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Exploring of endophytic *Bacillus subtilis* as an agent of biocontrol for walnut canker

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ABSTRACT

One endophytic bacteria was found with strong inhibition to walnut canker pathogen, *Botryosphaeriadothidea*, on medium PDA during dual culture. Field investigation within 2 years after scaled inoculation verified this bacteria can be explored as potential function agents of biocontrol for walnut canker disease, with 84.1% cured rate and 9.6% new occurred rateat 3 months later at Sept., and 3.2% new occurred rate at next Sept, compared with 60.5% cured rate and 24.5 % new occurred rate of fungicide tebuconazole at current year, while 25.6% of new occurred rate in next Sept. Sequences of 16S ribosome DNA showed this bacteria is identical with *Bacillussubtilis*. Functional gene of *Tas*A detection revealed it secret antimicrobe piptidase, which can inhibit pathogen sporulation and can be used in field for walnut canker suppression.

KEYWORDS

Bacillus subtilis;
Walnut canker disease;
Inhibition;
16SrDNA;
*Tas*A.

INTRODUCTION

Walnut (*Juglansregia*), an indigenous tree species in China, naturally distributed in the mountainous areas and sporadically in the country yard. In recent years, market demand has stimulated importation of man-made forest of walnut, amounts of walnut trees come down from the mountain to agriculture fields, and new cultivars from adjacent provinces, as well as from the USA and the EU, were imported hastily. Whereas, one most worst thing is the canker disease occurred on whatever seedlings or adult trees, symptoms included lesion girdling the basal of the seedlings, canker forming on the stems and brown to red-brownish exudates on seedling and adult tree stems. Tree vigour got weaken, and yield decreased seriously, even seedlings died at last. There are two most well-known diseases of walnut in China,*Valsa* canker and*Botryosphaeria*canker. The disease caused by *Valsajuglans* was called Black Water Disease by local people, often leads the plantation forestry catastrophic loss. The latter, *Botryosphaeria* canker is eventually found in the walnut plantation, and the pathogen is believed to be *Botryospharia dothidea*in China, while in Egypt is *Botryodiplodiatheobromae*[1], and in Greece is *B. ribis*[2]. Some authors consider *B.ribis* as synonym as *B. dothidea*[3,4]. In recent year, *B. parva* was believed the new pathogen of walnut in USA, Spain[5] and in China[6]. Disease management strategies for

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walnut canker typically include the explore of canker resistant varieties, integrate of select culture practices, application of chemical pesticides, wiping out spots by operative treatments, and combinations thereof. However, the use of some of these practices is not always viable, and the continuous use of fungicides has caused severe ecological and food-safe problems. Owing to their environment-friendly nature and sustainably colonization in the tissues of healthy plants, endophytes have emerged as an alternative to chemical treatment for plant disease control[7]. Several endophytic bacterial strains have been shown to have beneficial effects on their host plants by disease suppression, stress tolerance, growth promotion, providing fixed nitrogen or phosphate soluble to the plants[8-11]. The endophytic bacteria isolate used for the current study was obtained from walnut stem where canker spots covered. We isolated it and explore its potential for biocontrol of walnut canker disease for two years observation of indoor and in the field.

METHODS AND MATERIALS

Isolation and inhibition experiments of endophtyic bacteria

Samples of walnut canker were collected from adult trees in Wugong county, Northwest China, and were cultured on PDA following surface-sterilization in 1/1000g/L mercury bichloride for 10 seconds followed by washing in sterile water, and then incubated at 25°C in dark chamber. After 1 week, bacteria were sub-cultured from agar, and purified via streak culture, and an investigation of Koch's Postulates was applied for checking pathogensis of bacteria. Canker pathogens, *Botryosphae riadothidea*, were also isolated and purified by tissues culturing, and applied for further antagonism experiments by the dual culture technique as follow. Endophytic bacteria, dated as WB, was inoculated via streak culture on agar PDA firstly, then 4 agar plugs of *B.dothidea*were input on and incubated at the 25°C dark chamber for 2 weeks. Killed barrels and any other reactions, e.g. color changing, antagonism, were dated and taken photos.

