tested fungi, the bacterial cells (a) and supernatant (b and c) were inoculated at the same time and the antagonism was scored after plate's incubation for 2-6 days at 25 °C

The isolate (*9SRTS*) identified here as *B. amyloliquefaciens* had the most important antifungal activity in comparison to the other isolates and the commercialized type strains S499 and FZB42 and it reached 83 % of *F. oxysporum* growth inhibition. The identification of these isolates using the molecular analyses of the PCR amplified 16S r-DNA fragments showed that they clustered in two genera of the *Bacillacieae*, namely, *Bacillus* and *Paenibacillus.* The average similarity values of the *16S r-RNA* sequences from *Bacillus spp.* was 99.1% and the strains were indistinguishable from one another. However, the *gyr-A* gene sequences analysis clarified further the identification of the *Bacillus spp*. isolates. Thus, they were closely related to *B. amyloliquefaciens, B. subtilis sub sp spizezenii* and *B. velezensis*. The strain (18SRTS) was identified based on 16Sr-RNA as a *Panaebacillus polymyxa*. This strain had a very low sporulation yields. However, the other *Bacillus* isolates had high sporulation levels which varied between 0, 8 x$10^9$ and 2.5 x$10^9$ spores/ml (**Table 1**).

**Table 1. Production of spores by *Bacillus* strains in flasks cultivation**.

| Sampled site (number of antagonistic *Bacillus* isolates/ number of total *Bacillus* strains) | *Bacillus* isolates | Spore yields (×$10^8$ spores/ml) |
|---|---|---|
| Agricultural soil in Northern Burundi: (4/26 isolates) | (9SRTS) *B.amyloliquefaciens* | 25±1 |
| | (18SRTS) *Paenibacillus polymyxa* | ND |
| | (23SRTS) *B.subtilis sub sp spizezenii* | *8±2* |
| | (26SRTS) *B. velezensis* | *20±1* |
| Model type strain | (S499) *B. amyloliquefaciens* | 18±1 |

### *3.2. Identification of antifungal compounds produced by Bacillus isolates*

The *Bacillus* strains isolated in this work showed an important growth inhibition percentage of *Fusarium oxysporium* and *Botrytis Cinerea* (39 % - 83 %). The most important antifungal activity was observed in the *B. amyloliquefaciens (*9SRTS) and the *B. velezensis* (26SRTS) in comparison with the other *Bacillus* species. The LC/ESI-MS analysis of the *Bacillus* supernatant obtained after 72 h of cultivation in opt medium at 30 °C and 160 rpm showed that most of *Bacillus* isolates produced surfactin and iturin. Furthermore, two types of iturin were produced. The iturin(A) by the *B. amyloliquefaciens* (9SRTS) and the commercialized type strains (S499) and the Bacillomycin(D) by the *Bacillus velezensis* (26SRTS) and the commercialized type strains (FZB42) which were the only strains producing fengycins. However, the *Paenibacillus polymyxa* (18SRTS) didn't produce any type of lipopeptides. For the cell-wall degrading enzymes, all the *Bacillus* isolates here produced cellulase but the protease activity was found only in the *B. amyloliquefaciens* species (*9SRTS)* and (*FZB42)* and non strain produce chitinase (**Table 2**).

**Table 2. Antifungal activity, lipopeptides and cell-wall degrading enzymes production**

| | Fungal growth inhibition (%)[A] | | Lipopeptide homologues production[B] | | | Cell-wall degrading enzymes production | |
|---|---|---|---|---|---|---|---|
| *Bacillus* isolates | *F. oxysporium* | *B. cinerea* | Iturin | Fengycin | Surfactin | Protease activity[C] | Cellulase activity[D] |
| (9SRTS) *B.amylolyquefaciens* | 83 ± 2 | 65 ±2 | It A + | - | + | + | ++ |
| (18SRTS) *Paenibacillus polymyxa* | 66 ±3 | 66 ±4 | - | - | + | - | ++ |
| (23SRTS) *B.subtilis* sub sp spizezenii | 39 ± 2 | 48 ±2 | It A + | - | + | - | + |
| (26SRTS) *B.velezensis* | 60 ±2 | 61 ±2 | It B.D + | + | + | - | ++ |
| Commercialized type strain (FZB42) *B. amylolyquefaciens* | 68 ±4 | 66 ±2 | It B.D + | + | + | + | + |
| Model type strain (S499) *B. amylolyquefaciens* | 68±2 | 72 ±1 | It A + | - | + | - | + |

