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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

From *Piriformospora indica* to Rootonic: A review

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Piriformospora indica **(Hymenomycetes, Basidiomycota) is a cultivable endophyte that colonizes roots and has been extensively studied.** ***P. indica*** **has multifunctional activities like plant growth promoter, biofertilizer, immune-modulator, bioherbicide, phytoremediator, etc. Growth promotional characteristics of** ***P. indica*** **have been studied in enormous number of plants and majority of them have shown highly significant outcomes. Certain secondary metabolites from the fungus are reasons behind such promising outputs. Effect of** ***P. indica*** **has been studied on more than 150 plants. Promising outputs of laboraatory experiments and small field trials indicated the need for its mass cultivation and usage. For field trials, a formulation "Rootonic" was prepared by mixing** ***P. indica*** **biomass in magnesium sulphite. The quantity of formulation (Rootonic) to be used per acre of land for maximum productivity has also been standardized for about 50 plants.** ***P. indica*** **has proved to be highly beneficial endophyte with high efficacy in field. This article is a review on our journey from** ***P. indica*** **to "Rootonic".**

Key words: *Piriformospora indica*, Rootonic, Mycorrhizae, endophyte, biofertilizer.

INTRODUCTION

Piriformospora indica was discovered by Prof Dr. Ajit Varma and his colleagues in Thar Desert of Western India in 1992 from the root system of several xerophytic plants (Varma et al., 1999; Verma et al., 1998). It belongs to *Hymenomycetes, Basidiomycota*. A new family Sebacinaceae and new order Sebacinales Glomeromycota was created for this fungus due to its unique features (Weiß et al., 2004; Qiang et al., 2011). This is a very unique symbiotic fungus which not only promotes plant growth but also has other multifunctional activities such as plant growth promoter, bio-protectant, bio-pesticide, helps in enhanced flowering and fruiting etc. Its properties have been patented in Germany (European Patent Office, Muenchen, Germany, Patent No. 97121440.8-2105, Nov. 1998) dating back to 1997. *P. indica* is deposited at the Deutsche Sammlung fu¨r Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM 11827). It promotes plant growth, increases the resistance of colonized plants against fungal pathogens and increases their stress tolerance (Harman 2011; Varma et al., 2012a).

Characterization of *P. indica* has shown its multifunctional activities as plant growth promoter, biofertilizer, immune-modulator, etc (Figure 1). It significantly improves plant growth and overall biomass and can be easily cultivated on a variety of synthetic media (Varma et al., 2012a; Oelmüller et al., 2009). Till date about 150 plants have been reported with the fungus to show its effect. Plants

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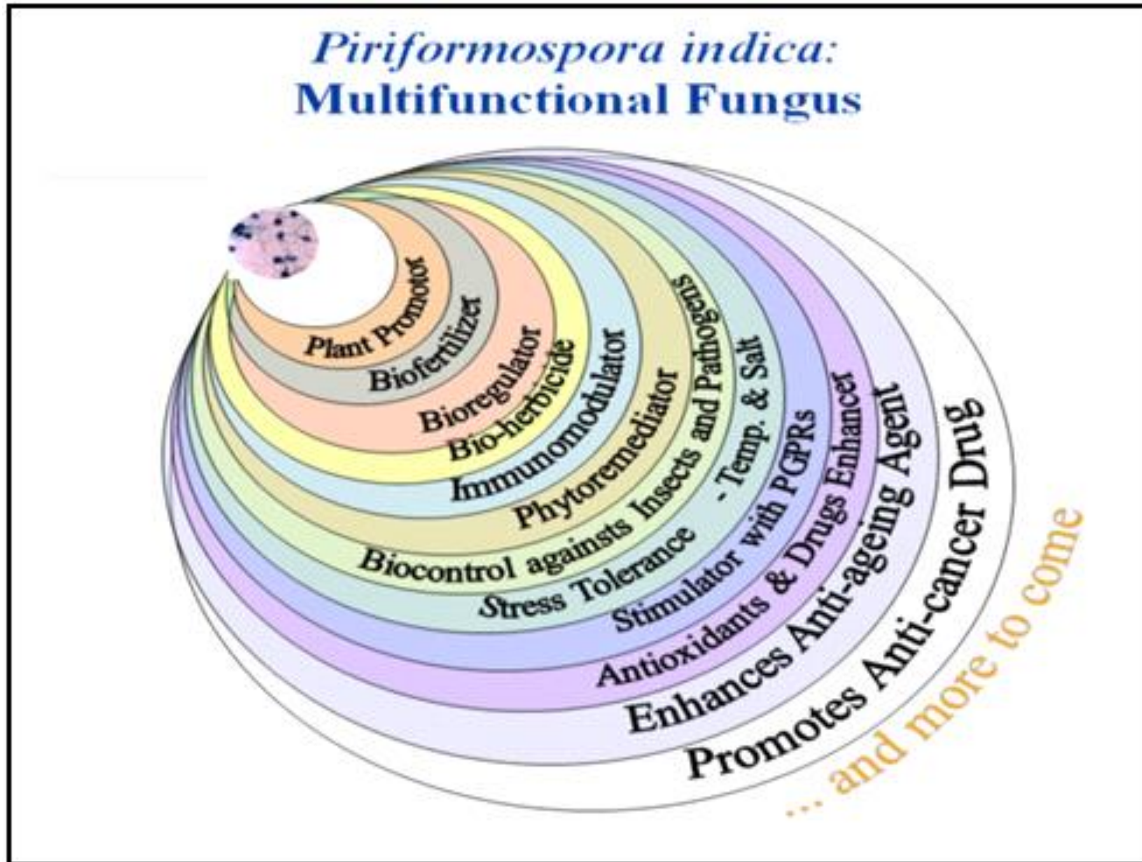

Figure 1. Functional characteristics of fungus (c.f. Varma et al., 2013).

belonging to Bryophytes, Pteridophytes, Gymnosperm and Angiosperm (both mono-dicots), include orchids (Singh and Varma, 2000). *Aneura pinguis L., Cicer arietinum, Adhatoda vasica* L., *Aristolochia elegans, Daucus carota* L., *Arachis hypogaea (*groundnut or peanut*), Petroselinum crispum* L., *Medicago sativa, Centella asiatica, Glycyrrhiza glabra, Cuminum cyminum, Abrus precatorius* L.*, Foeniculum vulgare, Mimosa pudica, Carum capticum, Vigna unguiculata, Coriandrum sativum, Glycyrrhiza glabra, Artemisia annua* L., *Acacia catechu, Spilanthes calva, Stevia rebaudiana, Prosopis chilensis Stuntz sys., Calendula officinalis, Prosopis juliflora, Arnica* spp. are few of the plants showing enhanced flowering, enhanced plant size, increased production of secondary metabolites etc. upon interaction with *P. indica* (Varma et al., 2012a, b, c).

Colonization by *P. indica* increases nutrient uptake, allows plants to survive under water, temperature and salt-stresses, confers (systemic) resistance to toxins, heavy metal ions and pathogenic organisms and stimulates growth and seed production. The valuable secondary metabolites excreted by *P. indica* influence early seed germination, better plant productivity, early flowering, etc. Use of *P. indica* to increase desiccation tolerance in higher plants has been studied by Varma and his colleagues (2012c) and significant increase tolerance was achieved.

Genome wide study revealed that its genome is assembled into 1,884 scaffolds containing 2,359 contigs with an average read coverage of 22 and a genome size of 24.97 Mb. The estimated DNA content of *P. indica* nuclei ranges from 15.3 to 21.3 Mb. To assess the genome completeness of *P. indica* a blast search was performed with highly conserved core genes present in higher eukaryotes (Zuccaro et al., 2009). *P. indica* can be stably transformed by random genomic integration of foreign DNA and that it possesses a relatively small genome as compared to other members of the Basidiomycota (Zuccaro et al., 2011).

P. indica is a model organism used in mycorrhizal research, and its research outputs has been published in highly recognized journals like Nature, PNAS, Plos Pathogen, JBC, Plant Physiology, Molecular Plant Pathology, etc. Extensive research on this organism has brought it to an appreciable state and made its field trials and marketing possible.

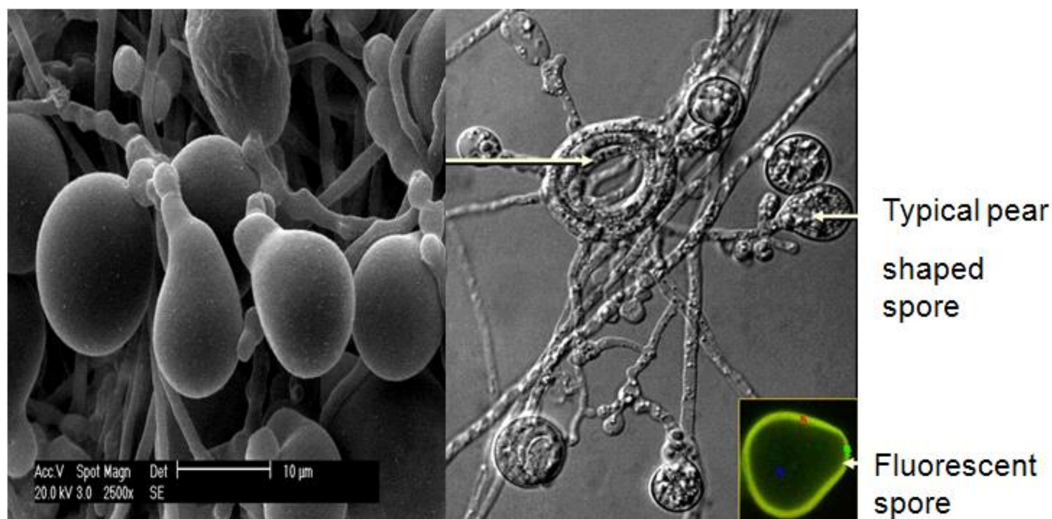

Figure 2. Morphological features of *P. indica* indicating hyphae and pear shaped spores. Scanning electron microscopy view spore (left) (c.f. Varma et al., 2013).

Objective of this review is to give a general view of journey of *P. indica* from laboratory to field and finally towards industrialization.

CULTIVATION AND MORPHOLOGICAL CHARACTERISTICS

Simple morphological features of *P. indica* contain hyphae and pear shaped large spores (Figure 2). It can be cultivated on basic defined medium at an optimum temperature of 25°C ± 2, pH 6.8. Maximum biomass production was obtained after 7 days incubation at 120 rpm (Varma et al., 2013).

FUNCTIONAL CHARACTERISTICS OF *P. INDICA*

Plant growth promotion

P. indica promotes growth of plants of forestry, horticulture and agriculture importance. Numerous plants have been tested for the effect of *P. indica* on their growth and interestingly majority of them have shown beneficial effect. Few results of its effect on sugarcane, Pinus and potato are shown in Figure 3. It is important to note that in addition to enhancement of plant growth, the fungus also helps in enhancement of active ingredients in plants. In the case of Ratoon crop of sugarcane it was seen that plants not associated with *P. indica* turned yellow due to iron deficiency, whereas plants subjected to *P. indica* treatment remained green, indicating that the fungus also helps in iron transport. Almost 39% enhancement in iron content and 16% increase in sugar content were recorded in *P. indica* treated plants (Table 1). Noticeable increase in plant size and tuber size was observed in the case of Pinus and potato, respectively.

Value addition in spices and plants of pharmaceutical importance

Effect of *P. indica* has been studied on large number of spices and plants of medicinal importance. To name few are *Curcuma longa, Spilanthus calva*, *Artemisia annua, Tridax procumbens, Abrus precatoriu, Bacopa monnieri, Coleus forskohlii, Adhatoda vasica, Withania somnifera, Chlorophytum tuberosum*, *Foeniculum vulgare*, *Linum album*, *Podophyllum* sp., etc (Das et al., 2012). The organism has shown significant increase in concentration of active ingredients like curcumin, artemisnin, podophyllotoxin and bacoside leading to value addition to the plant.

Interaction of *P. indica* with *C. longa* resulted in approximately 21, 19 and 13% increase in essential oil, Curcumin and rhizome yield. Field trials showed that increase in rhizome yield after treatment with *P. indica* would benefit a farmer with Rs. 16,000/ (US $ 280.00) per hectare of land. It is also probable that healthy and shiny rhizomes would fetch better price. Increase in plant size, secondary metabolite release and increase ability to fight against infections was observed upon interaction of *P. indica* with *A. annua*, *B. monnieri*, etc (Figure 4).

The tissue culture results obtained were evaluated for field trial and similar promising outputs were obtained. Field trial of *A. annua* was done in Central India. Improved plant growth and 1.6 fold increased concentration of active ingredient artemisinin was observed. In the case of *B. monnieri* 3.5 fold increase in bacoside concentration was observed.

Interaction of *Ephedra ciliata* (used for treatment of hay fever, asthma etc.) with *P. indica* also led to enhanced growth of treated plants (Varma et al., 2013).

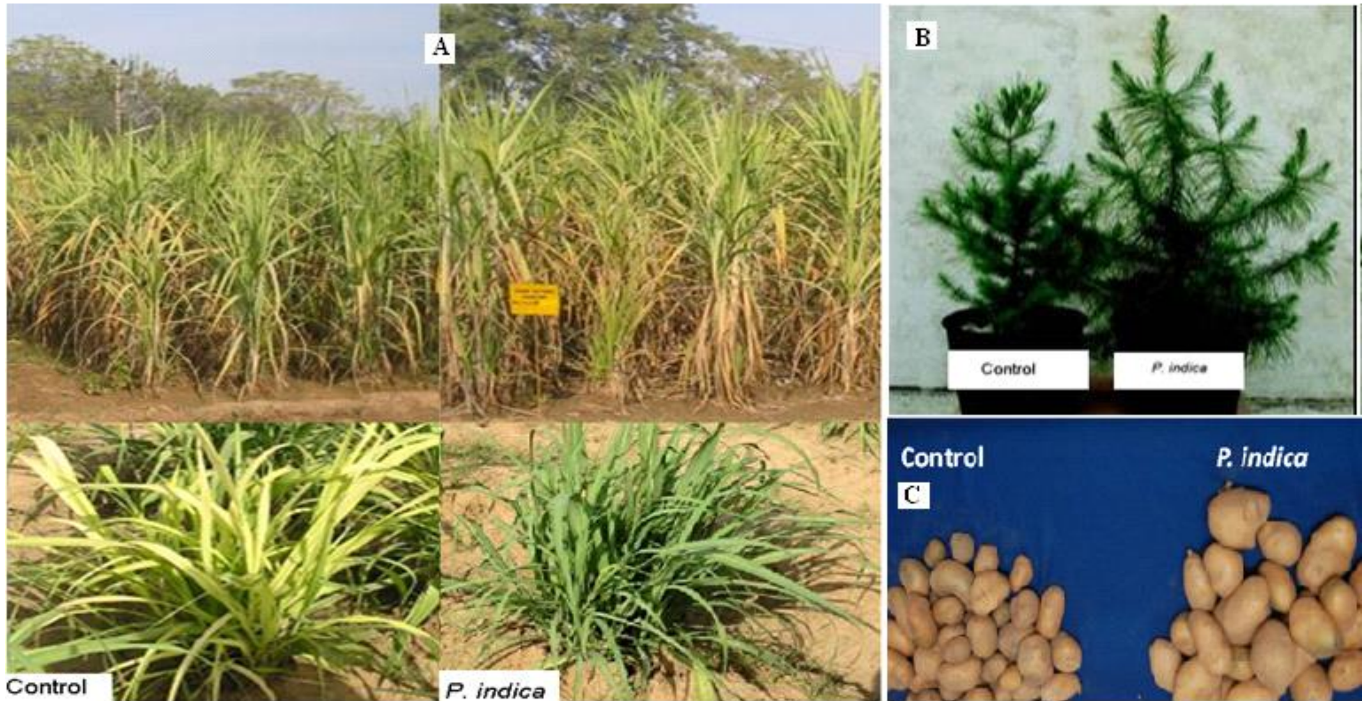

Figure 3. (A) Effect of *P. indica* on sugarcane (*Saccharum officinarum*) cultivated in field at Punjab. (B) Growth promotion of Cyprus plants (pinus) upon interaction with *P. indica* grown on rocky sand. (C) Increases size and improves texture of potato plants upon interaction with *P. indica*, cultivated in field at Punjab. (c.f. Varma et al., 2013).

Table 1. Enhanced iron and sugar content of the sugarcane upon interaction with *P. indica*. (c.f. Varma et al., 2013).

Parameter	Iron (ppm)	Sugar (°Bx*)
Control	202.2	18.35
P. indica	281.4	21.4

* °Bx (degree Brix) is the sugar content of an aqueous solution. One degree Brix is 1 g of sucrose in 100 g of solution.

P. indica as bioprotectant, rejuvenate fruiting, promoting early flowering

Experimental data suggests that *P. indica* suppresses the growth of a large number of pathogens like *Geaumannomyces graminis, Alternaria* sp., *Colletotrichum falcatum, Fusarium oxysporum, Fusarium udum, Rhizoctonia bataticola, R. solani, Sclerotinum rolfsii, Verticillium* sp. and many more (Dolatabadi et al., 2011; Ghahfarokhy et al., 2011). Field trials on *Lagenaria siceraria* and *Tagetes* sp. showed that interaction with *P. indica* suppressed the infestation by plant pathogens including viruses (Figure 5). *P. indica* protecting a *Tagetes* sp. from mite infection has also been recorded.

Rigorous fruiting was observed in kinnow (*Citrus reticulata*) plant after interaction with *P. indica* with a limitation that this fruiting was time dependant and seen only at the early stage. *P. indica* also possess unique characteristics of inducing early flowering. This property is seen in case of plants viz., tobacco, *Coleus*, *Brassica,* etc.. Orchids are well known ornamental plants and their cultivation is expensive. Another limiting factor to its growth is that among millions of tiny seeds, unfortunately 99% do not germinate and transform into fully grown and mature plants unless they establish contact with mycorrhiza. Its interaction with *P. indica* has shown increased seed germination. *P. indica* has also shown early seed generation and enhanced plant growth in highly valuable plants like *Jatropha curcas* and *Populus deltoides* (Kaldorf et al., 2005).