Biocontrol tests of walnut canker in the field with the endophytic bacteria

Twenty adult walnut trees of 10-year old in each of four repeats with *Botryosphaeria* canker were chosen for biological control at nursery of Northwest A.&F. University (N34° 16' 56.24",E+108° 4' 27.95) by endophytic bacteria isolate WB1&WB2 respectively, which were prepared in suspended liquid of Luria-Bertani (LB) medium in advance. Tree stems were scaled 0.3-0.5 cm depth at the canker spots by sharp knife. Bacteria suspended liquid with concentrate 1×10^6 per millimeter was brushed over the scaled line for 2 times, then one white plastic film was covered the scaled line for keeping humidity for 1 weeks. Two kinds of controls with 5 trees assay respectively were applied in this field experiments, fungicide group with tebuconazole (Shaanxi Sunger Colt.) of concentrate 2%, labeled as P-CK, were smeared at the canker spots, another control was the blank group, labeled as N-CK, canker spots was sprayed with sterilized water. Three months later at Sept., canker spot numbers were dated and analyzed by Excel 2010, and new canker spots at Sept. and next Sept. were also dated. Cured rate and new spot rate dated following the formula:

Cured rate= cured spot number at Sept./total canker spots before treatment*100%.

New spot rate 1 = new spot number at Sept./ total canker spots before treatment *100%.

New spot rate 2 = new spot number at next Sept./ total canker spots before treatment *100%.

Five treated canker spots were randomly selected for re-isolating endophytic bacteria and canker pathogens on agar PDA as above Method 1, as well as two spots from each control. All control data from sub-culture WB1 and WB2 are averaged for evaluation effect of bio-control by the endophytic WB.

Identification of endophytic bacteria

The two sub-cultures of isolate WB, strain WB1 & strain WB2, were used for identification and gene study. Two strains were cultured in the medium liquid of LB and PA (medium PDA without dextrose)at different pH value respectively, and checked morphology under microscopy after 1 week incubation

at 28℃ chamber. Genomic DNA of endophytic bacteria was extracted by 2% CTAB, and 16S rDNA of ribosome was amplified by universal primers 8F/1525R, 8F:5'-agagtttgatcctggctcag-3'/1525R:5'ttctgcagtctagaaggaggtgwtccagcc-3'.PCR reactions were carried out in a total mixture volume of 25μL containing a final concentrate of 1.5 mM $MgCl_2$, 0.5 mMdNTPs (Applied Biosystems), 10 pmol of each primer, 1.0 U AmpliTaq® DNA Polymerase (Applied Biosystems), and 50 ng of genomic DNA.Samples were incubated in a thermal cycler (PTC-100, MJ Research, USA) and followed the reaction protocol: 94°C for 3 min pre-denature, 94°C 2 min denature, 55°C anneal for 1 min 30 s,72°C extension for 1 min 30 s, after 10 cycles,continued as following: 93°C for 1 min, 51°C for 55 s and 72°C for 1 min, 25 cycles later, with a final extension at 72°C for 10 min 30 sec, and then stop reaction at 25°C for 10 minutes. PCR products were purified and sequenced by ABI3730xl sequencer in Senggong Colt., Shanghai, sequences were modified by deleting the gaps and redundant bases, and then blasted in the Genbank, related sequences were aligned with Clustal X 1.81 and a phylogenic tree was constructed by using neighbor-joining (NJ) analysis performed using Software Mega 5.1, and the isolate was identified according its taxonomic placement in the phylogeny tree. Morphologic traits and culture physiological characters were also used for assistant identification.