[A] The antagonism experiments were carried in replicates for each bacterial fungal combination on PDA medium. Data were expressed as the percentage of reduction of mycelium expansion compared with control plates without bacteria.
[B] Lipopeptides produced by the *Bacillus* isolates were assessed by LC/ESi-MS analyses of bacterial supernatants, obtained by the bacteria cultivation in opt medium during 72h at 30°C and 160rpm. It A: Iturin (A); It B.D: Iturin Bacillomycin (D).

[C] *In vitro* protease activity (plate assay): +represents hydrolysis; - represents no hydrolysis
[D] *In vitro* cellulase activity: +represents 10 - 15mm wide clear zone; ++ represents 15-20 mm wide clear zone; +++represents >20 mm wide clear zone.

### *3.3. Siderophores and IAA production*

The *Bacillus spp.* isolates (*9SRTS, 23SRTS and 26SRTS*) and the commercialized type strain FZB42 produced low concentrations of IAA (7 to 14 µg/ml) and high levels of siderophores (<10mm yellow orange zone diameter). However, the *Paenibacillus polymyxa* (18SRTS) didn't produce siderophores and produced higher concentrations of IAA which reached 53µg/ml (**Table 3**).

**Table 3. Phosphate solubilization and production of Indole-3-acetic acid (IAA) and siderophores**

| Bacillus strains | IAA (µg/ml)[A] | Siderophores production [B] |
|---|---|---|
| (9SRTS) *B. amylolyquefaciens* | 7±2 | +++ |
| (18SRTS) *Paenibacillus polymyxa* | 53±2 | - |
| (23SRTS) *B.subtilis sub-sp spizezenii* | 14±1 | +++ |
| (26SRTS) *B.velezensis* | 6±1 | +++ |
| (FZB42) *B. amylolyquefaciens* | 5±1 | +++ |

[A] Colorimetric scale of IAA production
[B] *In vitro* siderophores production: **-** represents the absence of siderophores production, **+++** represents >10 mm wide yellow- orange zone.

## 4. Discussion

In this study 15 % of the *Bacillus* strains isolated from the rhizosphere of *Osyris lanceolata* had the capacity to substantially inhibit growth of some phytopathogenic fungi like *Fusarium oxysporium, Botrytis cinerea, Aspergillus niger, Cladosporium cucumerinium* and *Alternaria alternata*. These bacteria were identified based on *16S r-RNA* and *gyr-A* gene sequences as *B.amyloliquefaciens*, *B.subtilis sub sp spizezenii*, *B.velezensis* and *Panaebacillus polymyxa.* Similar results were found by Nihorimbere (2014) [28]. Thus, the *Bacillus* strains isolated from the rhizosphere of *Osyris lanceolata* showed an antagonistic effect against *Rhizoctonia solani*, *Verticillium dahliae* and *Fusarium culmorum*. The strains were identified based on *16S r–RNA* sequences analyses as *B. subtilis, B. vallismortis, B. amyloliquefaciens and B. atrophaeus.*

*Bacteria* belonging to the *Bacillus subtilis spp group* produced 0, 8 x$10^9$ to 2.5 x$10^9$ spores/ml. However, the *Panaebacillus polymyxa* strain had a very low sporulation yield (**Table 1**). Previously, the spore yields in submerged optimized cultivation were lower and estimated at 8.3 x$10^8$ spores /ml [29]. The antifungal activity detected in this work can be explained by the capacity of the *Bacillus* isolates to produce the cyclic lipopeptides (cLPs) and cell-wall degrading enzymes as previously showed [30-31]. The cLPs were described previously [32] as a major class of *Bacillus* peptide antibiotics, including the surfactin, iturin and fengycin families which may vary in the type of amino acid residues, the nature of the peptide cyclization and in the nature, length and branching of the fatty acid chain. In this study, almost all *Bacillus* isolates produced surfactin and iturin while fengycin was only detected in the commercialized type strain FZB42 which was used as the model/commercial strain in this work. In previous works, it has been reported that a very limited number of strains are reported to co-produce fengycin homologues [33]. The production of the phytohormone (IAA) and siderophores by *Bacillus* species has been investigated in many studies, IAA stimulate the plant growth and siderophores chelate iron (Fe) and deprive the phytopathogenic fungi of it [34-35]. In this work, IAA and siderophores were produced by all the *Bacillus* strains studied. Our data show that such fungitoxicity traits of *Bacillus* isolates may thus also be extended to the fungi pathogenic strains *in planta* even within other plants.