Unique features of *P. indica*

P. indica shows striking unique features upon interaction with various plants. Field trials to investigate activity of the fungus have been conducted in North, Central and in extreme cold deserts (Ladakh-Leh) of India. *P. indica* mixed with frozen Leh soil (temperature -30°C) showed significant results where all the fifteen seeds of *Cichorium endivia* germinated within 12-25 days (Singh and Varma,

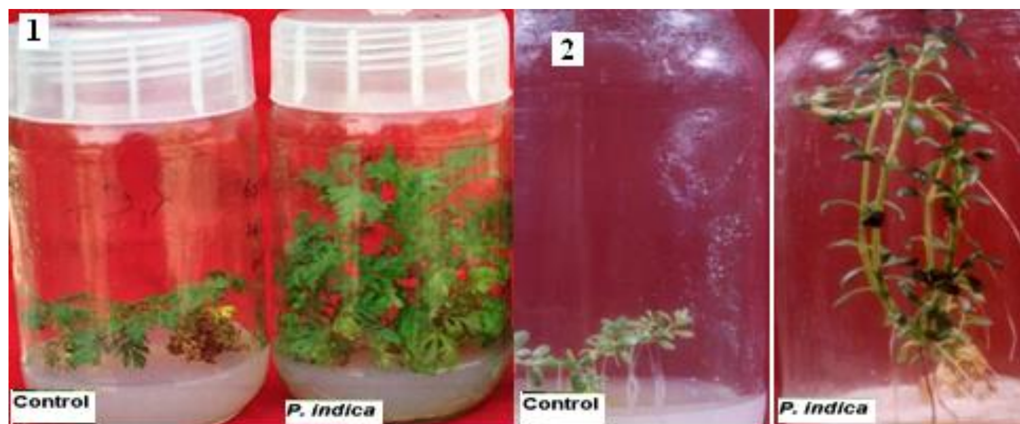

Figure 4. Co-cultivation of *Artemisia annua* and *Bacopa monnieri* in plant tissue culture with *P. indica* showing increased plant size (1 and 2 respectively) (c.f. Varma et al., 2013).

Figure 5. (1) Field trial on interaction of *P. indica* with *Lagenaria siceraria* showing how it suppresses the infestation by plant pathogens including viruses (upper); a magnified view of the same (lower). (2) Interaction of *P. indica* with plant of *Tagetes* sp. Note: Control plant with folded leaf due to mite infestation; no such symptoms were observed on treated plants (c.f. Varma et al., 2013).

2000). The germinated plantlets when transferred to macro-plots attained full growth and imparted better productivity than the control. In contrast, not a single seed broke dormancy in absence of the fungus was noticed.

Liquid fertilizer from *P. indica*

Culture filtrate (liquid fertilizer) of *P. indica* also acts as an excellent plant growth promoter. In order to prepare culture filtrate, *P. indica* was grown in broth and for 10 days followed by removal of biomass. Culture filtrate helped in early seed generation and early flowering as well (Figure 6). It was then interpreted that the fungus secretes some secondary metabolites that works as fertilizers (Bagde et al., 2010a, b, 2011).

Mechanisms behind the unique action of *P. indica*

The fungal interactions are characterized by increase in

Figure 6. (1) Interaction of *Phaseolus vulgaris* (rajma) with *P. indica*. (2) Co-cultivation of *Brassica oleracea* (Broccoli) with *P. indica*. (c.f. Varma et al., 2013).

efficiency of nutrient uptake from soil due to better hyphal penetration as compared to thicker root hairs. Plants deliver phosphor assimilates to fungus and during mycorrhizal associations; plants acquire phosphates from extensive network of extra radical hyphae. Interaction of *P. indica* with plant alters pathway for nitrogen metabolism, thereby helping plants to absorb more nitrogen. This phenolmenon gives higher resistance to water deficiency and makes plants drought tolerant. Enhanced growth of plants under mycorrhizal condition amplifies its starch requirement. This starch is obtained from deposition in root amyloplasts. Thus, it is interpreted that one of the major starch degrading enzymes, the glucan-water dikinase is activated by *P. indica* (Iris et al., 2010).

Uptake and transportation of important macronutrients like iron, zinc, manganese, copper, etc. are also regulated by the fungus. Along with this, beneficial phytohormones are synthesized by plants associated with *P. indica.* The cumulative effect of macro-micro-nutrients and phytohormones regulates plant metabolism leading to value addition, early flowering, plant growth promotion, etc. Massive proliferation of useful rhizospheric microorganisms sustains soil fertility (Varma et al., 2013).

STEP TOWARDS COMMERCIALIZATION

Laboratory scale data and field trials have evidently concluded that *P. indica* can be extensively used to increase plant growth quantitatively and qualitatively (Sahay and Varma, 1999, 2000; Rai et al., 2001). Main motive behind such huge previous experimental subject was further applying it in normal fields so that it is available to all. For making this target come true, a step towards commercialization was taken. Fungal biomass is mass cultivated in fermentor (Bagde et al., 2010b). These fungal biomass formulated with magnesium sulphite (carrier) was prepared (Figure 7). Most effective formulation was standardized to 2% (w/w). Moisture content and colony forming unit was maintained at 20% and 10^9, respectively. This formulation is named “Rootonic”. Seed treatment was done by mixing Rootonic to seed and incubating it under shade overnight. Protocol for seed treatment has been given as Figure 8.

Quantity of formulation required for seed treatment has been standardized for large number of plants. Details are included in Table 2.

Preliminary studies with nanoparticles

Nanotechnology has significant benefits on food and agriculture system. Preliminary work on interaction of nanoparticle embedded *P. indica* biomass with Broccoli has shown better growth promotional property as compared to the control (*P. indica* without nano material); as tested in our laboratory (Suman et al., 2010).

CONCLUSION

P. indica is a rewarding organism with its huge and distinguished properties. Colonization by *P. indica* increases nutrient uptake, allows plants to survive in drought, salt-stress and temperature stress. Excellent plant growth promotion, growth at extremes of climate and bio-protecting capability of the organisms has paved way for its varied field applications. Large field trials at

Figure 7. Steps for the preparation of the formulation (c.f. Varma et al., 2013).

various locations in India showed beneficial effects of *P. indica* on plant growth and development. Promising outputs of field trials showed that it should be used at large scale so that common farmers are benefited and finally countries economy is at profit. Increase in productivity of certain crop upon interaction with *P. indica*

Figure 8. Protocol for seed treatment (c.f. Varma et al., 2013).

will increase total land usage. Enhanced field usage of the microorganisms requires its mass production. Field trials of the same are done by formulating biomass with powder and inoculating the mixture into root of plants. The formulation is termed "Rootonic". The journey from *P. indica* to Rootonic is exciting and very fulfilling. Large scale production and application of the product is still under process and we are looking forward to its commercialization soon.

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Table 2. Quantity of formulation required for the seed treatment. (c.f. Varma et al., 2013).

Crops	Seed treatment (g/acre)
Tomato, chillies, brinjal, capsicum, cabbage, cauliflower	25.00
Muskmelon, watermelon, long melon, cucumber, bottle gourd, bitter gourd, sponge gourd, round melon	50.00
Sunflower, ladies finger, onion, spinach, fenugreek, mustard, cotton	100.00
Maize, paddy and millets (barley, pearlmillet, sorghum)	300.00
Pulses (gram, pea, lentil, mungbean, Uradbean, pigeonpea, cowpea, soyabean)	500.00
Potato, wheat, sugarcane	1,000.00

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Full Length Research Paper

Effects of cultivating orchid *Gastrodia elata* with the introduced *Armillaria* in a local ecosystem

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To characterize the potential effects of an introduced fungus in a local ecosystem, an experiment was performed in an abandoned farmland, where a traditional Chinese medicinal plant, *Gastrodia elata* had been continuously cultivated using the introduced *Armillaria* M2 and it had not been grown for the last 6 years. In this case, a newly introduced *Armillaria* strain M1 was used to cultivate orchid *G. elata* again. Inoculating strain M1 showed an infection rate of 95.56% on *G. elata*, much higher than that of without, 22.2%. Molecular evidence showed that all *Armillaria* re-isolated from *G. elata* in the farmland were genetically identical to the introduced ones. It suggested that the introduced *Armillaria* completely blocked the infection of natural ones on *G. elata*. Such an effect could persist several years even after stopping cultivation of *G. elata*. A total of 53 strains of *Armillaria* were obtained from a variety of isolation sources. Analysis of their intergenic spacer (IGS) sequences revealed that diverse and novel species of *Armillaria* existed in local forest. They are valuable resources for cultivating *G. elata* in future. Regarding ecological risk, utilization of the introduced *Armillaria* is not recommendable for local farmers.

Key words: *Gastrodia elata*, *Armillaria*, the introduced microbe, orchid, ecological risk.

INTRODUCTION

“Gastrodia”, the tuber of the orchid *Gastrodia elata* Blume, is a valuable traditional Chinese medicinal plant and has been widely applied for treating a variety of disease, including headache, dizziness, hemiplegia, rheumatism and epilepsy (Tang and Eisenbrand, 1992; Chen and Sheen, 2011). In the wild, *G. elata* has to obtain the nutrients

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through symbiosis with *Armillaria* species, because *G. elata* is an achlorophyllous and aphyllous plant (Xu et al., 1989). Generally, it takes three years to obtain the mature tubers from seeds of *G. elata*. In the first year, the seeds are able to germinate through the symbiotic fungus *Mycena osmundicola* Lange and grow into protocorms. The protocorms could be used to obtain the immature tubers after one year of cultivation with *Armillaria*. Finally, the immature tubers could grow into mature ones after further one year of cultivation with *Armillaria* (Zhang and Yang, 2007).

Approximately 40 *Armillaria* species are distributed worldwide (Ota et al., 2011) and at least 19 biological species have been reported in Asia (Wang, 2010). However, only a few have been proved to be conducive to the growth of *G. elata* (Wang and Guo, 2002), including *Armillaria ostoyae*, *Armillaria gallica*, *Armillaria jezoensis*, *Armillaria sinapina*, *Armillaria singular* and *Armillaria nabsnona* (Cha and Igarashi, 1995; Sekizaki et al., 2008).

Currently, there is a trend in artificial inoculation of commercially grown *G. elata* to ensure colonization by effective *Armillaria* species and strains (Hua, 2004). From the ecological aspect, more and more concerns in recent years have been paid on the effects of the introduced fungus on local ecosystems (Litchman, 2010; Cowan et al., 2011). For example, an invasive mycorrhizal fungus originating in the Western United States has become a novel symbiont of endemic plants in California (Pringle et al., 2009a), and this novel association could affect not only the success of the plant species, but also nutrient cycling and other ecosystem properties (Pringle et al., 2009b).

It is well-known that *A. mellea* is a sapro-parasitic basidiomycete which is able to survive in the soil for a long time on wood and root debris without any living host. In forests, *Armillaria* genets can even spread across large areas up to 15 ha (Smith et al., 1992). Coetzee et al. (2001) reported that the *A. mellea* introduced into Cape Town, Africa, from Europe, could expand its colonization more than 300 years, with a colonized area at least 345 m in diameter. Therefore, people wonder if introducing exotic *Armillaria* spp. for increasing growth of comer-cially grown *G. elata* could have significant negative impacts on the native fungal community.

Xiaocaoba, located at Zhaotong, Yunnan Province, is a place famous for its high quality of *G. elata* in China (Yuan et al., 2002). The introduced *Armillaria* have been commonly applied for cultivating *G. elata* in the farmland (unpublished data). However, there is little knowledge on the effect of the non-local commercial species of *Armillaria* on the infection of *G. elata* by native *Armillaria* species. In this case, a newly introduced *Armillaria* strain M1 was used to cultivate *G. elata* in an abandoned farmland, where *G. elata* had been continuously cultivated using the introduced *Armillaria* M2 and it had not been grown for the last 6 years, to determine (1) whether the application of non-local *Armillaria* deters the infection of endemic *Armillaria*; (2) whether the activity of the introduced *Armillaria* persists in soil after stopping cultivation of *G. elata*.

MATERIALS AND METHODS

Description of experimental site

Cultivation of *G. elata* was carried out in Xiaocaoba, Zhaotong, at about N27°, E104°, with an average altitude of 1700 m. Xiaocaoba receives annually 960-1300 mm of rainfall and has relatively high humidity (ranging from 76 to 85%). The average temperature of the coldest month is above -1°C and the highest below 25°C. This kind of condition is suitable for the growth of *G. elata* (Zhang and Yang, 2007). An experimental plot (50 × 50 m) was selected to cultivate *G. elata*. Based on the investigation of local history of cultivating *G. elata*, this area had once grown *G. elata* with the inoculation of *Armillaria* M2. Since 2004, however, this farmland has been abandoned (unpublished data).

The cultivation of *G. elata*

A standard cultivation of *G. elata* was carried out in September, 2010. Briefly, a total of 270 holes, each 0.5 (wide) × 1 (long) × 0.5 m (high), were dug on the experimental plot. Fresh tree branches were collected from forest, cut into nearly 50 cm length and were used as nutrients for the growth of *Armillaria*. In each hole, 5-10 fragments of fresh tree branches (about 10 kg) were placed as two layers. When necessary, a volume of solid culture of commercially produced *Armillaria* M1 (Zhaotong Shengnong Limited, China) was placed on the woods (see below). Then the holes were covered with soils to allow *Armillaria* to grow on the wood for two months. The inoculation volumes of *Armillaria* were arranged as zero, 250 and 500 g per hole, respectively, and each treatment has 90 repeats.

In November 2010, the top soils were uncovered and the immature tubers of *G. elata* were put on the bed of tree woods and then the top soils were put back into the hole again. In October 2011, at the end of cultivation of *G. elata*, the *Armillaria* rhizomorphs connected with mature tubers of *G. elata* were collected from the holes and were subject to re-isolate *Armillaria* strains.

Isolation of *Armillaria*

Generally, 3-5 different rhizomorphs were collected from each hole in which there are mature tubers. To compare the genetic difference, *Armillaria* rhizomorphs were also obtained from four other farmlands (A, B, C and D), in which *G. elata* was traditionally cultivated through inoculating wild *Armillaria* collected from local forest (Table 1). In addition, wild *Armillaria* rhizomorphs associated with local tree barks were also collected from the mixed coniferous and broad-leaved forest far away from farmland of *G. elata*. A standard strain *Armillaria mellea* was bought from Yunnan Microbiology Institute, Kunming, China, and used as a reference.

The collected rhizomorphs were brought back to the laboratory. Rhizomorphs were cut into 1 cm fragments, washed with water and rinsed with 2% sodium hypochlorite solution for 1 min and 75% ethanol for 1 min. Then the disinfected rhizomorph was placed on Potato Dextrose Agar medium (PDA), containing streptomycin sulfate (40 μg/ml) and penicillin sodium (20 μg/ml) to inhibit bacterial

Table 1. Haplotype distribution of *Armillaria* in this study.

Haplotype [a]	Strain[b]	Source
1 (2)	SCH	Tree bark of *Dipentodon sinicus*
	S3c	*G. elata* tuber of farmland D
2 (20)	391a, 391b, 391c; 392b, 392c; 396a, 396b, 396d, 396e; 409a; 420a, 420b, 420c;1059a,1059b; 397a, 397b	*G. elata* tuber of our farmland [c]
	Armillaria M1	A commercially obtained strain
	S4a, S4c	*G. elata* tuber of farmland A
3 (2)	YQJ, YQJs	*G. elata* tuber of farmland B
4 (25)	828a, 828b; 824b; 532a, 532b, 532c; 227b, 227c; 533a; 33a, 33b; 46a, 46b; 219b, 219c, 219d; 44a; 218a, 218b, 218c; 220a, 220c; 211d; 829c	*G. elata* tuber of our farmland [d]
	Armillaria M2	A commercially obtained strain
5 (1)	829a	*G. elata* tuber of our farmland [d]
6 (5)	YYT	Tree bark of *Cerasus serrula*
	HSS	Tree bark of *Pinus armandii*
	SLZ	Tree bark of *Aleurites moluccana*
	S2a, S2b	*G. elata* tuber of farmland C

[a] Number in parenthesis is no. of isolates in each haplotype; [b]The strains with the same number are those obtained from different tubers of *G. elata* in the same hole and are underlined; [c]This farmland was inoculated with *Armillaria* M1 when cultivating *G. elata*; [d]This farmland was not inoculated with *Armillaria* M1 in this study but had been inoculated with *Armillaria* M2 to cultivate *G. elata* 6 years ago.

proliferation. All plates were incubated in the dark at 28°C for 2-3 weeks and the pure mycelia were collected for phylogenetic analysis.

Phylogenetic analysis

The genomic DNA of strains was extracted with CTAB method (Zolan and Pukkila, 1986). Because the ITS sequences are highly conserved and similar among *Armillaria* species (Dunne et al., 2002; Sekizaki et al., 2008), the IGS regions was amplified with primers LR12R (5'-CTG AAC GCC TCT AAG TCA GAA-3') (Veldman et al., 1981) and O-1(5'-AGT CCT ATG GCC GTG GAT CAG AA-3') (Duchesne and Anderson, 1990). The PCR reactions were performed with a PCR-Cycler (Biometra). PCR products were then purified using DNA gel purification kit (Shanghai Sangon Biological Engineering Technology & Services Co.,Ltd). Sequencing was performed by the Beijing Genomics Institute (BGI).

To determine the phylogenetic position of all isolates, the sequences were aligned using the DNAStar software and manually edited in SeqMan (from DNAStar software package). The edited sequences were uploaded on GenBank for BlastN search (http://www.ncbi.nlm.nih.gov). The sequences with the highest similarities were downloaded and were subjected to construct phylogenetic tree using the method of Neighbor-Joining tree by software PHYLIP3.65. The bootstrap analyses (1000 replicates) were preformed to calculate the confidence intervals at the branch nodes. The sequences in this report were deposited in GenBank as accession number KC844229 to KC844234.

Data analysis

Unless *Armillaria* successfully infects, *G. elata* is not able to develop into a mature tuber. Therefore, the infection rate of *Armillaria* was defined as the percentage of the holes with mature tuber of *G. elata* at the end of experimental period. Statistical analysis was performed by using SPSS 13.0 for Windows (SPSS, Inc., 2004). Nonparametric test of two independent samples was used to evaluate differences of infection rate of *Armillaria* in artificial culture of *G. elata*. Mann-Whitney Test was used to determine significant variances between independent samples. Statistical significance was defined as $P < 0.05$, unless otherwise noted in the text.

RESULTS

Infection rate of introduced *Armillaria*

Among 90 holes without inoculation of *Armillaria* M1, only 20 holes were found to produce the mature tuber of *G. elata*, with an infection rate of 22.2%. However, among 180 holes being inoculated *Armillaria* M1 (half with an inoculation volume of 250 g and half with 500 g per hole), 172 holes were found to produce the mature tuber of *G. elata* and the infection rate of *Armillaria* was 95.56%. Therefore, artificial inoculation of *Armillaria* significantly

Table 2. Haplotype diversity of *Armillaria* strains obtained from different sources

Isolation source	Sample size	IGS haplotype (no. of isolates in each haplotype)
Farmland inoculated with *Armillaria* M1[a]	17	2(17) [d]
Farmland inoculated without *Armillaria* M1 [b]	25	4(24) [e]; 5(1)
Farmland A, B, C, D[c]	7	1(1); 2(2); 3(2); 6(2)
Tree barks	4	1(1); 6(3);
Total	53	

[a] This farmland was inoculated with commercial *Armillaria* M1 when cultivating *G. elata*. [b]This farmland was not inoculated with commercial *Armillaria* M1 in this study but had been inoculated with *Armillaria* M2 to cultivate *G. elata* 6 years ago; [c] These farmlands were inoculated with naturally infected *Armillaria*; [d] *Armillaria* M1 is included into this haplotype; [e] *Armillaria* M2 is included in this haplotype.

increased infection rate of *Armillaria* in the culture of *G. elata* (Mann-Whitney Test, p<0.001). However, inoculation volume of *Armillaria* (250 g/hole vs. 500 g/hole) has no influence on the infection of rate of *Armillaria* (94.4 vs. 96.7%, Mann-Whitney Test, p=0.471) but on the weight of *G. elata* tuber (data not shown).