*Tas*A gene amplify

One pair of primer of *Tas*A gene, fwd-5'-gaattcatgggtatgaaaaagaa-3'/rvd-5'-ctgcagttaattttatcctcgct-3', was designed by referring Sequence in GenBank (accession CP002468), and PCR reaction carried out on a thermal cycler (PTC-100, MJ Research, USA) by the following, 94°C for 3 min pre-denature, 94°C 2 min denature, 50°C anneal for 1 min,72°C extension for 1 min 30 s, 25 cycles,with a final extension at 72°C for 10 min 30 sec, and then stop reaction at 25°C for 10 minutes. PCR products were sent to Sengong Colt.as above Method 3, one bio-technologic firm in China, for sequencing.

Data analysis

Incidence of cured spot, new occurred spot under biological control, P-CK and N-CK was subjected to one-way analysis of variance (ANOVA). For percentage data of the relative incidence, data were first transformed using arcsine square root transformation before the ANOVA. Fusher's least significant difference (LSD) multiple comparisons were then used to separate the means among treatments. All the statistical analyses were performed using ProStat software (Poly Software International Inc., Pearl River, NY).

RESULTS

Inhabitation activity of Endophytic bacteria verse *B. dothidea*

Bacteria colonies, and walnut canker pathogen, *Botryosphaeria*, were isolated simultaneously. The isolates of bacteria showed no symptom of canker disease when they were inoculated on the stem, and so far were thought as endophytic bacteria. Isolate *B.dothidea* caused typical canker symptoms. At the first isolation of tissues, this bacteria isolate showed high activity of inhibition to pathogen *Botryosphaeria*, Figure 1-A. The sub-culture after purified, still showed high potential activity of inhibition to pathogen *Botryosphaeria* during the dual culture, Figure 1-B. The pathogen *Botryosphaeria*

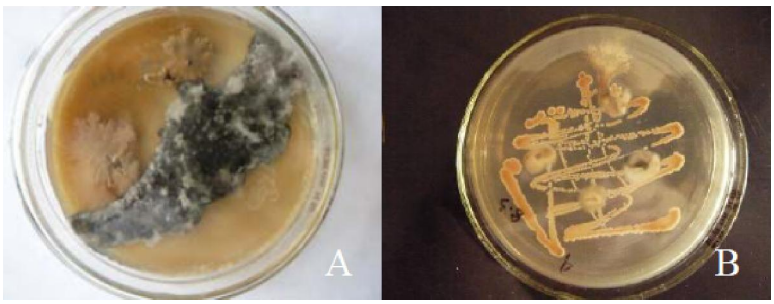

Figure 1 : Inhibition of endophytic bacteria to walnut canker pathogens on PDA medium assay

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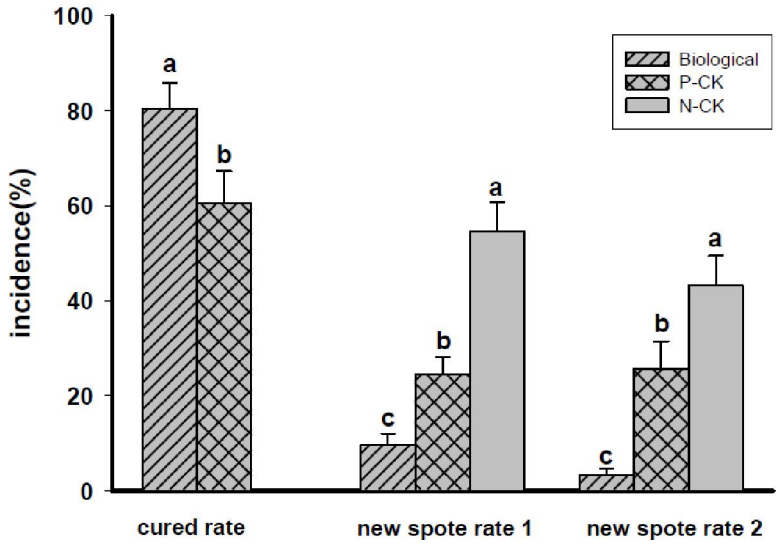

Figure 2 : Compare between biological controls and Chemical controls in the field

was almost shrink away after 2 weeks co-culturing with the endophytic bacteria. Endophytic bacteria show strong antagonisms to the pathogen *Botryosphaeria dothidea.*

A: antagonism between endophytic bacteria and *Botryosphaeriadothidea* during the process of isolation by tissue culturing. B: streak culture of isolate WB verse *Botryosphaeria* in dual culture assay.