## 5. Conclusion

*Bacillus* strains isolated from the rhizosphere of *Osyris lanceolata* have interesting biocontrol and plant growth promotion traits and high spore yields which enable them to be a feasible product that can be used to improve crop/plant systems. As this beneficial effect was observed against the phytopathogenic fungi inhabiting different locations, this suggests that the use of such bacteria can probably be extended to other regions of Burundi with similar climate to combat phytopathogenic threat wherever in other plantations. Such implementation of microbial biocontrol agents in those planting systems is even more important considering a socio-economic context where many farmers do not have access to conventional pesticides because these products are just not available or too expensive and are not well informed on the appropriate and safe use of these harmful chemicals. The authors are working to farther develop the *in planta* model protection of

that plant with medicinal interest and great economic importance.

## 6. References

[1] S. Girma, M. Giday, B. Erko & H.Mamo (2015). Effect of crude leaf extract of Osyris quadripartita on Plasmodium berghei in Swiss albino mice. *BMC Complementary and Alternative Medicine*, 15(184), 1-9.

[2] A. Mohamed M. & D. K. Musya (2005). The status of *Osyris lanceolata* Hochst. & Stendel "the money tree": An overexploited medicinal plant in southern drylands, Kenya. A preliminary report presented to National project coordinator (NPC)-Natural Forests, KEFRI.

[3] G. D. Pamplona & M. D. Roger (2000). Plants for the stomach. Encyclopedia of medicinal plants, vol 2.

[4] B.P. Muley, S.S. Khadabadi, N.B. Banarase (2009). Phytochemical Constituents and Pharmacological Activities of Linn (Asteraceae): A Review. *Tropical Journal of Pharmaceutical Research* 5, 455-465.

[5] S.C. Shrotri, J.S. Gupta & R.N. Srivastava (1985). Seed-borne fungi of *Calendula offcinalis* and their control. *Indian Phytopathol* 38, 142-143.

[6] D. Pieta (1991). Mycoflora of *Calendula officinalis* L. seeds. *Acta Agrobotanica* 44, 1-2.

[7] J.M. Whipps (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* 52, 487–511.

[8] S. Compant, B. Duffy, J. Nowak, C. Clément & E.A. Barka (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71,4951–4959.

[9] J.W. Kloepper, J. Leong, M. Teintze & M.N. Scrhorth (1980). Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature* 286, 885–886.

[10] B.R. Glick, D.M. Karaturovi & P.C. Newell (1995). A novel procedure for rapid isolation of plant growth-promoting pseudomonads. *Can. J. Microbiol* 41, 533-536.

[11] B.R. Glick, C.L. Patten, G. Holguin & D.M. Penrose (1999). Biochemical and genetic mechanisms used by plant growth promoting bacteria. *J Theor Biol* 190, 63–68.

[12] C. Dunne, J.J. Crowley, Y.M. Loccoz, D.N. Dowling, F. de Bruijn & F.O. Gara (1993). Biological control of Pythium ultimum by Stenotrophomonas maltophilia W81 is mediated by an extracellular proteolytic activity. *Microbiology* 143, 3921- 3931.

[13] B.R. Glick (1995). The enhancement of plant growth by free-living bacteria. *Can.J.Microbiol* 41, 109-117.

[14] V. Leclère, M. Béchet, A. Adam, B. Wathelet, M. Ongena, P. Thonart and P. Jacques (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organisms antagonistic and biocontrol activities. *Appl Environ Microbiol* 71, 4577–4584.