Phylogenetic analysis

A total of 53 strains of the *Armillaria* were isolated from the rhizomorphs and were divided into 6 haplotyes based on their IGS sequences (Tables 1 and 2). Seventeen isolates were obtained from 17 different tubers of *G. elata* collected from 7 holes inoculated with the commercial *Armillaria* M1. These strains were grouped into Haplotype 2, with the strain *Armillaria* M1. Similarly, twenty-five isolates from different tubers of *G. elata* collected from 13 holes without inoculation of *Armillaria* M1 were divided into 2 haplotypes. Among of them, 24 were grouped with *Armillaria* M2 into Haplotype 4 and remaining 1 isolate (829a) belonged to Haplotype 5.

Two isolates from farmland A were also grouped with M1 into Haplotype 2, but 2 from farmland B formed Haplotype 3. Isolate SCH, obtained from tree bark of *Dipentodon sinicus* was haplotype 1. Three strains YYT, HSS and SLZ, which were isolated from tree barks of *Cerasus serrula*, *Pinus armandii* and *Aleurites moluccana* in local forest, respectively, were grouped into haplotype 6. In addition, strains S3c (from farmland C), S2a and S2b (farmland D) were grouped with above wild strains (Table 2).

Phylogenetically, these strains were different from *A. mellea*. Haplotype 2 was close to *Armillaria cepistipes* Velen, and Haplotype 3 to *Armillaria calvescens* Bérubé & Dessureault and *Armillaria sinapina* Bérubé & Dessureault. However, Haplotype 1, 4, 5, and 6, were phylogenetically distinct from those reported *Armillaria* species (Figure 1).

DISCUSSION

Currently, the *Armillaria* used in the artificial cultivation of *G. elata* is dependent on two sources. One is the naturally infected tree bark or branches of *Armillara.* This method benefits from low cost but is limited to low availability and production of wild *Armillaria.* The other one is the commercial *Armillaria*, in which the *Armillaria* were generally propagated in a laboratory and most of them are introduced into local farmland by a company. From the ecological perspective, potential risks for the introduced fungi are highly concerned in recent because the introduction of exotic fungi could have negative impacts on the native community (Litchman, 2010; Cowan et al., 2011). Our results showed that inoculating commercial *Armillaria* completely blocks the infection of natural genets of *Armillaria* on *G. elata* in soil, because the holes newly inoculated by commercial *Armillaria* M1 showed a very high infection rate (95.56%) and all of the strains isolated from different tubers of *G. elata* collected from different holes are grouped into one haplotype with strain M1 (Haplotype 2) (Tables 1 and 2). The holes that were not inoculated with the newly introduced *Armillaria* M1 also showed a certain degree of infection rate of *Armillaria* (22.2%). Interestingly, because all isolates obtained from these holes, with the exception of strain 829a, were phylogenetically identity to the *Armillaria* M2, a strain which had been used to inoculate *G. elata* in this experimental field but had not been applied for the last 6 years (Table 2), it further demonstrated that once the non-local *Armillaria* was introduced into an area, it was difficult for local wild *Armillaria* to infect *G. elata*. Moreover, this result indicated that the capacity of the introduced *Armillaria* for developing a symbiotic relationship with *G. elata* and blocking the infection of wild *Armillaria* on the *G. elata* could persist in soil several years even after stopping cultivation of *G. elata*.

Although many fungi are able to produce lots of spores, so as to easily disperse over a long distance by the wind, most fungi infect their hosts endemically (Taylor et al., 2006; Lumbsch et al., 2008), which may be partially due to dispersal barriers (Litchman, 2010; Peay et al., 2010). In nature, most of *Armillaria* species have been also considered to be endemic (Keča and Solheim, 2011). In this case, however, two strains (S4a and S4c) obtained

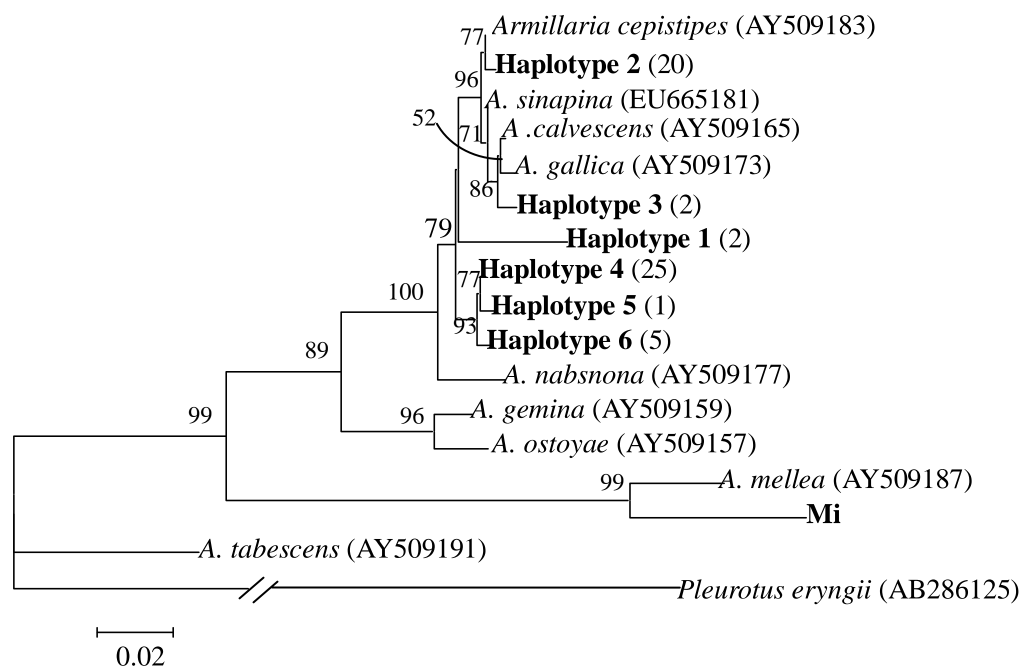

Figure 1. Neighbour-joining tree based on the IGS sequence of *Armillaria* in this case and highly similar sequences from GenBank. The occurrence time of each haplotype is indicated in parentheses. GenBank accession numbers of reference sequences are shown in parentheses. Bootstrap values are indicated on the tree branches (only those >50% are shown) and scale bar represented 2% of the genetic distance between the isolations. *Pleurotus eryngii* was used as an outgroup. Mi is a reference strain of *Armillaria mellea*.

from farmland A, in which cultivating *G. elata* was used to infect tree bark or branches of *Armillara* directly collected from local forest, were grouped with the introduced *Armillaria* M1 (Table 1). It suggested that those *Armillaria* being able to support the growth of *G. elata* should be easily dispersed into local forest through the facilitation of human force. Regarding potential risk on local ecosystem of exotic *Armillaria*, it is very important for local governments to supervise and control utilization of the introduced fungi.

On the other hand, *Armillaria* obtained from farmland B, C and D, and those from local tree species *Cerasus serrula*, *Pinus armandii* and *Aleurites moluccana*, were grouped into three different haplotypes which are distinct from *Armillaria* M1 as well as M2 (Tables 1 and 2). Interestingly, these strains are phylogenetically different from those *Armillaria* that were previously reported to be symbiotic with *G. elata* (Cha and Igarashi, 1995; Sekizaki et al., 2008), as well as those not (Figure 1). These data suggested that there are diverse and novel species of *Armillaria* in local forest and their application for cultivating *G. elata* should be encouraged for local organization and farmer. In addition, because different strains play a different role in the production of cultivating *G. elata* (Ning and Yu, 2008; Rong and Cai, 2010), it is necessary to explore their effects on the yield of *G. elata* in the Xiaocaoba areas in future.

Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial activity of fungi isolated from the water of the sky high, Recife-PE supply against bacteria of clinical interest system

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Water supply assumes an important role, since it can serve as a vehicle for microorganisms such as viruses, bacteria and pathogenic fungi. The presence of a group of microorganisms (fungi) that cause deterioration of water quality is related to the production of secondary metabolites, such as antibiotics and toxins. One of the reasons of carrying out tests with these organisms is, among other things, to verify their antimicrobial activity against other pathogens. From this, five fungi species isolated from the Water Supply System of High Heaven (*Pestalopsis palestris*, *Cladosporium cladosporioides*, *Trichoderma pseudokoningii*, *Curvularia lunata* (50) and *Penicillium* sp. (45)) were tested against the bacteria of clinical interest (*Staphylococcus aureus* (UFPEDA 01), *Pseudomonas aeruginosa* (UFPEDA 39), *Mycobacterium tuberculosis* (UFPEDA 71)) and three oxacillin-resistant *S. aureus* (ORSA UFPEDA 709, 730, 733) using the disc diffusion method. It was observed that the extract of *Penicillium* sp. showed antibacterial activity against *S. aureus* (730 ORSA).

Key words: Test activity, filamentous fungi, *Staphylococcus aureus* (ORSA).

INTRODUCTION

Microbiological quality is expressed by the amount of bacteria present in a given volume of water (Hageskal and Skarar, 2008), but in recent decades, viruses and other parasites have also been accepted as quality parameters. Some studies have reported the occurrence of filamentous fungi in drinking water, but their quantification is still small compared to bacteria (Paterson et al., 2005). But, there are already reports of fungi causing nosocomial infection with water as a vector. Pereira et al. (2009, 2010) reported the occurrence of several species of fungi in various sources of drinking water. In addition to this study, 49 different species of fungi were identified as

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producers of toxins and metabolites.

Secondary metabolites in nature are important for microorganisms that produce them; they work as sex hormones, ionophores, competitive weapons against other organisms, are symbiotic agents and have effects of differentiation and unknown activities (Demian and Adrio, 2008)

In nature, organisms are organized in biofilms known to be composed of complex communities of organisms, including aerobic and anaerobic bacteria, amoeba, protozoa, nematodes, and fungi. The microorganisms that reside near and synergistically live in biofilm support the fungi or nutrient source for bacteria, thus contributing to biofilm formation (Fachin et al., 2001). Many organisms comprising biofilms produce metabolites with other organizations, using antibiosis as a defense mechanism of the microbial community. These metabolites can inhibit the growth or kill the organism that causes damage to the biofilm structure (Silva et al., 2006; Wilson et al., 2011.)

The bacteria, *Staphylococcus aureus* is an important pathogen because of its virulence, antimicrobial resistance and association with several diseases, including life-threatening systemic diseases, skin infections, opportunistic infections and food poisoning. Antimicrobials are drugs that have the ability to inhibit the growth of or kill pathogenic microorganisms, without being toxic to the host (Tortora, 2000; Griffin, 1993).

Despite the availability of a large number of antibiotics in the last generation, it is very important to seek compounds that may act as new drugs for fighting diseases caused by bacteria (Bills et al., 2013). Numerous studies have reported the antimicrobial resistance of *S. aureus* bacteria. Thibaut et al. (2010) reported that *S. aureus* and *Escherichia coli* isolated from food bacteria were resistant to antibiotics group β - lactams and macrolides. Ferreira et al. (2011), investigating the presence of methicillin- resistant *S. aureus* on the surface of various objects in an intensive care unit, found that 29 samples (60.4%) were resistant to methicillin. The authors emphasized that the surface area of the infusion pump and aprons was infected by 60 and 75% respectively of the isolated samples. Catão et al. (2013) documented the rate of hospitalized patients with various infections in a hospital in Campina Grande, Paraíba, Brazil. Of the 1056 charts reviewed, 26 reported infections caused by *S. aureus*. Of these, 17 reported cases of methicillin-resistant *Staphylococcus*.

Another determining factor is the optimization of the culture media for the production of secondary metabolites by microorganisms isolated from diverse environments. One technique currently used is the experimental design, an effective tool for statistical optimization of fermentation processes. Some authors have reported the use of this methodology for process improvement. De Paris et al. (2012) reported the use of an experimental design (CCR) with twenty two runs for optimization of the culture medium of *Aspergillus niger* in solid state fermentation.

Given all, the above aims to evaluate the antimicrobial activity of fungi against bacteria of clinical interest, using an optimized culture medium.

MATERIALS AND METHODS

Microorganisms

Fungi

The five fungi, *Pestalopsis palestris* URM 04; *Cladosporium cladosporioides* URM 06; *Trichoderma pseudokonigii* URM 35; *Curvularia lunata* URM 50 and *Penicillium* sp. URM 45 isolated from the main supply system of the High Sky in Recife were used (Table 1). These organisms were identified by micro-morphological fungal structures (conidiophores and vegetative structures) observation as well as by biochemical tests; and they were subsequently deposited in the Culture Collection of the URM Culture Collection UFPE.

Bacteria

In order to test the antibacterial activity of the metabolites produced by the fungi, gram positive bacteria (G+); *S. aureus* (UFPEDA 01) and *S. aureus* (ORSA UFPEDA 709, 730, 733); and Gram negative bacteria (G-) (*Pseudomonas aeruginosa* UFPEDA 39) were used. *Mycobacterium tuberculosis* (UFPEDA 71) and alcohol resistant acid (ARA) of clinical interest were acquired from the Culture Collection of the Federal University of Pernambuco, Department of Antibiotics (UFPEDA) (Table 2).

Selection of microorganisms

Before the selection was done, fungal spores were inoculated separately in the center of the Petri dishes containing Sabouraud culture; they were incubated at 30°C for 48 h to obtain the mycelial mass.

In order to test the fungi that produce metabolites against better clinical bacteria, agar discs of approximately six millimeters (6 mm φ/1.5 g/L) and yeast previously grown were inoculated in two ways in potato dextrose liquid culture (PD - 200.0 g Potato, 15.0 g glucose and 1.000 mL of distilled water , pH 6.8 to 7.0) and Sabouraud (SAB - 50.0 g peptone, 40.0 g glucose, 1000 ml distilled water; final pH adjusted to 5.6). They were incubated under static conditions for five days. After this period, the filtrate of growth culture was obtained by filtration and tested for antimicrobial activity using the method of Kirb et al. (1966). In this, paper discs (6 mm φ) were soaked in 10 µL filtrate of growth culture of the fungus and placed in a Petri plate previously seeded with the standard solutions at 0.5 McFarland scale of bacteria (10^8 UFC/mL) to be tested. The samples were incubated at 37°C for 24 h. After this period, zones of inhibition (mm) were measured. At this stage the five, fungi were tested against *S. aureus* (UFPEDA 01), *P. aeruginosa* (UFPEDA 39) and *M. tuberculosis* bacteria (UFPEDA 71).

Influence of aeration on the production of metabolites

In order to observe the influence of aeration on the production of metabolites, agar discs (approximately six millimeters) of fungus selected in the previous step were inoculated in the culture medium showing the best conditions for production of metabolite in a 250 mL Erlenmeyer flasks subjected to 180 rpm at 37°C. Every 24 h, aliquots were removed for measurement of pH potentiometer, Model HSP-3B and biomass dry weight by observation method. The influence of stirring was examined by observing the formation of inhibition zones by the methodology described above against oxacillin-resistant *S. aureus* (ORSA).

Table 1. Fungi.

Fungi	Identification number (URM)
Pestalopsis palestris	04
Cladosporium cladosporioides	06
Trichoderma pseudokonigii	35
Curvularia lunata	50
Penicillium sp.	45

Table 2. Bacteria.

Bacteria	No. of collection (UFPEDA)
Staphylococcus aureus (G+)	01
S. aureus ORSA (G+)	709
S. aureus ORSA (G+)	730
S. aureus ORSA (G+)	733
Pseudomonas aeruginosa (G-)	39
Mycobacterium tuberculosis (AAR)	71

Table 3. Coded matrix of 2^2 factorial design with three center points for the fungus selected.

Tests	Glucose (g/L)	Inoculated
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	- 1.41	0
6	+1.41	0
7	0	-1.41
8	0	+1.41
9	0	0
10	0	0
11	0	0

Optimization of conditions for production of metabolites

In order to optimize the conditions for the production of metabolites, Statistical Experimental Design methodology was used. With a trend that showed better results in the previous assay, an experimental design was performed in order to obtain the best operating conditions. To this, a central composite design (CCD) was applied, through a full factorial design (22) with levels of -1 and +1, four axial points (-1.41 and +1.41) and three central points (zero level). The variables studied are pH and agitation (independent variables) and antimicrobial activity (dependent variable). The design consisted of 11 experiments (Table 3) and for its realization, we used the Statistic 6.0 Software.

Statistical analysis

To verify that there was a significant difference between the pH values and biomass inhibition halo of tested fungi, analysis of variance was performed using the software Statistic 6.0.

RESULTS AND DISCUSSION

Selection of microorganisms

Pestalopsis palestris, *Cladosporium cladosporioides*, *Trichoderma pseudokonigil*, *Curvularia lunata* and *Penicillium* sp. were fermented in two liquid media in static conditions for a period of five days. In the fermentation process, a pH of 6.05 to 7.54 is demonstrated in the Potato Dextrose (PD) medium, which is close to neutral; while in half Sabouraud (SAB), the pH is 6.76 to 8.76, which is neutral to alkaline (Figure 1). After the analysis of variance done on the pH values tested, it was observed that there was no significant difference among the five pH values obtained with $p = 0.9233$. The pH is a parameter that should be taken into consideration because of its importance for the continual viability of the fungus during the fermentation process. Pimenta et al. (2008) tested in shake flasks, fungal activity of *Hypholoma fasciculare*, *Saccharomyces cerevisiae* yeast (PYCC 4455), *Kluveromyces marxianus* and *Candida tropicalis* (PYCC 3886T 3097T PYCC), taking into account the variation of pH and temperature. They observed that pH around neutrality (5.6 to 5.8) favored the production of secondary metabolites by the filamentous fungi tested. Bhimba et al. (2012) evaluated the activity of 34 endophytic fungi against various bacteria in liquid medium using the disc diffusion method; they observed that there was increased fungal activity when fermented with acidic pH.

In the production of metabolites, pH is also important in maintaining the environment of the microorganism, whether natural or synthetic. Many fungi present in biofilms in water systems tend to acidify, creating conditions for their survival. Gao et al. (2013) isolated a fungus, *Aureobasidium pullulans* from marine biofilm and observed after stimulation, the aquatic environment has a tendency to acidify the saline.