Walnut canker controlled by endophytic bacteria in the field

Biological: average incidence of isolate WB. P-CK: fungicide group of positive contrast of tebuconazole. N-CK: blank group of negative contrast with sterilized water.

Of 20 trees, two hundred thirty five canker spots were applied bacteria suspended liquid, and 198 canker spots were cured after 3 months at Sept., almost took on 84.1% of total tested canker spots. The positive control with fungicide tebuconazole cured 60.5% canker spots, and the negative control with no cured spots, but newly increased 54.6%. New appeared canker spots on endophytic bacteria treated trees were much least than controls, only 9.6% of total tested canker spots verse 24.5 % of the fungicide group (P-CK) and 54.6% of the blank group (N-CK), Figure2. The next Sept., new spots incidence of biocontrol group was 3.2%, contrasted to the 25.6% of fungicide group and 43.1% of the blank group. Strain WB1 showed little stronger canker inhibition than strain WB2, however, both strains exhibited function of nearly curing the diseased trees without exudatesany more, but N-CK and P-CK were not, Figure3. There was no canker pathogen isolated from bacteria treated spots except endophytic bacteria after 3 months, whereas, canker pathogens can be easily re-isolated from the fungicide tebuconzole group and the blank group.

A: N-CK: without inoculation but spraying water at June; B: scaled inoculation with isolate WB1; C: smeared with tebuconazole

Identification of the endophytic bacteria

Colonies of strain WB1 and WB2 are both with white-yellow and rough milk surface. WB1 grew well in the LB liquid, but not well in PA liquid. WB2 can grew well in both LB liquid and PA liquid. Both are preferred to alkaline medium. Morphology of both strains is identical, with rode-shape body and an endospore at the middle or near the top. 16S ribosome DNA sequence of both two strains show 99% homomgensis and 98%, and 99% coverage respectively with *Bacillus subtilis*, and placed the nearest location of *B. subtilis* in the phylogeny tree (Figure 4). So they were identified as *Bacillus subtilis* (GenBank accession:KF591602-KF591603). The isolates used in this study are maintained in the culture collection of the Forestry College, Northwest A. & F. University, China, as culture collection numberWB1 & WB2.

Figure 3 : Walnut canker controlled by endophytic bacteria in the field

*Tas*A gene amplify

There was one specific band about 800bp amplified from both strain WB1 and WB2, Figure 5. Sequence blasting (accession KF800001-KF800002) showed there are 99% homogensis and 99% coverage with *Tas*A gene of *B.subtilis* (accession FJ713581.1). *Tas*A gene was believed one peptidase gene, coding a translocation-dependent antimicrobial spore component (Stein, 2005). Both WB1 and WB2 can secreted antimicrobial agents and inhibit the growth of pathogen *B.dothidea*, and showed strong inhibition in the dual culture on medium PDA, as well as in the field control.