[15] R. Aslantas, R. Cakmakci, F. Sahin (2007). Effect of plant growth promoting rhizobacteria on young apple tree growth and fruit yield under orchard conditions. *Sci. Hort* 111, 371-377.

[16] R. Lolloo, D. Maharaih, J. Görgens & N. Gardiner (2010). A downstream process for production of a viable and stable *Bacillus* cereus aquaculture biological agent. *Applied Microbiology and Biotechnology* 86,499-508.

[17] T.C. Palit & R.R. Belanger (2001). Biological control in greenhouse systems. *Annu. Rev. Phytopathol* 39,103-133.

[18] Z.A. Siddiqui & L. Mahmood (1999). Role of bacteria in the management of plant parasitic nemathodes. A review. *Bioressource Tech* 69,167-179.

[19] L. Seldin, J.D. van Elsas & E.G.C. Penido (1983). *Bacillus* nitrogens fixers from Brazilian soils. Plant Soil 70,243-255.

[20] Y. Toure, M. Ongena, P. Jacques, A. Guiro & P. Thonart (2004). Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by Botrytis cinerea on apple. *J Appl Microbiol* 96,1151-60.

[21] P. Garry, J.L. Vendeuvre & M.N. Bellon-Fontaine (1998). Surface properties and adhesion of *Bacillus cereus* and *Bacillus subtilis* to polyurethane- influence of growth temperature. *J. Dispersion Sci. Technol* 19,1175–1197.

[22] M. Ventura, M. Elli, R. Reniero & R. Zink (2001). Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiology Ecology* 36, 113-121.

[23] M.S. Roberts, L.K. Nakamura & F.M. Cohan (1994). *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int. J. Syst. Bacteriol*, 44, 256–264.

[24] H. Ariffin, N. Abdullah, M.S. Umi Kalsom, Y. Shirai, M.A. Hassan (2006). Production and

characterisation of cellulase by *Bacillus pumilus*EB3. *Int. J. Eng. Technol* 3, 47-53.

[25] V. Nihorimbere, H. Cawoy, A. Seyer, A. Brunelle, P. Thonart & M. Ongena (2012). Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens*S499. *FEMS Microbiology Ecolog* 79,176–191.

[26] E. Glickmann & Y. Dessaux (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol* 2,793-796.

[27] E. Husen (2003). Screening of soil bacteria for plant growth promoting activities *in vitro.* Indones. J. Agric. Sci 4, 27-31.

[28] V. Nihorimbere (2014). Functional significance of microbial communities in soil. In: P. Singh (Ed.): Trends in Soil Microbial Ecology, 385 p. Studium Press LLC, Huston, USA, 1-21. ISBN : 1-62699-036-0.

[29] V. Nihorimbere & M. Ongena (2017). Isolation of Plant growth-promoting Bacillus Strains with Biocontrol Activity In vitro. Merit Res. J. Microbiol. Biol. Sci. 5(2), 013-021.

[30] J. Sasse, E. Martinoia & T. Northen (2017). Feed your friends:do plant exudates shape the root microbiome? *Trends Plant Sci*. 23, 25–41. doi: 10.1016/j.tplants. 2017.09.003

[31] S. Khodavaisy, M. Nabili, B. Davari, & M. Vahedi (2011). Evaluation of bacterial and fungal contamination in the health care workers' hands and rings in the intensive care unit. *J. Prev. Med. Hyg*., 52, 215-218.

[32] M. Ongena, A. Adam, E. Jourdan, M. Paquot, A. Brans, Joris B, J.L. Arpigny & P. Thonart (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology* 9,1084-1090.

[33] P. Jacques, Hbid C, J. Destain, H. Razafindralambo, M. Paquot, E. Pauw & P. Thonart (1999). Optimization of biosurfactant lipopeptides production from *Bacillus subtilis* S499 by Plackett– Burman design. *Appl Biochem Biotechno* l77, 223–233.

[34] Y. Subhash ( 2011). Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. *Journal of Basic Microbiology* 51, 98–106.

[35] A. Beneduzi, D. Peres, P.B. Da Costa, M.H.B. Zanettini (2008). Genetic and phenotypic diversity of plant growth- promoting bacilli isolated from wheat fields in southern Brazil. *Res Microbiol* 159, 244-250.