Another factor that must be observed in the processes of production of secondary metabolites by filamentous fungal is that biomass is produced. It must be considered that, as these substances are fruits of secondary metabolism and not directly related to growth, it is more interesting to get a smaller amount of biomass. Throughout the growth stages, there was a higher yield of biomass of the fungus in the culture medium (PD) with values ranging from 0.80 to 4.24 g/L, showing a good affinity for growth in this medium; whereas in the culture medium (SBA), yield was lower with growth ranging from 0.10 to 1.4 g/L (Figure 2). This behavior is due to the alkaline pH which is not a good condition for growth of fungi. The analysis of variance between the biomass values showed no statistically significant difference between the masses of the five fungi with $p = 0.0451$.

Prabha et al. (2009), who also conducted tests on fungi,

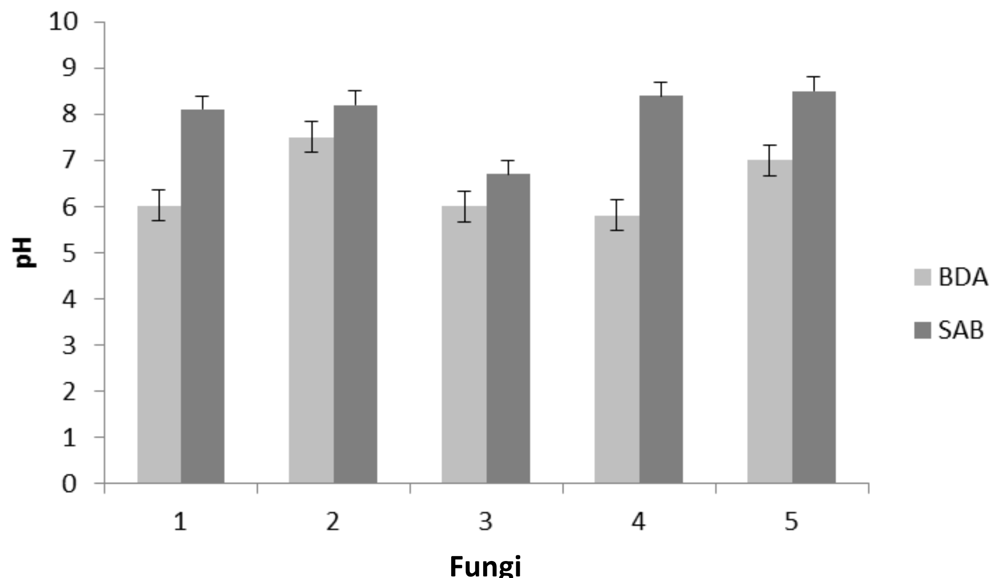

Figure 1. Value of pH in Sabouraud (SAB) and potato dextrose (PD) of *Pestalopsis palestris* (1), *Cladosporium cladosporioides* (2), *Trichoderma pseudokonigii* (3), *Curvularia lunata* (4) and Penicillium sp. (5) after five days of fermentation.

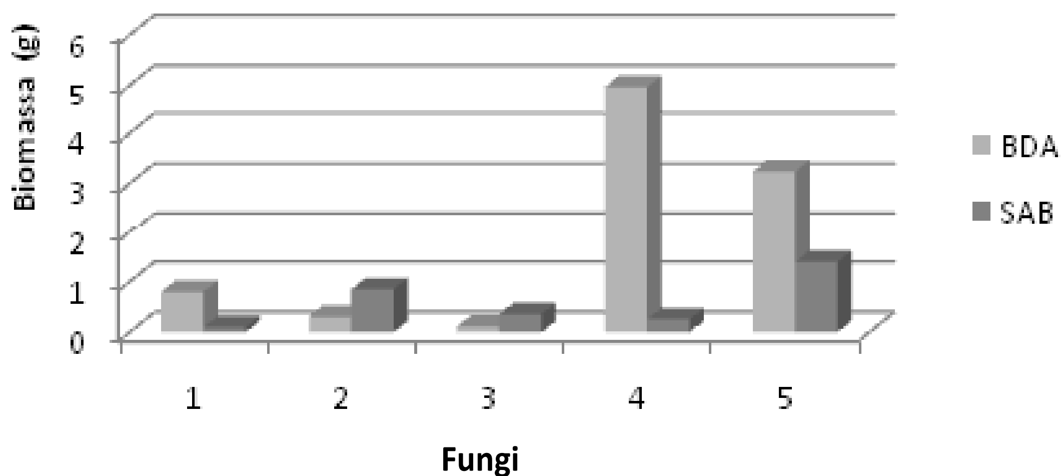

Figure 2. Biomass value produced by fungis *Pestalopsis palestris* (1), *Cladosporium cladosporioides* (2), *Trichoderma pseudokonigii* (3), *Curvularia lunata* (4) and *Penicillium* sp. (5) in mediums tested after five days.

observed the growth of *Penicillium chrysogenum* and *Aspergillus* sp. These species were grown in liquid fermentation for biomass and secondary metabolites featuring four typical stages of growth: the lag phase (0-7 days) where the physical balance between the microorganisms and the environment showed little growth of 8 to 15°C. In the log phase, growth occurred with increase of biomass mainly on the 15th day. In the stationary phase of growth, there was altered cell growth medium through the substrate consumption and excretion of secondary metabolites. This is followed by reduction of the biomass in the culture medium, indicating cell death- the death phase. Also observed was a strong anti-bacterial activity of citrinin from *P. aeruginosa* bacteria and *Vibrio cholera*.

The tested *Penicillium* sp. strain produced metabolites with antimicrobial activity and inhibition zone of 6.5 mm after 24 h against *S. aureus*; while other fungal extracts showed no activity against the microorganisms tested. So this fungus was selected to be used in later steps.

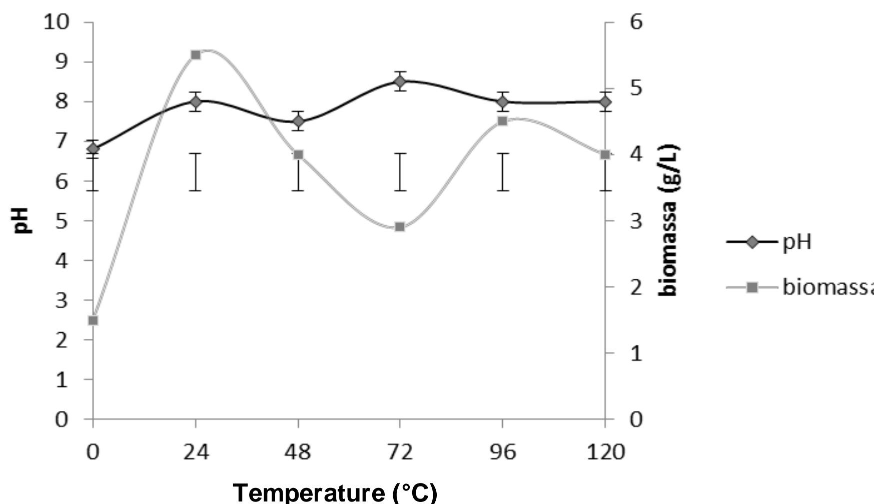

Figure 3. Values of pH and biomass of fermentation liquid in PD broth of *Penicillium* sp. under agitation condition.

Table 4. Inhibition zone of metabolites produced by *Penicillium* sp. in PD broth.

Growth period (time)	Average of inhibition zones (mm) against *S. aureus* UFPEDA		
	709	730	733
24 h	_	19,73±1	_
48 h	_	20,21±1	_
72 h	_	21,80±1	_
96 h	_	22±0.8	_
124 h	_	22±0.8	_
148 h	20.25±0.05	21±0.8	16±0.87
Vancomicina ($C^{+)}$	18.3±0.05	14.2±0.02	22.09±0.05
Caldo BD (C^{-})	0	0	0

(-) No activity.

Penicillium species are a group of microorganisms which synthesize high amount of secondary metabolites; in some cases, they have 73% production more than the other classes of microorganisms. The production of these substances by fungi intrinsically depends on the conditions used for their growth and development (Pepper et al., 2008). In addition, *Penicillium* species produce a diverse range of active secondary metabolites, including antibacterial, immunosuppressive agents and cholesterol reduction (Petit, 2009). As described since 1929 when Alexander Fleming observed growth inhibition of *S. aureus* in a Petri dish, there was a culture of *Penicillium notatum* contaminant describing penicillin and its effects on Gram- positive microorganisms (Bauer et al., 1966).

From the results obtained, *Penicillium* sp. was selected for fermentation in PD broth under stirring condition and by the observation of the influence of this parameter at the middle. After the experiment, it was found that it favored the growth of the fungus. This is because agitation promotes homogenization and increases availability of nutrients from the medium. At the end of the stirring, the fungus grew from 3.96 to 5.03 g, with an anticipation to get better growth at pH 8.5 for 72h (Figure 3). Fitsum et al. (2014) tested three species of fungi against *Colletitrichum lindemunthianum*, the fungus which causes anthracnose of beans. They observed that the activity was higher under agitation conditions (150 rpm) than in static condition. The authors attributed the activity to a better distribution of nutrients in the culture medium.

Table 4 shows the activity of the fungus *Penicillium* sp. against *S. aureus* (UFPEDA 709, UFPEDA 730 and UFPEDA 733) tested and their respective standard deviations. The *Penicllium* sp. was active against *S. aureus* (UFPEDA 730) in the first twenty-four hours in culture while maintaining an increase in this activity over 148h with inhibition zones ranging from 19.73 to 22 mm. Although *Penicillium* sp. has not been shown to be active

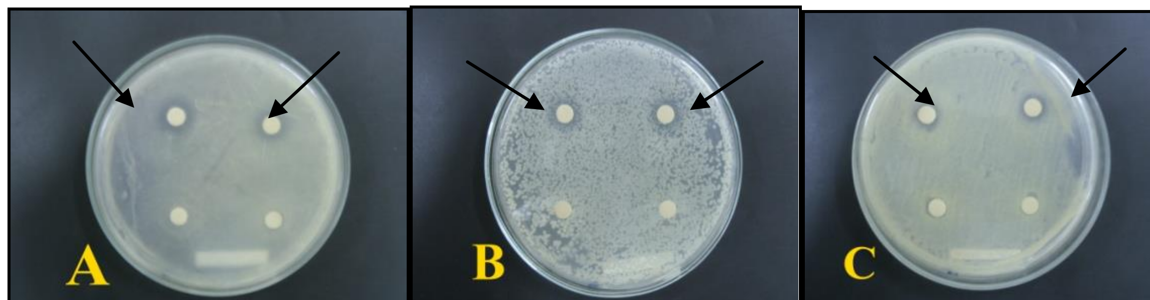

Figure 4. Inhibition formed by the metabolite produced by the fungus *Penicillium* sp. on PDA against *S. aureus* 733. Experiments 9, 10 and 11 equivalent to the midpoint, respectively.

in the early hours against *S. aureus* 709 and 733, there was a significant activity with halos of 20.25 mm against *S. aureus* 709; while for *S. aureus* 733, the halo was 16 mm after 148 h.

Cortez (2011) reported the activity of two strains of fungi, *P. chrysogenum* and *Diplodia* sp. against Gram positive and Gram negative bacteria in fermented PD broth after 72 h under stirring of 180 rpm. The author relates the presence of the activity with the pigment produced by the organism. Corroborating this work, He et al. (2013) reported the isolation of a substance produced by *Aspergillus* spp., it was active against various micro-organisms in the soil. The authors describe the substance isolated as an Anshamicyna and highlight its effectiveness in combating fungi and actinomycetes, human pathogens. Unlike this work, Kuephadungphan et al. (2014) reported the activity of the extract of the fungus *Gibellula pulchra* EPF083 against methicillin resistant *S. aureus* SK - 1 in Sabouraud broth under static condition for 48 h. The authors highlight the importance of this kind of fungus because it is a pathogen of invertebrates.

The secondary metabolism of microorganisms is extremely diverse, providing the discovery of new compounds and new classes of compounds (Prabha et al., 2009). However, the production of secondary metabolites by the fungus depends intrinsically on the conditions used for their growth and development (time of incubation, culture medium composition, temperature, pH) (Petit, 2009). *Penicillium* sp. was grown in liquid medium under stirring, at pH 7.0 with incubation time of 5 days. Glucose and inoculum component experiment was varied so that the line was subjected to growth and production of metabolites in different conditions. Thus, multivariate methodology was used to vary growth conditions and to verify how these variations influence the production of secondary metabolites by the fungus strain. Once the incubation period was complete, the culture medium for each experiment was filtered separating the biomass from the liquid medium. The analyses were performed using antimicrobial test, tested with 3 strains of *S. aureus* (ORSA). It was observed that for the 11 runs, only the races of the central points (9, 10 and 11) showed zone of inhibition of 10.5, 11 and 10.8 mm respectively for *S. aureus* 709 14, 25; 14 and 13.9 mm respectively for *S. aureus* 730 and 9.75, 10 and 10.2 respectively for *S. aureus* 733 (Figure 4). The results verified that race 10 is the only one that showed a pH of 7.7 lower than the other races. It was remaining an alkaline environment that favors the production of secondary metabolites, inhibiting the growth of the test microorganism. The response surface graph explains the low activity of growth on PDA medium, since the glucose present in the medium usually has an inhibitory effect on repressor and use of other carbon sources of subsequent metabolism of other compounds of this medium, causing retardation of fungal growth. However, the PDA proved to be a good medium when the culture biomass formation was desired. Based on the results, it can be suggested that the PDA medium has a minimal amount of glucose (< 0.1) and variation of inoculum (< 2) , where the main goal is to deliver growth metabolite within a short space of time. However, if the aim is to analyze reproductive structures, it is suggested that PDA is employed, because it permits rapid sporulation.

One explanation for the inhibition of growth of *Penicillium* was influenced by the independent variables, as carotenoid biosynthesis (fungi have a significant potential for the biotechnological production of carotenoids, because yellow pigments accumulate during growth) naturally changes the pH of the medium. The pH is one of the most important environmental parameters that influence growth and product formation.

The results of the optimization of the experimental design (Figure 5) are expressed in Statistic 6.0 ANOVA; as noted, the concentrations of the dependent and independent variables are done in eleven repetitions. The Pareto chart shows how the variables influence the process, either positively or negatively.

In this case, the inoculum and glucoses variables influence negatively the process, which means that their low amount is best for antibiotic production by the fungus. The response surface figures show the trend in which the process should be conducted (Figure 6).

In this case, it is observed from the graphical response

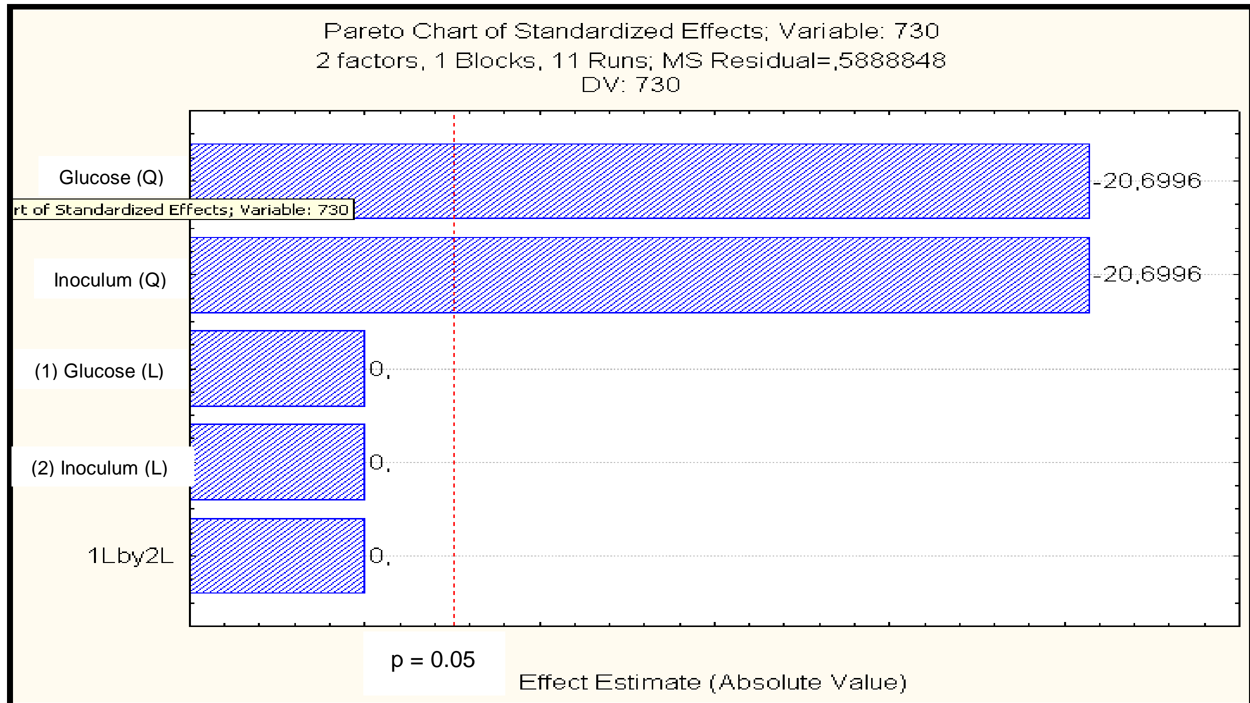

Figure 5. Pareto and standardized effects using glucose and inoculums which bustle as the dependent variable by *Penicillium* sp.

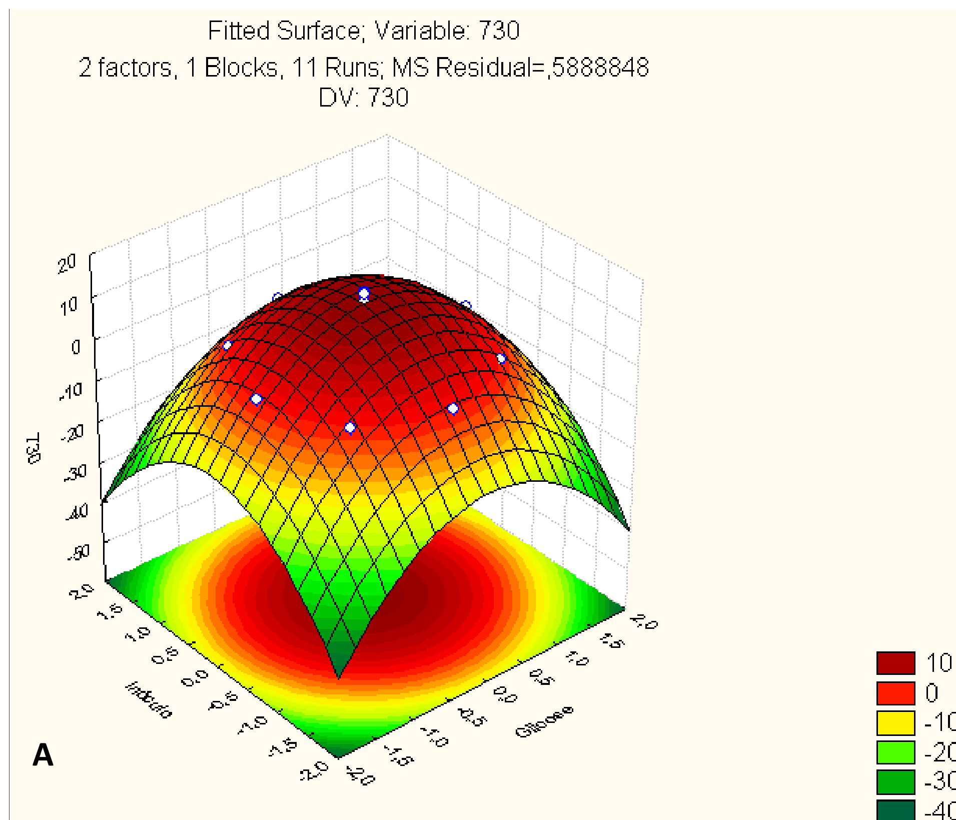

Figure 6. Surface response optimization means for PD production of bioactive compounds by *S. aureus* (ORSA, A - 730, B-733 and C-709).