DISCUSSIONS

Species of the genus *Bacillus*, e.g. *B. amyloliquefaciens* and *B. subtilis*, have been reported to produce a range of antimicrobial dipeptides and cyclic lipopeptides[12,13]. Endophytic*B. amyloliquefaciens* (isolate PEBA20) from poplar has been applied for control *Botryosphaeria* canker of poplar in vitro assay and in cut shoots assay. Two genes, *Tas*A and *aiiA*-2 associated with antagonistic activity, were detected, and showed the potential to serve as a biological control agent for the poplar canker disease[14]. In this study, Strain WB1 & WB2 of *B. subtilis* were both isolated from walnut canker tissues, and they both showed strong antagonistic activity against pathogen *Botryosphaeria dothidea*, and *Tas*A gene was both amplified from their genome. The control effective of the isolate was higher than that of the fungicide applied in this study, and showed sustainable control effective. In vitro of agar medium, we can found the colony of *B.dothidea* almost completely shrink when dual culturing with *B. subtilis*. Endophytic strains tested in this study are from walnut host, and they can parasite easily on walnut stems.There are many reports showed most endophytic microorganisms have wide range of host and so far were applied for different host plants[14-19], however, there are still documents emphasized the original species plants are the best hosts for endophytes applying by possessing a close relationship and by possessing greater adaptability for colonization[20-21]. These two strains of *B. subtilis* were both obtained from walnut, which makes them a natural candidate for biocontrol of walnut canker. We applied these two strains of *B. subtilis* for biocontrol in the field for 2 years from June to September in northwest China, when *Botryosphaeria* canker is prevailing, and we found they showed strong heat-resistance and strong parasite after scaled inoculating. *B. subtilis* still can be re-isolated from the cured canker spots after 3 months without any pathogenesis.Strobel and Daisy ever indicated the relationship between endophytes and their hosts is variable[22], which for several reasons can shift from symbiosis or mutualism, to saprophytism or opportunist plant pathogen, thus the measurements of utilization of endophytes is never underestimation, the inoculation time, the concentrate and inoculation way therefore can verify the functional effect of endophytes in practice. Scaled inoculation was first report here, and the data from this study showed this *B. subtilis* can sustainably control the walnut canker incidence without pathogenesis, but heat-resistance and strong parasite, and so is here recommended as a good candidate of biocontrol agent for future.

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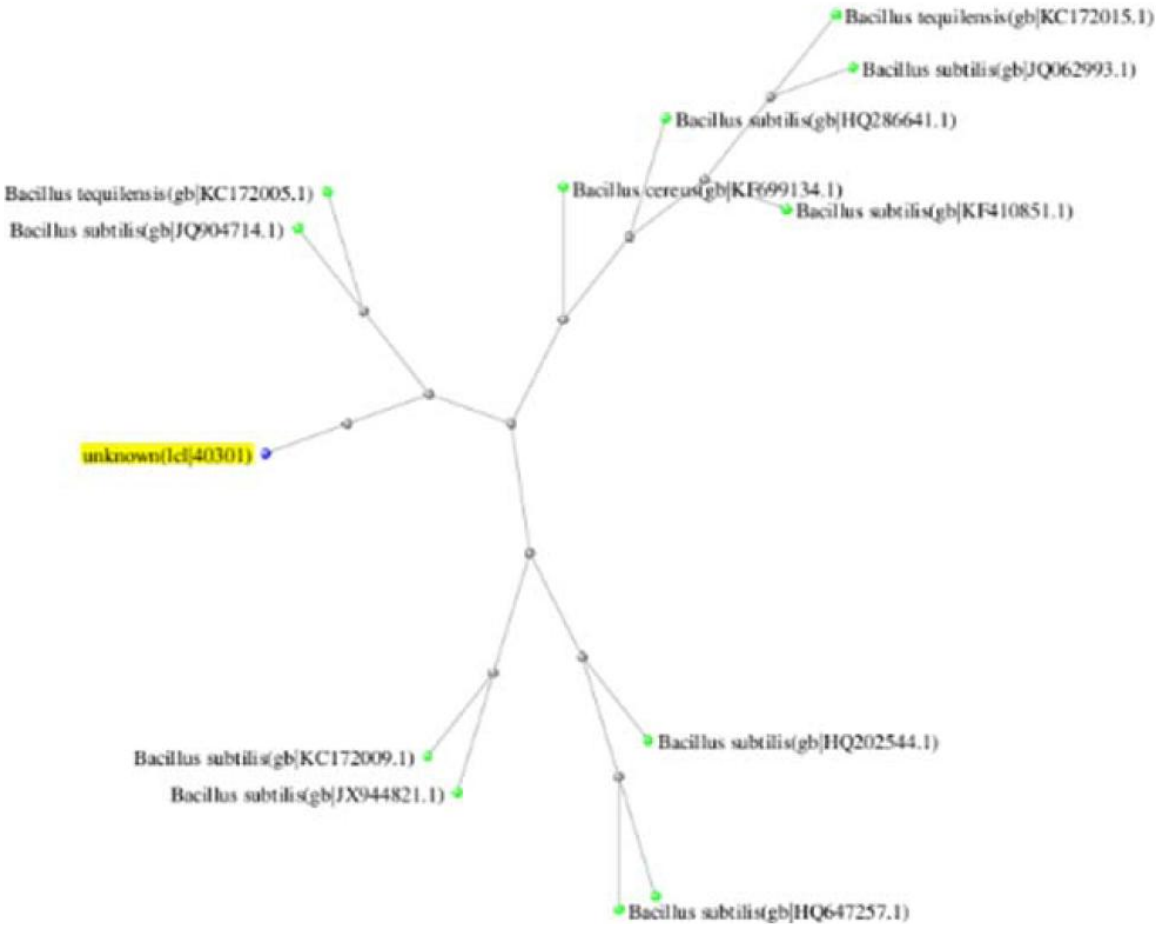