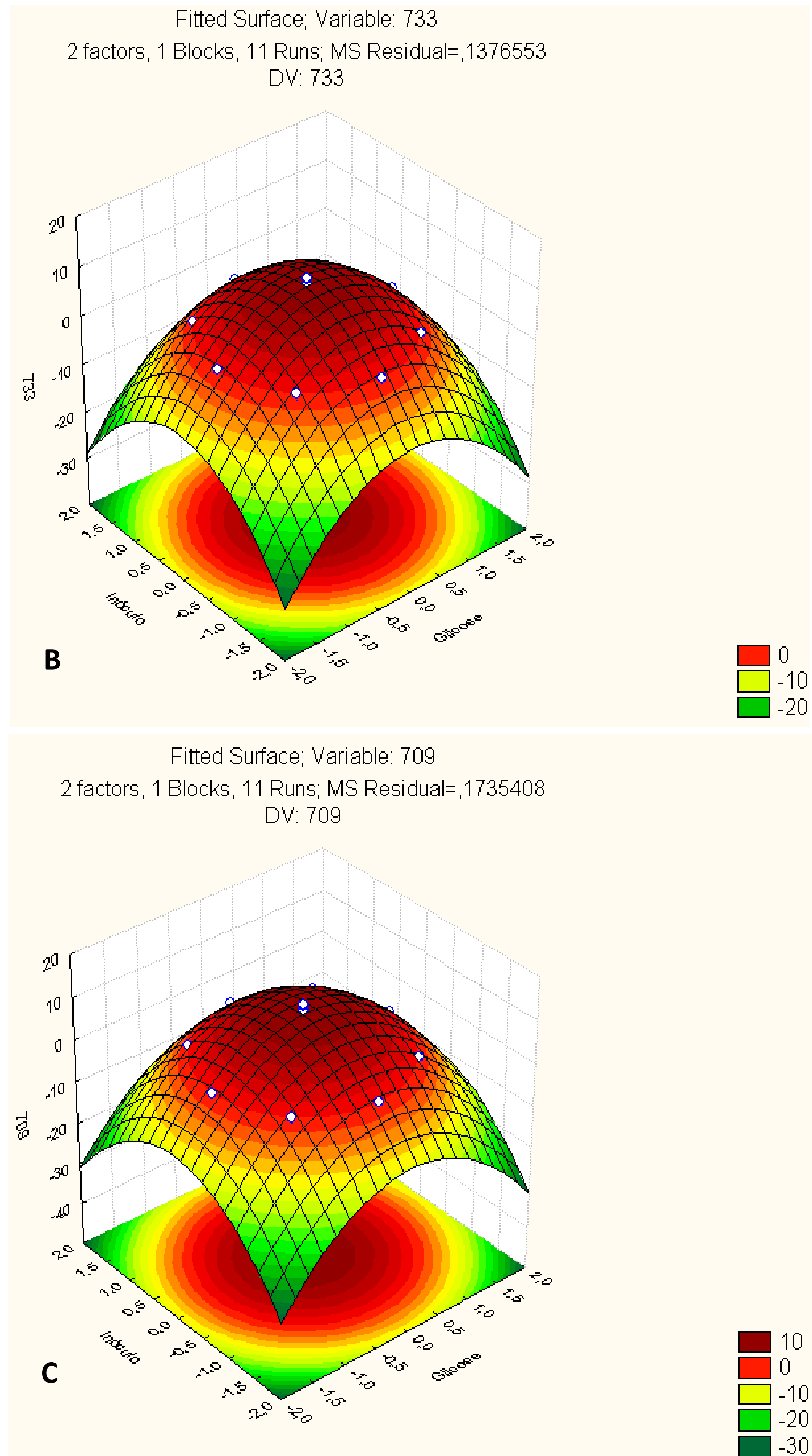

Figure 6. Contd.

surface that lower amount of glucose and inoculum will lead to a better production of the metabolite. It can be seen in the figure that in response variable surface 733 (arrow) greater halo (red top) is present when glucose is between zero and 0.5, likewise the inoculum.

Conclusion

Filamentous fungi showed a preference for growth in PD broth having a good biomass yield and sporulation. Penicillium sp produce secondary metabolites that inhibit the growth of *S. aureus* (ORSA). Glucose amounts greater than 0.1 g had an inhibitory effect on the growth and production of metabolite by the fungus.

Conflict of Interests

The authors have not declared any conflict of interests.

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Activity of the desert truffle *Terfezia boudieri* Chatin, against associated soil microflora

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This research focuses on the activity of the desert truffle *Terfezia boudieri* Chatin against its associated microflora. It consists of the enumeration and pre-identification of soil microflora associated with ascocarps of *T. boudieri* Chatin, in contact with fungus ascocarps and at 10 cm deep. Enumeration reveals that the number of some microorganisms is higher when in contact with ascocarps of *T. boudieri* than at 10 cm deep. Also, bacteria are more dominant than fungi and actinomycetes (especially *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Streptococcus* sp., *Proteus* sp.). The number of bacteria is estimated to be 8.3 - $8.6x10^7 \pm 0.5x10^7$ CFU/g of dry soil, while fungal genera (with a dominance of *Penicillium* sp., *Aspergillus* sp., *Mucor* sp. and *Alternaria* sp.) are between $8.6x10^4 \pm 0.8x10^4$ and $4.5x10^5 \pm 0.8x10^5$ CFU/g and actinomycetes (especially *Streptomyces* sp.) are between $8.2x10^2 \pm 0.3x10^2$ and $2.2 \times 10^3 \pm 0.3 \times 10^3$ CFU/g. Microorganisms included in the nitrogen cycle are represented by free nitrogen fixing bacteria (Azotobacters) ($2.2x10^1 \pm 0.8x10^1$MPN/g), ammonifying bacteria ($4x10^3 \pm 1.0 \times 10^1$ MPN/g), nitric bacteria (between $6.8X10^3 \pm 1.0x10^3$ and $0.6x10^1 \pm 1.0x10^1$ MPN/g), and nitrous bacteria (between $4x10^1 \pm 1.0x10^1$ and $0.3X10^1 \pm 1.0x10^1$ MPN/g). Bacteria included in the degradation of organic matter are mainly proteolytic (between $1.2x10^6 \pm 0.8 \times 10^6$ and $2.4X10^4 \pm 0.8 \times 10^4$ CFU/g), amylolytic (between $6.8 \times 10^3 \pm 0.2 \times 10^3$ and $0.6 \times 10^1 \pm 0.2 \times 10^1$ CFU/g) and cellulolytic bacteria (between $1.3x10^4 \pm 0.7x10^4$ and $1.5x10^3 \pm 0.7x10^3$ MPN/g) but lipolytic and denitrifying bacteria are absent. These results suggest that *T. boudieri* Chatin stimulate soil microflora in contact with their ascocarps.

Key words: *Terfezia boudieri* Chatin, terfez soil, microorganisms, enumeration.

INTRODUCTION

Desert truffles or terfez are hypogeous ascomycota that grow in arid and semi-arid regions in Mediterranean and Middle Eastern countries (Awameh, 1981; Fortas and Chevalier, 1992; Roth- Bejerano et al., 1990; Bratek et al., 1996; Slama et al., 2006; Mandeel and Al-Laith, 2007; Trappe et al., 2008). A few species are found in Asia (China and Japan), America (Mexico, USA), Australia and southern Africa (South Africa, Madagascar) (Callot et al., 1999;

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Figure 1. Ascocarps of the desert truffle, *Terfezia boudieri* Chatin taken with a digital still camera DSC-W.

Ferdman et al., 2005; Kovacs et al., 2008; Trappe et al., 2008). They include several genera: *Balsamia*, *Delastria*, *Eremiomyces*, *Elderia*, *Kalaharituber*, *Leucangium*, *Mycocleandia*, *Mattirolomyces*, *Phaeangium*, *Picoa*, *Reddelomyces*, *Terfezia*, *Tirmania* and *Ulurua* (Morte et al., 2008; Trappe et al., 2008; Jamali and Banihashemi, 2013).

Terfez are valued for their nutritional values; they contain proteins, amino acids, fibers, fatty acids, minerals and carbohydrates (Bokhary and Parvez, 1992; Omer et al., 1994; Hussain and Al- Ruqaie, 1999; Dabbour and Takruri, 2002; Murcia et al., 2002; Ahmed, 2013; Hamza et al., 2013; Stojković et al., 2013). They contain antioxidant substances (Murcia et al., 2002; Pervez-Gilabert et al., 2005b; Al-Laith, 2010; Dundar et al., 2012; Hamza et al., 2013; Gouzi et al., 2013a and b) and contain enzymes with medical and industrial interest (Perez-Gilabert et al., 2005a). Their therapeutic properties have been used for centuries in traditional medicine (Loizides et al., 2012).

Their antimicrobial activities have been the subject of numerous studies like those of Rougieux (1963), Chellal Lukasova (1995), Janakat et al. (2004 and 2005), Akyuz et al. (2010), Neggaz and Fortas (2013) and Stojković et al. (2013). Terfez are also a source of compounds with anti-inflammatory characteristics, immunosuppressants, anti-mutagenic and anti- carcinogenic properties (Hannan et al., 1989).

Desert truffles are mycorrhizal fungi establishing an association mainly with annual or perennial Cistaceae plants belonging to the genus *Cistus* and *Helianthemum* (Dexheimer et al., 1985; Fortas and Chevalier, 1992; Morte and Honrubia, 1997; Gutiérrez et al., 2003; Slama et al., 2010, 2012; Zitouni- Haouar et al., 2014) and also with some species of *Pinus* and *Quercus* (Diez et al., 2002; Morte et al., 2008; Zitouni-Haouar et al., 2014). They can form ectomycorrhiza, endomycorrhiza or ectendomycorrhiza with their host-plants; such mycorrhiza depend on the nature of the substrate, growing conditions, plant species and terfez species (Kagan-Zur and Roth-Bejerano, 2008; Kagan-Zur et al., 2008; Slama et al., 2010 and 2012; Zitouni-Haouar et al., 2014).

Soil microflora of truffles (for example, *Tuber*) has been the subject of numerous studies where there are abundance of bacteria, actinomycetes, yeasts and filamentous fungi (Mamoun and Olivier, 1989; Zacchi et al., 2003).

The soil microflora of desert truffle (terfez) has been rarely studied. Only the studies of Rougieux (1963) and Bokhary and Parvez (1992) which addressed the antibiotic and stimulating activity of *Terfezia* against soil microflora are known to the authors.

Our study focuses on the activity of the soil microflora associated with the desert truffle *Terfezia boudieri* Chatin. This work shows: Enumeration of total soil microflora and other microbial groups included in nitrogen cycling and organic matter degradation, located at point of contact of ascocarps and at 10 cm deep, in order to demonstrate the stimulating effect of terfez on the development of some telluric and saprophytic bacteria; Isolation of the dominant forms of bacteria, actinomycetes and fungi, from soil terfez and their pre-identification on the basis of macroscopic, microscopic and biochemical characteristics.

MATERIALS AND METHODS

Ascocarps and soil samples of *T. boudieri*

Ascocarps of desert truffle were collected during the month of February in 2010, from a semi-arid region, in the north of Algeria, near their natural host plant (*Helianthemum ledifolium*). They were subglobose (4-14 cm) with smooth brown peridium (surface) (Figure 1). When light and electronic microscopy were used (Figures 2 and 3), the spherical shape of ascospores and the presence of warts with rounded ends show that ascocarps belong to *T. boudieri* Chatin according to Diez et al. (2002) and Slama et al. (2006).

Soil samples were taken with the ascocarps above, at contact (sample A) and at 10 cm deep from ascocarps (sample B), using sterile Petri dishes. The soil was a composite sample derived by mixing soil from sampling 14 points. The soil samples were stored at 4°C until processing within 24 h (Pochon and Chan, 1948).

pH and dry weight of soil samples of *T. boudieri*

Soil pH of each soil sample (A and B) was measured using a soil-water mixture 1:5 (w/v); after 72 h of decantation, the pH of the supernatant was measured with a pH meter. The dry weight of each soil sample was determined by oven drying of 10 g of fresh sieved soil (a sieve with a porosity of 2 mm) for 24 h at 105°C (Davet, 1996).

Enumeration of the soil microflora associated with *T. boudieri*

Two grams of each soil sample (samples A and B) was aseptically sieved (sieve with a porosity of 2 mm), suspended in 18 ml of sterile saline solution and agitated in a stirrer type Fisherbrand to obtain an homogeneous suspension (Girard and Rougieux, 1967). Serial

Figure 2. Asci of *Terfezia boudieri* containing 8 ascospores observed under light microscope (bar = 25 µm).

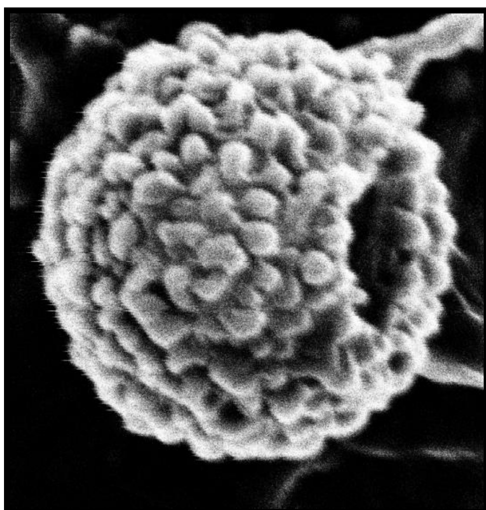

Figure 3. Ascospore of *Terfezia boudieri* observed with scanning electron microscope showing warts with rounded ends (3500 X) (personal photos).

dilutions were performed in tubes containing 9 ml of sterile saline solution until dilution 10^{-10}; 1 ml of each suspension- dilution tube was used as inoculum for plate count of colony forming units (CFU) and for the most probable number technique (MPN).

Total microflora, heterotrophic and aerobic bacteria, proteolytic, lipolytic and amylolytic bacteria then fungi and actinomycetes were counted on solid culture media by the CFU method (colony forming units) according to Girard and Rougieux (1967) and Larpent and Larpent- Gourgaud (1985). 1 ml of inoculum was inoculated in mass of culture media (the culture media corresponding to the group of microorganism researched). Three Petri dishes (20 ml of medium per Petri dish) were prepared for each medium and each dilution. All bacterial cultures were incubated at 37°C for 2 days.

Actinomycetal and fungal cultures were incubated for 7 days, respectively, at 28 and 25°C. Total microflora and heterotrophic-aerobic bacteria was enumerated using the medium M1 (5 g peptone, 5 g NaCl, 15 g agar, 1000 ml distilled water (pH 7)). Proteolytic bacteria were enumerated using the medium M8 (5 g peptone, 3 g yeast extract, 5 g NaCl, 40 g gelatin, 15 g agar, 1000 ml distilled water (pH 7)) and the Frazier reagent (15 g $HgCl_2$, 20 ml 10% HCl, 100 ml distilled water) by counting bacterial colonies having a transparent zone due to hydrolysis of gelatin around bacterial colonies by proteases. Lipolytic bacteria were counted using the medium M10 (10 g peptone 5 g NaCl, 0.1 g $CaCl_2$,12 g agar, 1000 ml distilled water, 10 ml Tween 80 (pH 7)) by counting bacterial colonies having a transparent halo due to hydrolysis of Tween 80 by lipases.

Amylolytic bacteria were enumerated using the medium M11 (2 g yeast extract, 10 g peptone, 2 g starch, 12.5 g agar, 1000 ml distilled water (pH 7)) and 2 ml of Lugol by counting bacterial colonies having yellow coloration around bacterial colonies due to hydrolysis of starch by amylases.

Fungi were detected by their filamentous colonies and counted using the medium M12 (5 g yeast extract, 30 g sucrose, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$ (II), 1 g K_2HPO_4, 13 g agar, 1000 ml distilled water (pH 4)).

Actinomycetes were detected by their specific colonies and enumerated using the medium M13 (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 18 g agar, 1000 ml distilled water (pH 7.2)) according to Boughachiche et al. (2005).

Ammonifying, denitrifying, cellulolytic, nitrous, nitric and free nitrogen fixing bacteria (Azotobacters) were enumerated in liquid culture media according to Girard and Rougieux (1967) by using the most probable number technique (MPN) (Halvorson and Ziegler, 1933). 1 ml of inoculum was inoculated in liquid culture media (the culture media corresponding to the group of microorganism researched). Three tubes (9 ml of liquid culture medium per tube) were prepared for each medium and each dilution. All tubes were incubated at 37°C for 2 days. Ammonifying bacteria, were enumerated by using the culture medium M7 (2g asparagine, 1g K_2HPO_4, 10g KH_2PO_4, 0.5g $MgSO_{4,}7H_2O$, 8ml glycerol, 1000 ml distilled water (pH 7)) and by using the Nessler's reagent after growth.

Denitrifying bacteria were counted by the culture medium M6 (2 g KNO_3, 10 g glucose, 5 g $CaCO_3$, 50 ml saline standard solution (mixture of solutions A and B; Solution A: 5 g K_2HPO_4, 2.5 g $MgSO_4$,$7H_2O$, 900 ml distilled water. Solution B: 0.05 g $FeSO_4$, 0.05 g $MnSO_4$, 100 ml distilled water), 950 ml distilled water (pH 7)). After growth, some drops of sulfuric diphenylamine reagent were added to indicate the presence of nitrate (blue coloration).

Free nitrogen fixing bacteria (Azotobacters) were enumerated by using the culture medium M3 (10 g glucose, 50 ml standard saline solution (mixture of solutions A and B mentioned above), 950 ml distilled water (pH 7)).

The enumeration of cellulolytic bacteria was done in the medium M9 (0.5 g $NaNO_3$, 1 g K_2HPO_4 , 0.5g $MgSO_{4,}7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$,$7H_2O$, 0.5 g yeast extract, 1000 ml distilled water (pH 7.3)) by detecting pigmentation on the strips of filter paper immersed in the culture medium. Nitrous bacteria were enumerated by using the culture medium M4 (0.5 g $(NH_4)_2SO_4$, 1 g $CaCO_3$, 50 ml standard saline solution (mixture of solutions A and B mentioned above), 950 ml distilled water (pH 7)). After growth, sulfuric diphenylamine reagent was added.