Figure 4 : Placements of strain WB in the phylogeny tree

Note: the yellow is sequence of strain WB

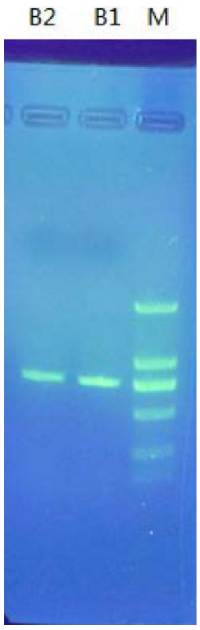

Figure 5 : Products of *Tas*A gene electrophysis on 1.5% agar

B1: strain WB1, B2: strain WB2, M: mar ker (2000bp,1000bp,750bp,500bp,250bp,100bp)

CONCLUSION

Bacillus subtilis, one endophyticbacteria from walnut tree, with peptidase *Tas*A gene, can explored for canker disease biocontrol in the field. It is heat-resistant, and good parasitic and good adaption with host walnut. Scaled inoculation is a good way for this bacteria applying in the field. After inoculation with *B. subtilis* in field for two years, walnut canker caused by *Botryosphaeria*can be suppressed successfully.

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REFERENCES

[1] W.M.Hagsag, A.Rayya, N.E.Kasim; "First report of a canker disease of walnut caused *by Botryodiplodiatheobromae*in Egypt", Disease Notes, DOI:10.1094/PDIS-92-2-0226B,2007.

[2] I.C.Rumbos; "Twig and branch dieback of walnut trees induced by *Botyosphareriaribis*", Plantpathology, **36**, 602-605 **(1987)**.

[3] J.A.Von Arx, E.Muller; *Die Gattunten der amerosporen Pyrenomeceten*, Beitraigezur Kryptogamenflora der Schweiz, **(1954)**.

[4] J.L.Maas, F.A.Uecker; "*Botryosphaeriadothidea* cane canker of thornless blackberry", Plant Disease, **68**, 720-726 **(1984)**.

[5] T.J.Michalidis, S.F.Chen, B.Coates, D.Morgan, R.Puckett, J.Hasey, K.Anderson, R.Buchner, C.DeBuse, E.Fichtner, W.Bentley; "Managing anthracnose blight and *Botryosphaeria* and *Phomopsis* cankers of Walnut", Walnut Research Reports, **12**, 367-381 **(2012)**.

[6] Z.D.Yu, G.H.Tang, S.B.Peng, H.Chen; "*Neofusicoccumparvum* causing canker of seedlings of *Juglansregia* in Chin", Journal of Forestry Research (in Pressed), **(2014)**.

[7] M.L.Daughtrey, D.M.Benson; "Principles of plant health management for ornamental plants", Annual.Review Phytopathology, **43**, 141-149 **(2005)**.

[8] M.Beyler, C.Keel, P.Michaux, D.Haas; "Enhanced production of indole-3-acetic acid by a genetically modified strain of *Pseudomonas fluoresces* CHA0 affects root growth of cucumber, but does not improve protection of the plant against *Pythium* root rot", FEMS Microbiology Ecology, **28(3)**, 225-233 **(1999)**.

[9] S.Timmusk, B.Nicander, U.Granhall, E.Tillerg; "Cytokinin production by *Paenibacilluspolymyxa*.Soil biology and biochemistry", **31(13)**, 1847-1852 **(1999)**.

[10] B.Reiter, H.Burgmann, K.Burg, A.Sessitsch; "Endophytic*nif*H gene diversity in African sweet potato", Canadian Journal of Microbiology, **49(9)**, 549-555 **(2003)**.

[11] R.S.Redman, Y.O.Kim, C.J.D.A.Woodward; "Increased fitness of rice plants to abiotic stress via habitar adapted symbiosis: a strategy for mitigating impacts of climate change", PLoS ONE, Article ID e14823, **6(7)**, **(2011)**.

[12] T.Stein; "*Bacillus subtillis* antibiotics: structures, syntheses and specific functions", Molecular Microbiology, **56**, 845-857 **(2005)**.

[13] D.Romero, A.De Vicente, R.H.Rakotoaly, S.E.Dufour, J.W.Veening, E.Arrebola, F.M.Cazorla, O.P.Kuiper; "The iturin and fengycin families of lipopeptides are key factors in antgonism of *Bacillus subtilis* towards *Podosphaerafusca*", Molecular Plant Microbe Interaction, **20**, 430-440 **(2007)**.

[14] X.T.Yin, L.N.Xu, L.Xu, S.S.Fan, Z.Y.Liu, X.Y.Zhang; "Evaluation of the efficacy of endophytic*Bacillus amyloliquefaciens* against *Botyosphaeriadothidea* and other phytopathogenic microorganisms", African Journal of Microbiology Research, **5(4)**, 340-345 **(2011)**.

[15] H.S.A.Silva, J.P.L.Tozzi, C.R.F.Terrasan, W.Bettiol; "Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust", Biological Control, **63**, 62–67 **(2012)**.

[16] Z.Khan, G.Guelich, H.Phan, R.Redman, S.Doty; "Bactrial and yeast endophytes from poplar and willow promote growth in crop plants and grasses", International Scholarly Research Network, ISRN Agronomy, Article ID90280,11PAGES. DOI:10.5402/2012/890280, **(2012)**.

[17] R.L.Melnick, N.K.Zidack, B.A.Bailey, S.N.Maximova, M.Guiltinan, P.A.Backman; "Bacterial endophytes: *Bacillus spp.*from annual crops as potential biological control agents of black pod rot of cacao", Biological Control, **46**, 46-56 **(2008)**.

[18] N.L.Miller, C.M.Ocamb; "Relationships between yield and crown disease of sweet corn grown in the Willamette Valley of Oregon.Online", Plant Health Progress, doi:10.1094/PHP-2009-0831-01-RS, **(2009)**.

[19] H.C.Evans, K.A.Homes, S.E.Thomas; "Endophytes and mycoparasites associated with an indigenous forest tree, *Theobromagileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases", Mycological Progress, **2(2)**, 149–160 **(2003)**.

[20] Y.Chen, R.Mei, L.Liu, J.W.Kloepper; *The use of yield increasing bacteria (YIB) as plant growth-promoting rhizobacteria in Chinese agriculture*, In: *Utkhede, R.S., Gupta V.K.(Eds.), Management of Soil-borne Diseases*, Kalyani Publishers, Ludhiana, **(1996)**.

[21] A.M.Van Buren, C.Andre, C.A.Ishimaru; "Biological control of the bacterial ring rot pathogen by endophytic bacteria strains from potato", Phytopathology, **83**, 1406-1409 **(1993)**.

[22] G.Strobel, B.Daisy; "Bioprospecting for microbial endophytes and their natural products", Microbiology and Molecular Biology Reviews, **67**, 491–502 **(2003)**.