Nitric bacteria were enumerated by using the culture medium M5 (1 g $NaNO_2$, 1 g $CaCO_3$, 50 ml standard saline solution (mixture of solutions A and B mentioned above), 950 ml distilled water (pH 7)). After growth, sulfuric diphenylamine reagent and 50 mg of urea were added.

Statistical analysis was performed using the SAS System for

Table 1. pH and dry weight of the soil samples A and B of *Terfezia boudieri.*

Soil samples / Values	A	B
pH	7.85	7.55
Dry weight (g)	9.571	9.732

Windows V7 by SAS Institute Inc., Cary, NC, USA.

Isolation of the dominant bacteria, actinomycetes of the soil microflora associated with *Terfezia boudieri*

Well-isolated colonies obtained after soil dilution plate-count technique (CFU) were chosen from each plate and streaked on fresh plates. Isolates were checked for purity by re-streaking into corresponding culture media. Isolates were inoculated to agar slants; after growth they were stored at 4°C.

Bacterial and actinomycetal isolates were obtained using respectively the solid culture media M1 and M13 (compositions cited above). Isolation of bacteria and actinomycetes was performed by streaking 0.1 ml of inoculum on solid culture media in Petri dishes. The cultures were incubated at 37°C for 3 days (for bacteria) and at 28°C for 7 days (for actinomycetes).

Fungi were isolated on the medium M12 (cited above) from mycelial fragments collected using a sterile scalpel. The cultures were incubated at 25°C for 7 days (Larpent and Larpent-Gourgaud, 1985).

Pre-identification of bacteria, actinomycetes and fungi isolated

Cultural bacterial characteristics (shape, elevation, color, margin, surface of bacterial colony) and microscopic characteristics (shape, cell arrangement of the bacteria, Gram staining) were studied according to Hart and Shears (1997).

The main biochemical characteristics studied for Gram negative bacteria were: presence of the enzyme cytochrome oxidase and the growth in the culture media: MacConkey, TSI, mannitol-mobililty, urea-indole (Girard and Rougieux, 1967; Larpent and Larpent-Gourgaud, 1985). Heavy bacterial suspension of 24 h was prepared and inoculated to culture media. Incubation was at 37°C for 24 h. Also, API 20 NE strip was used according to Barr et al. (1989). Catalase and bacterial endospore were searched for the Gram positive bacteria according to Lachapelle (2004).

Actinomycetes were identified under stereomicroscope by observing colonies (morphology, color and shape), growth changes, colors of culture medium (Dastager et al., 2006). Their hyphae and Gram staining were observed under light microscope (Shirling and Gottlieb, 1966).

For fungi, some cultural characteristics were determined in particular the speed of growth, the colors of colony (and reverse of colony), the surface of mycelium. Microscopic observation (spores and mycelium) was done under light microscope according to Guiraud and Galzy (1980).

RESULTS AND DISCUSSION

Soil pH and dry weight

Table 1 shows the pH, the dry weight of the samples soil A and B, respectively in contact and at 10 cm deep of ascocarps. The soil pH is slightly alkaline, such as for certain species of desert truffles. Others like *Kalaharituber pfeilii* grow at pH values between 5.5 and 6.5 (Kagan- Zur and Roth- Bejerano, 2008). The pH of the soil is relatively higher in the sample A as compared to the sample B. These results confirm those of Hashem and Obaïd (1996) where the soil pH is higher (pH = 8.3) when in contact with the ascocarpes of *T. claveryi*.

Enumeration of the soil microflora associated with *T. boudieri*

The results of enumeration of the soil microflora (samples A and B) are shown in Table 2. The total number of microflora is higher in contact with ascocarps than at 10 cm deep. These results confirm those of Rougieux (1963) and indicate that the desert truffles can excrete substances that stimulate the development of these microorganisms. The abundance of these microorganisms has also been reported in the soil of *Tuber borchii* (Sbrana et al., 2002; Barbieri et al., 2007).

The number of actinomycetes in soil of *T. boudieri* is low; Rougieux (1963) did not detect their presence in soils. Sbrana et al. (2002) obtained a high number of actinomycetes in contact with mycorrhiza of *Tuber borchii* Vitt. while Barbieri et al. (2007) isolated a very small number of actinomycetes in contact with ascocarps of *Tuber magnatum* (3% of the number of bacteria isolated).

The number of fungi in the sample A is similar to that of sample B. Similar results were reported by Zacchi et al. (2003) in the soil in contact with ascocarps of *Tuber*. Mamoun and Olivier (1989, 1990) reported that the densities of the fungal flora of the rhizosphere of hazelnut trees producing truffles and of soil without vegetation are approximately the same.

Free nitrogen fixing bacteria (Azotobacters) are present in reduced number in contact with ascocarps (sample A) which appears to stimulate their growth. Similar results were obtained by Rougieux (1963) which demonstrated that the addition of the aqueous extract of *T. boudieri* Chatin into Azotobacters culture improves the development of these bacteria and has concluded that this species of terfez contains a substance which, diffusing into the soil, stimulates the development of these bacteria. According to Dommergues (1978), the number of free nitrogen fixing bacteria in the soil is low (2.2×10^1 CFU/g dry soil).

The nitrifying bacteria (nitrous and nitric) are more numerous in the soil in contact with the ascocarps (sample A). Rougieux (1963) reported that they are absent in soil of *T. boudieri*. According to Davet (1996), these bacteria develop in well aerated soils. Indeed, *T. boudieri* grows in soil with sandy clay texture which would explain the presence of these bacteria.

Table 2. Average numbers of soil microflora in samples A and B.

Soil microflora (per 1 g of dry soil)	Soil samples	
	A	B
Total microflora (CFU/g dry soil)	$8.8x10^{10} ±0.8x10^{10}$	$9.6x10^{8}±0.8x10^{8}$
Heterotrophic and aerobic bacteria (CFU/g)	$8.3x10^{7}±0.5x10^{7}$	$8.6x10^{7}±0.5x10^{7}$
Actinomycetes (CFU/g)	$8.2x10^{2}±0.3x10^{2}$	$2.2x10^{3}±0.3x10^{3}$
Fungi (CFU/g)	$8.6x10^{4}±0.8x10^{4}$	$4.5x10^{5}±0.8x10^{5}$
Free nitrogen fixing bacteria (MPN/g)	$2.2x10^{1}±0.8x10^{1}$	$1.0X10^{3}±0.8x10^{3}$
Ammonifying bacteria (MPN/g)	$4x10^{3}±1.0x10^{3}$	$4x10^{1}±1.0x10^{1}$
Nitrous bacteria (MPN/g)	$4x10^{1}±1.0x10^{1}$	$0.3x10^{1}±1.0x10^{1}$
Nitric bacteria (MPN/g)	$6.8x10^{3}±1.0x10^{3}$	$0.6x10^{1} ±1.0x10^{1}$
Denitrifying bacteria (MPN/g)	0	0
Proteolytic bacteria (CFU/g)	$1.2x10^{6}±0.8x10^{6}$	$2.3x10^{4}±0.8x10^{4}$
Lipolytic bacteria (CFU/g)	0	0
Amylolytic bacteria (CFU/g)	$6.8x10^{3}±0.2x10^{3}$	$0.6x 10^{1}±0.2x10^{1}$
Cellulolytic bacteria (MPN/g)	$1.3x10^{4}±0.7x10^{4}$	$1.5x10^{3}±0.7x10^{3}$

The density of ammonifying bacteria is very low in the soil sample A. According to Rougieux (1963), the desert truffle stimulates the development of those micro-organisms.

The denitrifying bacteria are not detected in the two soil samples, although that Rougieux (1963) showed a clear stimulation of these microorganisms by the desert truffle. According to Ewaze and Al- Naama (2006), the mycelia and ascocarps of *Terfezia* sp. and *Tirmania* sp. of Iraq are able to reduce nitrate.

The number of proteolytic microflora is important in the soil in contact with ascocarps of *T. boudieri*. These results are similar to those of Zacchi et al. (2003) who showed that almost all *Cryptococcus humicolus* strains isolated from soil in contact with ascocarps of *Tuber aestivum* have proteolytic activity. According to our results, the desert truffle seems to favor the development of these microorganisms which can give the necessary products in the nutrition of the fungus. It should be noted that this group of microorganisms isolated in contact with ascocarps of desert truffle, would probably be involved in the decomposition of these ascocarps which contain a significant amount of protein (about 20-27% of dry matter) (Hussain and Al- Ruqaie, 1999; Dabbour and Takruri, 2002; Morte et al., 2008).

The lipolytic bacteria are totally absent in the soil of *T. boudieri* although the desert truffles contain lipids (Hussain and Al- Ruqaie, 1999; Morte et al., 2008; Kagan-Zur and Roth- Bejerano, 2008). The absence of these bacteria is probably due to the production of antimicrobial substances by the fungus. Many studies have shown that extracts of desert truffles has an inhibitory effect on certain microorganisms (Rougieux 1963; Chellal and Lukášová, 1995; Hussain and Al-Ruqaie, 1999; Janakat et al., 2004; 2005; Neggaz and Fortas, 2013). Others have shown that desert truffles possess an antioxidant activity of lipids (inhibition of lipid oxidation) (Morte et al., 2008; Dundar et al., 2012; Gouzi et al., 2013 a, b).

The amylolytic bacteria are present in higher number in contact with ascocarps; these microorganisms are substantially stimulated in the presence of the desert truffle. Similar results were reported by Rougieux (1963).

Cellulolytic bacteria are abundant in both samples A and B; these results were reported by Rougieux (1963). This abundance of cellulolytic microbial community is due to the richness of the soil sampled with plant residues (Bokhary and Parvez, 1994).

Pre-identification of some bacterial isolates, actinomycetes and fungi

Identification of some bacterial isolates was based on the appearance of their colonies on culture media and their microscopic and biochemical characters. The results allowed us to identify three Gram positive bacterial genera: *Micrococcus* sp., *Streptococcus* sp., *Bacillus* sp. and five Gram negative bacterial genera belonging to: *Pseudomonas* sp., *Acinetobacte*r sp., *Enterobacter* sp., *Proteus* sp. and *Serratia* sp.

We noticed a slight predominance of *Pseudomonas* and *Bacillus* in the microflora of both soil samples (A and B). These results are consistent with those of some authors where the soil microflora of truffle is stable and specialized and is mainly represented by *P. fluorescens* and *Bacillus* (Sbrana et al., 2002; Barbieri et al., 2005, 2007).

Furthermore, according to Hartmann et al. (2009), *Pseudomonas* and *Bacillus* have been consistently

Table 3. Macroscopic and microscopic aspects of fungi isolated.

Fungal isolate	Macroscopic appearance	Microscopic appearance
Penicillium sp.	Mycelium is powder, fluffy, green and yellow	Single conidiophores, variable length, diverging phialides bearing long chains of globose conidia
Aspergillus sp.	Aerial mycelium, white, masked by black points. White reverse	Rigid conidiophores, long and not-septated with black phialides bearing globose conidia
Mucor sp.	Aerial mycelium, grey-brown. Yellow reverse	Simple sporangiophores, isolated or grouped on stolon with black sporangia of variable size
Alternaria sp.	Aerial mycelium, dark green. Beige reverse	Articulated conidiophores bearing dense spores, solitary or arranged in two. Spores are brown and articulated with variable shapes (spherical or elliptical)

reported in the rhizosphere. These bacteria are used as plant growth promoting rhizobacteria (PGPR) (Tarkka and Frey-Klett, 2008; Mehta et al., 2010), as mycorrhiza helper bacteria (MHB) (Lingua et al., 2008), as solubilizing phosphate agents (Richardson et al., 2009; Mehta et al., 2010) and as biocontrol agents (Hartmann et al., 2009).

Actinomycetes colonies appeared after 7 days of incubation at 28°C. They were compact, white to beige, powdery, tough, leathery colonies that adhered to the agar surface. There was the production of a strong odor from the mature colonies of all strains; this may be due to the presence of a volatile substance called geosmin produced by the genus *Streptomyces* according to Wenke et al. (2010). They were recognized by the Gram staining (Gram positive) and the presence of filamentous hyphae.

We isolated from the two soil samples of *T. boudieri*, 4 dominant fungal genera: *Penicillium* sp., *Aspergillus* sp., *Mucor* sp. and *Alternaria* sp. The macroscopic and microscopic characters are summarized in Table 3.

Penicillium species are cosmopolitan and are found in all types of soils (Dommergues and Mangenot, 1970; Davet, 1996; Giri et al., 2005b). Their presence has been reported in many soils of truffles (Luppi-Mosca et al., 1970; Mamoun and Olivier, 1990; Bokhary and Parvez, 1992) and in the ascocarps of *T. aestivum* and *Tuber melanosporum* (Rivera et al., 2010). The *Penicillium* species are able to use monosaccharides, disaccharides, starch, cellulose and/or chitin (Dommergues and Mangenot, 1970; Bokhary and Parvez 1994; Vassilev et al., 2006). Some species like *Penicillium notatum* produce antibiotic (penicillin) (Dommergues and Mangenot, 1970; Zhong and Xiao, 2009), others excrete siderophores and organic acids solubilizing phosphate (Gyaneshwar et al., 2002; Vassilev et al*.*, 2006; Richa et al., 2007; Rahi et al., 2009; Richardson et al., 2009), others produce phytohormones (Kuhad et al*.,* 2004). According to Chandanie et al. (2006), *Penicillium simplicissimum* and *Glomus mosseae* used as biocontrol agents induce systemic resistance of cucumber to anthracnose (fungal disease).

Aspergillus species is also cosmopolitan. They are present in the soils and ascocarps of terfez (Bokhary and Parvez, 1992) and *Tuber* (Mamoun and Olivier, 1989 and 1990). Some *Aspergillus* species have specialized enzymes to solubilize complex substances (Dommergues and Mangenot, 1970; Souchie et al*.,* 2006; Richardson et al., 2009). Others species are used as biological control agents against nematodes (Siddiqui and Akhtar, 2009) and against some phytopathogenic fungi such as *Fusarium oxysporum* f.sp. *melonis* (Suarez -Estrella et al., 2007).

The genera *Mucor* and *Alternaria* were also reported in the rhizospheres of some mycorrhizal fungi (Summerbell, 2005) and of truffles (Luppi-Mosca, 1973; Bokhary and Parvez, 1992).

Conclusion

This study focuses on the enumeration and pre-identification of the soil microflora associated with *Terfezia boudieri* Chatin. Enumeration reveals that the number of some microorganisms in contact with ascocarps of *Terfezia boudieri* is higher than at 10 cm deep. So, *T. boudieri* Chatin stimulate soil microflora in contact with their ascocarps. Bacteria are more dominant and more diverse than fungi and actinomycetes. Their numbers are estimated to be $8.3x10^7$ - $8.6x10^7$ CFU/g for bacteria, $8.6x10^4$ - $4.5x10^5$ CFU/g for fungi and $8.2x10^2$ - $2.2x10^3$ CFU/g for actinomycetes.

The increase in the number of free nitrogen fixing bacteria (Azotobacters) ($2.2x10^1 \pm 0.8x10^1$MPN/g), ammonifying bacteria ($4x10^3 \pm 1.0x10^3$MPN/g), nitrifying bacteria ($4x10^1 \pm 1.0x10^1$MPN/g for nitrous bacteria and $6.8x10^3 \pm 1.0x10^3$MPN/g for nitric bacteria), proteolytic bacteria ($1.2x10^6 \pm 0.8x10^6$CFU/g), amylolytic bacteria ($6.8x10^3 \pm 0.2x10^3$CFU/g) and cellulolytic bacteria ($1.3x10^4 \pm 0.7x10^4$MPN/g) in contact with the ascocarps of *Terfezia boudieri*, except lipolytic bacteria and denitrifying bacteria, suggests that *T. boudieri* stimulates

a definite microflora.

The macroscopic, microscopic and biochemical characteristics, allowed us to pre-identify six most dominant bacterial genera: *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Streptococcus* sp., *Proteus* sp., *Serratia* sp. and an actinomycete species (in particular *Streptomyces* sp.). Four fungal genera were identified as *Penicillium* sp., *Aspergillus* sp., *Mucor* sp. and *Alternaria* sp. on the basis of their macroscopic and microscopic characters.

These isolated and pre-identified microorganisms may be associated with inoculum (from spore suspension) of *T. boudieri* in mycorrhizal synthesis between terfez and their host-plant. They can also be used to test their *in vitro* stimulatory effect on the difficult germination of ascospores of *T. boudieri*. Also, the result obtained from this study can help in the production of high quality and quantity of desert truffles ascocarps.

Conflict of Interests

The authors have not declared any conflict of interests.

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Bovine vaginal lactobacilli and their adherence to mucus in different phases of the estrous cycle

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The aim of the study was to isolate vaginal latobacilli from heifers and cows with healthy reproductive tract and to test their adherence to vaginal mucus in different phases of the estrous cycle. Moreover, the strains were tested to produce hydrogen peroxide and organic acids and *in vitro* antagonistic activity against pathogenic indicator strains. Five strains were selected (from 244 samples of 122 heifers and cows). They were identified to the species level; the strains 5/K, 24S8 were allotted to the species *Lactobacillus büchneri* and the strains 29S8, 9/K, 5/Kb to the species *Lactobacillus mucosae.* Except nonadherent strain *L. büchneri* 24S8, the strains adhered to vaginal mucus in the follicular phase of the estrous cycle. All strains produced hydrogen peroxide in detectable amounts, lactic acid as the main organic acid and showed inhibitory activity against indicator bacteria. They were negative or with slight production (5 nmol) of enzymes connected with some disorders (β-glucuronidase, α-chymotrypsin, trypsin and N-acetyl-β-glucosaminidase). On the other hand, reaction for β-galactosidase as the enzyme helping e.g. in lactose digestion was positive (30 nmol). *L. mucosae* 5/Kb was deponed to the Czech Culture Collection of Microorganisms (CCM, Brno, Czech Republic) as CCM 7697. Preliminary results suggest that selected lactobacilli could be used in a probiotic preparation to prevent and treat diseases of reproductive tract.

Key words: *Lactobacillus* spp.,probiotic, reproductive tract, cow.

INTRODUCTION

Metritis produced by pathogenic microorganisms is the main reason for conception failures in the different herds (Sheldon et al., 2008). Infertility in dairy farms produces severe economical losses (Gilbert et al., 2005).This

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problem could be propably diminished by the use of probiotic strains (Otero et al., 2006). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) has defined probiotics as live microorganisms which administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). In October 2013, the Consensus Panel on Appropriate Use of the Term Probiotic was convened including 10 academic experts who agreed with the up to now used definition and explained all implementations concerning the word probiotic. Microorganisms used as probiotics have to be defined as Generally Recognized As Safe (GRAS) and at least transiently colonize the reproductive tract. Although the selection of probiotic microorganisms should be initially performed through the application of *in vitro* characteristics, the application to animal model and clinical *in vivo* effects have to be evaluated (Otero et al., 2006).

Administration of a well-characterized probiotic strain could overcome problems related to drug-resistant strains, chronic toxicity, the loss of obligatory microbiota (Otero et al., 2006) and residues in milk, which has to be discarded (Sheldon et al., 2004). Kummer et al. (1997) described the stimulation of the cell defense mechanisms of the bovine endometrium by inoculation of dairy *Lactobacillus* strain. Mucus plays an essential role in the reproductive process of all mammals. The primary function of reproductive tract mucus is its action as mechanical barrier against microbial infection of the uterus (Rutllant et al., 2005). *Lactobacillus* spp. protects the host against the onset of pathogenic strains by inhibition of pathogens‘ adhesion to surfaces and competition for nutrients (Otero and Nader-Macías, 2007). This consequently improves bovine female fertility. The aim of this study was to test the adherence of vaginal lactic acid bacteria (LAB, isolated from the vagina of heifers and cows with healthy reproductive tract and characterized by us) to mucus in different phases of the estrous cycle.

Lactobacilli are recognized as an important component of the normal vaginal microbiota of mammals, which may prevent colonization of pathogenic microorganisms. Therefore, the reduction or disappearance of lactobacilli may lead to the onset of infection. Vaginal mucus can bind bacteria and may thus influence the initial attachment and subsequent colonization by beneficial or pathogenic microorganisms (Otero et al., 2006). To select probiotic strains, production of antagonistic substances such as organic acids (Juárez Tomás et al., 2003b), hydrogen peroxide (H_2O_2) (Juárez Tomás et al., 2003a)or bacteriocins (Otero et al., 2006) is commonly tested (Aroutcheva et al., 2001; Strus et al., 2006). The lactic acid (LA) produced by lactobacilli is responsible for low pH level in the vagina (Aroutcheva et al., 2001). Therefore, the aim of this study was to select suitable strains with beneficial properties for their further use to prevent/treat diseases of reproductive tract.

MATERIALS AND METHODS

Strains isolation and selection

Two hundred forty-four vaginal swabs of 122 healthy heifers and cows were sampled from 4 localities (Dobšiná, Vlachovo, Kokava and Rimavicou and Plachtince) in Slovakia. Sampling was provided with the agreement of local Veterinary Administration and farmers following the guides for animal handling. The vulvar area was washed with povidone-iodine and water. A disposable speculum was inserted into the vagina to swab the posterior area. Vaginal swabs were placed into the Amies agar gel with charcoal (DispoLab, Copan Italia, Brescia, Italy). Samples were treated by the standard microbiological method (ISO); diluted in saline solution and appropriate dilutions were spread onto MRS agar (De Man, Rogosa and Sharpe; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Plates were incubated at 37°C for 48h under anaerobic conditions (Gas Pak Plus, BBL Microbiology systems, Cockeysville, USA). Randomly picked up colonies (20) were tested for their purity and growth characteristics (Styková et al., 2012). Pure colonies were examined for the following tests: enzyme production, H_2O_2 and organic acids production test, their antagonistic activity, and adherence to vaginal mucus in different phases of estrous cycle. Negative strains were excluded from each further testing step.

Strains identification

Selected bacteria were phenotyped by BBL™ Crystal™ Anaerobe Identification System (Becton Dickinson, Cockeysville, MD) (Styková et al., 2012). Species identification was confirmed by genotypization (rep-PCR with primer GTG5) in the Czech Culture Collection of Microorganisms (CCM, Brno, Czech Republic). Moreover, for the strains 29S8, 9/K and 5/Kb was performed sequencing of the gene pheS (phenylalanyl-tRNA synthase alpha subunit) in LMG Bacteria Collection, Ghent University (Belgium). Strains were maintained using microbank tubes (Pro-Lab Diagnostics, Neston, Wirral, UK) at -70°C.

Sampling and preparation of mucus for adherence testing

The tested mucus was collected from the vagina of three slaughtered heifers and nine slaughtered cows with healthy reproductive tract by gently scraping the mucosa with a rubber spatula. Twelve mucus samples were prepared.Volume of the mucus sample was 5-10 mL from each animal depending on the phase of the estrous cycle. Phase of the reproductive cycle was determined by inspection of the ovaries. Glass beads were put into 50 mL test-tubes (Nunc International, Roskilde, Denmark) and the mucus was prediluted with 0.15 mol/L phosphate-buffered saline (PBS; pH 7.2) in a 1:1 ratio. Test-tubes were vigorously shaken on a shaker during 15-20 min at room temperature. Before use, the mucus was filtered through a glass filter (Papírna Perštejn spol. s r.o. Keseg and Rathouský, Pernštejn, Czech Republic) using vacuum and then through the membrane filter (Millipore, 0.22 µm, 47 mm, Fisher Scientific Ltd., Dublin, Ireland). A vacuum filtration kit was used for large volumes of mucus. Syringe filters and manifold connected to a vacuum were used for small volumes of mucus. Mucus was further diluted after filtration with PBS to ratio 1:30. Homologicity of diluted mucus was experimentally verified in technical experiments on the Synergy™ 4 Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) as previously described in Styková et al. (2013).

Adherence testing

Modified microtiter plate binding assay was used to test adherence ability of the isolates (Štyriak and Ljungh, 2003). Bacterial cultures were adjusted to have the optical density (OD_{640}=0.5, Spekol EK, Jena, Germany) which was in accordance with cells concentration approximately 1x10^8 CFU/mL. Cell counts were controlled by plating of culture on MRS or BHI agar. Microtiter 96-well plates (Greiner ELISA 8 Well Strips, 350 µL, Flat Bottom, Medium Binding; Cruinn Diagnostics Ltd., Dublin, Ireland) were used.The absorbance values ($A_{580\ nm}$) were determined in a Synergy™ 4 Multi-Mode Microplate Reader (BioTek Instruments Inc., USA). Bacteria were classified as strongly adherent ($A_{580\ nm}$ ≥ 0.25), weakly adherent ($A_{580\ nm}$ 0.15–0.24), or nonadherent ($A_{580\ nm}$< 0.15), according to the methods of Štyriak and Ljungh (2003).

Enzymatic activity of lactobacilli

Isolated strains were tested for enzyme production using the API-ZYM system (BioMérieux, France) following the manufacturer's recommendations. The inocula (65 µL) of the McFarland standard 1 suspensions were pipetted into each well of the kit. Enzyme activities were evaluated after 4 h of incubation at 37°C and after the addition of Zym A and Zym B reagents. Colour intensity values from 0 to 5 and their relevant value in nanomoles were assigned for each reaction according to the colour chart with the kit.

Production of H_2O_2 and organic acids

Ability of lactobacilli to produce H_2O_2 was determined in Synergy^{TM}4Multi-Mode Microplate Reader (BioTek Instruments Inc., USA) by fluorescent assay kit Fluoro H_2O_2^{TM} (Bachem, St. Helens, UK).

Strains were grown in MRS broth (pH 6.5; Carl Roth GmbH + Co.KG, Germany) at 37°C for 16h.1 mL of each sample was diluted in 50 mL of deionized water and filtered through filter paper. A sample of 30 µL was used for analysis of short chain fatty acids. The concentration of formic, lactic, acetic, propionic, succinic and acetoacetic acids was determined by capillary isotachophoresis (ITP). The measurements were conducted using an Isotachophoretic analyser ZKI 01 (Radioecological Institute, Košice, Slovak Republic). The leading electrolyte of the following composition was used in the pre-separatory capillary: 10^{-2} M HCL+2.2x10^{-2} M ε-aminocaproic acid+0.1% methyl-hydroxyethylcellulosic acid, pH=4.3. The solution of 5x10^{-3} M caproic acid+2x10^{-2} M histidine was used as a finishing electrolyte. This electrolytic system worked at 250µA in the pre-separatory and 50µA in the analytic capillary.

Determination of antagonism *in vitro*

Pathogenic indicator strains used in this study are listed in Table 1. The method according to Jacobsen et al. (1999) with some modifications was performed. Lactobacilli were grown overnight in MRS broth (pH 6.5; Carl Roth GmbH + Co.KG, Germany) at 37°C.Sterile paper discs (6 mm diameter, BBL Microbiology systems, USA) containing 20 µL culture of lactobacilli were spread on MRS agar.The optical density at 640 nm of the bacterial culture was adjusted to 0.5 to yield approximately 1x10^8 colony-forming units/mL using the Specol EKspectrophotometer (Carl Zeiss, Jena, Germany). Additionally, the number of colonies for each bacterial culture was established using agar plates appropriate for each bacterial strain. Agar plates were incubated under anaerobic conditions (BBL Microbiology systems, USA) at 37°C for 24h.Paper discs were removed after incubation (strains were grown there). Indicator pathogenic strains were grown overnight in PYG (peptone yeast glucose; pH 6.9; 5 g/L bacteriological peptone, 10 g/L yeast extract, 5 g/L enzymatic digest of casein, 10 g/L glucose) at 37°C.Agar plates with grown tested lactobacilli were overlaid with 3 mL of agar containing 0.3 mL of the indicator bacteria (1×10^{8}colony-forming units/mL). The plates were incubated either anaerobically or aerobically depending on the growth characteristic of each indicator strain. Two controls were used: blank disc and discs inoculated with 20 µL of sterile MRS.After 48h of incubation, inhibition zones were determined. Antagonism of lactobacilli was evaluated by the size of inhibition zones: (-) no zone, (+) inhibition zone in the range 0.5 - 6 mm, (++) inhibition zone in the range 7- 12 mm, (+++) inhibition zone larger than 12 mm (PereaValez et al., 2007). Each test was performed five times.

RESULTS AND DISCUSSION

Among 20 colonies isolated from 244 vaginal samples of 122 healthy heifers and cows (tested for adherence to vaginal mucus in different phases of the estrous cycle, enzyme production, production of H_2O_2, organic acids, and for *in vitro* antagonistic activity against pathogenic indicator strains), five strains were taxonomically allotted to two different species; two strains (5/K, 24S8) were allotted to the species *L. büchneri* and three strains (29S8, 9/K, 5/Kb) were allotted to the species *L. mucosae.* Phenotypic properties (fermentation of disaccharides, esculin, NH_3 formation from arginine, etc., Table 2) were in agreement with those presented for the type strains in Bergey´s Manual (2009). They were also in accordance with their preliminary characterization (the growth phase) reported previously by Styková et al. (2012). Strains *L. büchneri* (5/K, 24S8) grew at 15°C and strains *L. mucosae* (29S8, 9/K, 5/Kb) grew at 45°C.

Otero et al. (1999) also reported occurrence of *L. büchneri* species in the vaginal microflora of healthy heifers and cows. The occurrence of *L. mucosae* in the mares' vagina was described by Fraga et al. (2008). As it was mentioned, microorganisms with beneficial effects on health of vaginal biocenosis should adhere to mucus of reproductive tract, survive there, and produce inhibitory substances against pathogens of reproductive tract (Martín et al., 2008).

Absorbance values A_{580nm} for mucus collected from vagina of healthy heifers and cows in the follicular phase were in the range from 0.09 to 0.42. Strongly adherent strains were *L. büchneri* 5/K and *L. mucosae* CCM 7697, 9/K (Table 3). *L. mucosae* 29S8 was weakly adherent. *L. büchneri* 24S8 was nonadherent. Absorbance values A_{580nm} for luteal phase ranged from -0.16 to 0.27. All strains, except nonadherent *L. büchneri* 24S8 were weakly adherent. Absorbance values A_{580nm} for mucus collected in anestrus ranged from -0.08 to 0.04. All strains were nonadherent in the luteal phase of estrous

Table 1. Pathogenic indicator strains used in antagonism determination test.

Strains	Host	Site of isolation	Growth medium	Incubation
CCM 7316 *Streptococcus equi* subsp. *zooepidemicus*[a]		Goat cheese	Columbia agar[f] with 5% sheep blood	48 h at 37°C, anaerobic conditions[b]
CCM 5753 *Arcanobacterium pyogenes*[a]	Cow	Mammary gland, pyogenic mastitis	Medium 2 blood agar[c] with 5% sheep blood	48 h at 37°C, anaerobic conditions[b]
CCM 5982 *Fusobacterium necrophorum*[a]	Bovine	Liver abscess	Brain heart infusion agar[d]	48 h at 37°C, anaerobic conditions[b]
CCM 6221 *Gardnerella vaginalis*[a]	Human	Vagina	VL medium[e] with 10% sheep blood	48 h at 37°C, anaerobic conditions[b]

[a]Strain was obtained from the Czech Culture Collection of Microorganisms Brno, Czech Republic. [b]BBL Microbiology Systems. [c]pH 6.9, Imuna Pharm a.s., Šarišské Michaľany, Slovak Republic.[d]pH 7.0, BBL Microbiology Systems, with 0.5 g of L-cystein HCL, 10 mL of hemin solution, 0.2 mL of vitamin K_1. [e]pH 7.4, Imuna Pharm a.s. [f]pH 7.3, Carl Roth GmbH + Co., KG.

Table 2. Phenotypic properties of lactobacilli when compared with Bergey´s Manual (BM) type strains (2009).

Acid formation from	24S8	5/K	BM *L. büchneri*	29S8	9/K	5/Kb= CCM 7697	BM *L. mucosae*
Arabinose	+	+	+	+	+	+	d
Mellibiose	+	+	+	+	+	+	d
Cellobiose	-	-	-	-	-	-	-
Galactose	+	+	d	+	+	+	d
Saccharose	+	+	d	+	+	+	+
Raffinose	+	+	d	+	+	+	d
Ribose	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-
Esculin	-	-	d	+	+	+	+
NH_3 formation from arginine	+	+	+	+	d	d	+

+ positive, - negative, d- dubious;24S8- *Lactobacillus büchneri* 24S8, 5/K- *Lactobacillus büchneri* 5/K, 29S8- *Lactobacillus mucosae* 29S8, 9/K- *Lactobacillus mucosae* 9/K, 5/Kb=CCM 7697- *Lactobacillus mucosae* 5/Kb=CCM 7697.

Table 3. Adherence of vaginal lactobacilli to vaginal mucus collected in different phases of the estrous cycle.

	Follicular phase	Luteal phase	Anestrus phase
L. büchneri 24S8	0.09 ± 0.03	Negative	Negative
L. mucosae 29S8	0.26 ± 0.06	0.10 ± 0.03	Negative
L. büchneri 5/K	0.35 ± 0.07	0.16 ± 0.01	Negative
L. mucosae 9/K	0.37 ± 0.06	0.16 ± 0.05	Negative
L.mucosae 5/Kb= CCM 7697	0.42 ± 0.05	0.27 ± 0.02	0.04 ± 0.009

Negative adherence values occurred when control values were subtracted from the values measured for each strain. Results are expressed as A_{580nm} values.24S8- *Lactobacillus büchneri* 24S8, 29S8- *Lactobacillus mucosae* 29S8, 5/K- *Lactobacillus büchneri* 5/K, 9/K- *Lactobacillus mucosae* 9/K, 5/Kb=CCM7697- *Lactobacillus mucosae* 5/Kb=CCM 7697.

cycle. The ability of lactobacilli to adhere is a crucial property for colonization capability of probiotics (Otero et al., 2006). To our knowledge, this is the first study on cows' vaginal lactobacilli adherence to mucus collected in different phases of the estrous cycle from heifers and cows with healthy reproductive tract. The mucus plays an essential role in the reproductive process of all mammals. The primary function of reproductive tract mucus is its

action as mechanical barrier against microbial infection of the uterus. The rheological, physical and chemical properties of vaginal fluid show cyclical changes under hormonal control (Rutllant et al., 2002). The vaginal fluid varies in consistency, texture and colour, depending on the phase of the estrous cycle, the presence of infection, certain drugs, genetic factors and diet (Borecki et al., 2010). The mucus becomes more plentiful, watery, less viscous and easier to traverse by spermatozoa in the follicular phase of the ovarian cycle, as well as under estrogen administration. During estrus in the cow, in contrast to most mammalian females, the production of vaginal fluid is so copious that up to 100 mL of it may accumulate in the vagina (Lofstedt et al., 1991). In the luteal phase of the cycle or under progesterone administration, this mucus becomes scanty, opalescent, viscous and consequently not favourable to sperm passage (Rutllant et al., 2005). Adherence ability of lactobacilli could be explained through their cell wall composition. Their wall is composed of different macromolecules together determining the strain-specific properties that include adaptation to the changing host environment and interaction with host immune receptors and epithelial cells. The thick multilayered peptidoglycan layer is decorated with teichoic acids, proteins and exopolysaccharides (Delcour et al., 1999). In contrast to coccoid bacteria, peptidoglycan and wall teichoic acid biosynthesis and protein secretion via the general secretion machinery appear to occur in helical patterns around the cell surface of rod-shaped bacteria such as *Bacillus subtilis* (Formstone et al., 2008). Such a helical pattern of cell wall biosynthesis, although not yet documented, can also be postulated for *Lactobacillus* rods (Lebeer et al., 2008).

In the study on variations in the vaginal microflora at different phases of the estrous cycle in cows, Otero et al. (1999) reported the highest number of lactobacilli during the estrus period. Dominated lactobacilli were *L. büchneri* and *L. brevis.* Strains have to be considered as GRAS (Dini et al., 2012). *L. mucosae* 29S8 and 9/K did not produce the enzyme β-glucuronidase, *L. mucosae* CCM 7697, *L. büchneri* 5/K, 24S8 produced only 5 nmol of this enzyme. Reactions for α-chymotrypsin, trypsin or N-acetyl-β-glucosaminidase were negative in the tested strains. Since bacterial β-glucuronidase is known for its substrate specificity and hydrolysis of different glucuronides, it seems to be associated with initiation of colon cancer (Lidbeck et al., 1992). Having in mind the possible probiotic potential of the isolated lactobacilli, the strains with positive enzymatic activities (more than 5 nmol) which are usually associated with intestinal diseases (α-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase) should be eliminated from probiotic studies. On the other hand, β-galactosidase helps in lactose digestion. Lactobacilli showed positive reaction for this enzyme (30 nmol). Reaction for the other tested enzymes was negative or only slightly positive (5-10 nmol).

The value of H_2O_2 produced by *L. mucosae* 5/Kb (CCM 7697) was 0.233 mmol/L, by *L. mucosae* 9/K 0.376 mmol/L and by *L. mucosae* 29S8 0.227 mmol/L. The strains of *L. büchneri* 24S8 and 5/K produced 0.101 and 0.038 mmol/L of H_2O_2 respectively. Fontaine and Taylor-Robinson (1990) showed that the culture conditions can influence the production of H_2O_2.In that study, only one strain of the vaginal lactobacilli produced detectable levels of H_2O_2 in nonagitated cultures (0.65 mmol/L). However, under agitated conditions, H_2O_2 production reached the range 4.85 - 9.11 mmol/L. In our study, H_2O_2 produced by selected vaginal lactobacilli was similar to those reported for other vaginal lactobacilli (Strus et al., 2006). Otero and Nader-Macías (2006) studied production of H_2O_2 by vaginal lactobacilli in cattle; they reported the inhibitory effect of *Lactobacillus gasseri* CRL1421 against *Staphylococcus aureus* as the result of the combined effect of H_2O_2 and LA. The highest inhibition was obtained under aerobic conditions where the highest amount of H_2O_2 was produced. Moreover, the antimicrobial activity of lactobacilli is a summation of various inhibitory mechanisms in which H_2O_2 plays some but not a crucial role, in addition to other substances (Strus et al., 2006). It is obvious that *in vitro* conditions do not occur *in vivo* and most probably the small amount of H_2O_2 produced by lactobacilli can only down regulate but not eliminate the pathogenic strains. It is highly probable that multiple antioxidant mechanisms present on vaginal epithelium surface also neutralize H_2O_2 activity *in vivo* (Basu et al., 1990). O'Hanlon et al. (2010) showed that under the hypoxic conditions that generally prevail in the vagina, H_2O_2 production by vaginal lactobacilli is undetec-table (detection threshold 10 nmol). Even with extended aerobic exposures *in vitro*, the mean H_2O_2 concentration achieved by lactobacilli in vaginal fluid is approximately 100-fold lower than the concentration of H_2O_2 achieved by lactobacilli under aerobic *in vitro* conditions in the absence of antioxidants. Furthermore, the vaginal fluid has sufficient antioxidant activity to block the microbicidal activity of H_2O_2 even when H_2O_2 is supplied at concentrations much higher than lactobacilli are capable of producing. Under optimal, anaerobic growth conditions, physiological concentrations of H_2O_2 produced no detectable inactivation of either bacterial vaginosis associated bacteria or vaginal lactobacilli. Moreover, at very high concentrations, H_2O_2 was more toxic to vaginal lactobacilli than to bacterial vaginosis associated bacteria (O'Hanlon et al., 2011).

Lactic acid (LA) was the main organic acid produced by the tested lactobacilli (Table 4). The highest amount of LA (34.83 mmol/L and 26.31 mmol/L) was produced by *L.büchneri* (24S8 and 5/K) respectively. LA (the characteristic fermentative product of lactobacilli) can reduce pH to the level in which the growth of pathogenic flora is inhibited. Low pH leads to decrease in the activity

Table 4. Production of organic acids by vaginal lactobacilli.

Organic acid (mmol/L)	29S8	5/K	24S8	9/K	CCM 7697
Formic acid	2.68	4.09	6.85	4.70	2.08
Lactic acid	11.48	26.31	34.83	17.45	10.47
Acetic acid	5.37	6.64	6.04	7.52	5.97
Propionic acid	3.69	3.89	3.83	4.70	4.16
Succinic acid	3.42	4.03	3.69	2.82	2.55
Acetoacetic acid	4.83	5.37	4.30	5.37	5.37

29S8- *Lactobacillus mucosae* 29S8, 5/K- *Lactobacillus büchneri* 5/K, 24S8- *Lactobacillus büchneri* 24S8, 9/K- *Lactobacillus mucosae* 9/K, CCM 7697- *Lactobacillus mucosae* 5/Kb=CCM 7697.

Table 5. *In vitro* antagonistic activity of vaginal lactobacilli against pathogenic indicator strains.

Strains	*A. pyogenes*	*G. vaginalis*	*S. equi* subsp. *zooepidemicus*	*F. necrophorum*
L. büchneri 5/K	35 ± 0.030	9 ± 0.050	0	21 ± 0.250
L. büchneri 24S8	35 ± 0.030	9 ± 0.050	0	21 ± 0.250
L.mucosae 5/Kb=CCM 7697	36 ± 0.028	8 ± 0.009	0	20 ± 0.020
L. mucosae 29S8	36 ± 0.035	11 ± 0.120	10 ± 0.007	22 ± 0.230
L. mucosae 9/K	33 ± 0.034	7 ± 0.007	0	20 ± 0.250

The different degree of growth inhibition is expressed in mm as the mean of 20 measurements ± SD. Antagonism of tested lactobacilli was evaluated by the inhibition zones: (-) no zone, (+) inhibition zone in the range 0.5 - 6 mm, (++) inhibition zone in the range 7 - 12 mm, (+++) inhibition zone larger than 12 mm (PereaValez et al., 2007); 5/K- *Lactobacillus büchneri* 5/K, 24S8- *Lactobacillus büchneri* 24S8, 5/Kb =CCM 7697- *Lactobacillus mucosae* 5/Kb =CCM 7697, 29S8- *Lactobacillus mucosae* 29S8, 9/K- *Lactobacillus mucosae* 9/K, *A. pyogenes*- *Arcanobacterium pyogenes*-Czech Culture Collection of Microorganisms (CCM), Brno, Czech Republic, *G. vaginalis*-*Gardnerella vaginalis*-CCM Brno, Czech Republic, *S. equi* subsp. zooepidemicus-Streptococcus *equi* subsp. *zooepidemicus*-CCM Brno, Czech Republic, *F. necrophorum*- *Fusobacterium necrophorum*- CCM Brno, Czech Republic.

of metabolic enzymes and its denaturation. Only few bacterial strains are able to grow in pH lower than the threshold for lactobacilli (Juárez Tomás et al., 2003b). Acetic acid (AA) is both smaller and more lipid soluble than LA; it is expected to acidify the cytosol more rapidly than LA and it is more rapidly bactericidal than LA. Antimicrobial action of LA is not based simply on cytosolic acidification. LA also functions as a permeabilizer of the Gram-negative bacterial outer membrane and may act as a potentiator of the effects of other antimicrobial substances (Alakomi et al., 2000). In our study, LA values are in agreement with those in other studies (Zalán et al., 2010; Tejero-Sariñena et al., 2012). The highest production of LA was produced by *L. büchneri* 24S8 (Table 4). Our results support the hypothesis that vaginal bacteria are the primary source of lactic acid in the vagina (Boskey et al., 2001). An increase in vaginal pH is detrimental to the survival of lactobacilli; therefore, local acidification with LA or lactobacilli is useful for restoration of the vaginal ecosystem also in cattle (Beckwith-Cohen et al., 2012).The highest amount of AA was produced by *L. mucosae* 5/Kb (CCM 7697) (7.52 mmol/L) followed by *L.büchneri* 5/K (6.64 mmol/L) and *L.büchneri* 24S8 (6.04 mmol/L). O'Hanlon et al. (2011) found in humans no detectable effects of AA until its concentration increased to 5%. However, various studies in humans suggested that H_2O_2 produced by lactobacilli may be more relevant than acid production in inhibiting the growth of bacterial species causing vaginal infection (Kaewsrichan et al.,2006; Falagas et al., 2007). More recent studies determined that in contrast to H_2O_2, LA at physiological concentrations was microbicidal against bacterial vaginosis associated bacteria, but had no effect on vaginal lactobacilli. Furthermore, the presence of vaginal fluid blocked the microbicidal activity of H_2O_2 but not of LA (O'Hanlon et al., 2011).

Lactobacilli showed an inhibition zone at least against three of five indicator strains (Table 5) reaching diameter of the inhibition zones up to 39 mm. *A. pyogenes* was the most sensitive strain. Only *L. mucosae* 29S8 showed antagonistic activity against *Streptococcus equi* subsp. *zooepidemicus*. The ability of lactobacilli to inhibit bacterial species associated with metritis (*A. pyogenes, F. necrophorum, G. vaginalis* and *S. equi* subsp.

zooepidemicus) is in accordance with various authors (Bondurant, 1999; Otero et al., 2006). *A. pyogenes* and *F. necrophorum* are most frequently isolated from cows with endometritis and during reconvalescence after placental retention (Williams et al., 2005). *G. vaginalis* and *S. equi* subsp. *Zooepidemicus* are also etiological agents of reproductive diseases of horses (Wittenbrink, 2012). *G. vaginalis* is particularly important in the research focused on harmoni-zation of the biocenosis of the reproductive tract because its mechanism of action is based on the disharmonization of the normal biocenosis (Atassi et al., 2006). Fraga et al. (2008) showed that lactic acid bacteria (lactobacilli and enterococci) isolated from mare‘s vagina produced antagonistic effects against *Staphylococcus aureus* as a representative of Gram-positive bacteria and *Escherichia coli* as a representative of Gram-negative bacteria. Antagonistic effects of lactobacilli against both Gram-negative and Gram-positive pathogenic strains were determined also in the study of Dini et al. (2012).

Conclusion

Based on the ability of the isolates to adhere to vaginal mucus in different phases of the estrous cycle, to produce hydrogen peroxide, organic acids and to inhibit pathogenic indicator bacteria *in vitro,* five strains (among 20 colonies from 244 vaginal samples of 122 healthy heifers and cows) were taxonomically allotted to two different species; the strains 5K, 24S8 were allotted to the species *L. büchneri* and the strains 29S8, 9/K, 5/Kb to the species *L. mucosae.* The strains adhered to vaginal mucus in the follicular phase of the estrous cycle (except nonadherent strain *L. büchneri* 24S8). *L. mucosae* 5/Kb was deponed to the Czech Culture Collection of Micro-organisms (CCM, Brno, Czech Republic) as CCM 7697. The tested strains also possessed inhibitory activity against pathogenic bacteria (causative agents of metritis in cows). Preliminary results indicated the possibility to use selected lactobacilli in a probiotic preparation to prevent and treat diseases of reproductive tract. However, their effectivity *in vivo* has to be checked during a double-blind randomized, placebo-controlled trial.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of resource conservation practices and conventional practices on population dynamics of *Meloidogyne graminicola* (Golden and Birchfield, 1965) under rice-wheat cropping system

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Study on effect of resource conservation practices and conventional practices on population dynamics of *Meloidogyne graminicola* under rice-wheat cropping system revealed that the fields with the intervention of resource conservation practices showed high population densities, root knot index and narrow nematode to root biomass ratio of *M. graminicola* as compared to conventional ones. It was also found that those tillage practices where residues were left on the field (with residues) has high population, root knot index and narrow nematode to root biomass ratio as compared to practices where crop were harvested from ground level without leaving residues in the field. Contrarily, zero till rice-zero till wheat + *Sesbania* sp. showed low population densities, root knot index and wide nematode to root biomass ratio and thus may serve as a better option for the management of *M. graminicola*.

Key words: *Meloidogyne graminicola*, resource conserving technologies, nematode-to-root biomass ratio, root knot index.

INTRODUCTION

The rice-wheat cropping system is the most important food grain production system in India. This cropping system is practiced by farmers of the Indo-Gangetic plains, over an area of about 2.7 million hectare. The resource-conserving technologies (RCTs) such as zero- or no-tillage and reduced tillage systems have been found beneficial in improving soil health, water use, crop productivity and farmers' income (Gupta and Seth, 2007). In addition, it also allow early and timely sowing of wheat (Tomar et al., 2006) and reduce the cost of production through less use of fossil fuels and herbicides, etc. Zero tillage is widely adopted by farmers in the Northwestern Indo-Gangetic plains of India, particularly in areas where rice is harvested late. As in zero- or no-tillage and

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Table 1. Size and biomass of different stages of root knot nematode recovered from roots of rice plant.

Stages	Length			Width			Size (µm)	Nematode biomass (µg)
	Min (µm)	Max (µm)	Mean (µm)	Min (µm)	Max (µm)	Mean (µm)		
Eggs	89.96	96.88	93.07	37.84	41.28	38.40	93.07×38.40	0.085
Second stage juvenile	385.28	412.80	397.66	15.57	17.30	16.78	397.66×16.78	0.069
Adult male	1183.36	1348.48	1281.05	27.68	31.14	29.23	1281.05×29.23	0.68
Developing female	357.76	467.84	410.50	137.60	165.12	146.77	410.50×146.77	5.52
Adult female	550.40	660.48	597.93	288.96	440.32	370.26	597.93×370.26	31.69

reduced tillage, wheat crops are planted with minimum disturbance of the soil by placing the seeds in a narrow slit (3-4 cm wide and 4-7 cm deep) without any land preparation. In both conventional and resource conservation technologies, rice or wheat are harvested either from the ground level (without residues) or from the top, leaving residues on the field itself (with residues). Zero tillage and reduced tillage has impact on soil biota especially soil borne pathogens, insects, and nematodes. Leaving plant debris on the surface or partially buried in the soil in resource conservation technologies may allow a number of pathogens to overwinter or survive until next crop is planted (Sumner et al., 1981). One of the most important soil-borne biotic factors is the nematode trophic groups. The nematode community structure varies in time and space, both in zero/minimum tillage and in conventionally tilled soil (Anonymous, 2000; Dabur, 2001). About 300 nematode species belonging to 35 genera have been reported infesting rice. Among them *Meloidogyne graminicola* (Golden and Birchfield., 1965) is one of the most damaging pests of rice and affects rice production by causing rice root knot and it has recently emerged as a major pest in rice-wheat cropping system (Soriano et al., 2000; Singh et al., 2006; Singh and Singh., 2009). However, detail studies on effect of resource conservation practices and conventional practices on population dynamics of *M. graminicola* under rice-wheat cropping system have not been conducted. In view of this, the present studies were made.

MATERIALS AND METHODS

Soil from the long term established (5-6years) wheat fields following rice-wheat cropping system adopted under the resource conservation practice and conventional practice showing patches of stunted plants caused by *M. graminicola* were collected in zig zag pattern at mature stage from different villages of Ballia district of Eastern Uttar Pradesh (India) during the month of March, 2011.

Different types of tillage practices chosen for the study

a) Conventional practices includes: i) Conventional tilled puddle transplanted rice (CTTPR) – conventional tilled broadcasted wheat (CTBCW) without residues; ii) CTTPR –CTBCW with residues

b) Resource conservation technologies include: iii) CTTPR – zero till (ZT) wheat without residue; iv) CTTPR- ZT wheat with residue; v) reduced till direct seeded rice (DSR) - ZT wheat without residue; vi) DSR- ZT wheat with residue; vii) zero till rice- zero till wheat (Double ZT) without residue; viii) Double ZT with residue and ix) Double ZT with sesbania.

All the collected soil samples were then bulked and about five kg soils was kept separately in polyethylene bags and brought to the laboratory. Then part of soil (500 g) were processed as per technique of Christie and Perry (1951) to estimate the initial population of 2nd stage juvenile from each option and remaining soil was separately filled into the earthen pots for further studies. Each treatment had three replications. The rice seeds (var.MTU-7029) were sown in each pot with 30 seeds at equal space. After 30 days of sowing of rice in earthen pots, observations were recorded on root knot index of rice seedlings and 2nd stage juvenile population of pot soil (500 g). From each of the representative pots 15 plants were uprooted gently, collected in polyethylene bags and brought to the laboratory and were then washed with running tape water on the same day. Special care was taken to avoid any chance of loss of the roots by placing them in a plastic tray having sufficient water in order to facilitate the loosening of the soil adhered with the roots. Plants were then taken for the estimation of the different growth and disease parameter. Plant height was measured in centimeter (cm). Shoot weight was estimated in milligram (mg) using the electronic balance (make-Sartorius). The root gall index was assessed following the scale given by Prot and Matias (1995) [1 = no gall, 2 = 1-25% roots with galls, 3 = 26-50% roots with galls, 4 = 51-75% roots with galls and 5 = > 76% roots with galls]. Thereafter, root systems of each plant were placed in boiling 0.1% (w/v) acid fuchsin in lactic acid, glycerol and distilled water (1:1:1) for staining roots (Bridge et al., 1981). Whenever possible, root galls of each root system were cut with a sharp blade and placed on a slide containing 2-3 drops of clear glycerol solution (equal parts glycerol and water). Then these galls were teased with the help of two fine needles for release of the different developing stages of the nematode. After removing the gall debris, the recovered developing stages including the eggs were taken for their measurement (Table 1). Then mean nematode biomass was calculated using formula given by Andrassy (1956) with slight modification.

$$G = \frac{a^2 b}{(1.6)\ (1{,}000{,}000)}$$

Where, G = biomass (µg); a = the greatest body width (µm); b = body length (µm); 1.6 = constant for correcting volume of nematode; and 1,000,000 is a factor for converting µm^3 to µg

For calculating the biomass of females, biomasses of neck and head and of the body were calculated separately. The width of the female body below the neck was measured at three points

Table 2. Population study of *M. graminicola* in rice plants grown on soil samples collected from wheat fields adopted under various resource conservation practices and conventional practices in rice-wheat cropping system under pot condition.

RCTs† options	Year of adoption of RCTs options	Initial population of 2nd stage juvenile (500 g soil)	Population of 2nd stage juvenile, 30 DAS ø (500 g soil)	Nematode biomass (µg)	Net root biomass (µg)	NB : RB ratio	Root knot index (RKI)
CTTPR- CTBCW (Without residue)	2004	65	138	703.200	70956.800	1:101	1.4
CTTPR- CTBCW (With residue)	2003	400	968	747.021	64552.979	1:86	2.3
CTTPR- ZT wheat (Without residue)	2004	918	3873	2018.067	62681.933	1:31	4.1
CTTPR- ZT wheat (With residue)	2005	982	3780	2369.114	78130.886	1:33	4.4
RTDSR- ZT wheat (Without residue)	2004	768	3264	1460.260	66289.740	1:45	4.5
RTDSR- ZT wheat (With residue)	2004	1030	3965	4414.204	77015.796	1:17	5.0
ZT rice- ZT wheat (Without residue)	2006	824	4317	2426.517	156973.483	1:65	5
ZT rice- ZT wheat (With residue)	2005	886	4102	1639.294	60110.706	1:37	5
ZT rice- ZT wheat with sesbania	2005	296	800	786.913	79043.087	1:100	2.5
CD at 5%	-	16.42	15.28	17.21	15.52	-	-
SEM	-	5.52	5.14	5.79	5.22	-	-

NB : RB = Ratio of nematode biomass to root biomass; root knot Index, 1) No gall, 2) 1-25% gall, 3) 26-50% gall, 4) 51-75% gall, 5) >75% gall (Prot and Matias,1995); **†**, resource conservation technologies; ******, 1) Conventional tilled transplanted puddle rice (CTTPR) – Conventional tilled broadcasted wheat (CTBCW) without residues (farmers' practice); 2) CTTPR–CTBCW with residues (farmers' practice); 3) CTTPR-zero tillage (ZT) wheat without residue ; 4) CTTPR-ZT wheat with residue; 5) reduced tillage direct seeded rice (RTDSR) – ZT wheat without residue; 6) reduced tillage direct seeded rice (RTDSR) – ZT wheat with residue; 7) ZT rice –ZT wheat (double ZT) without residue; 8) ZT rice - ZT wheat (double ZT) with residue; 9) ZT rice-ZT-wheat (Double ZT) with brown manuring (sesbania); ø, population of 2nd stage juvenile estimated 30 days after sowing.

and the biomass was calculated with the average width to get the realistic value.

In order to calculate nematode-to-root biomass ratio, measurements and biomass of the developing nematode and associated roots were taken. The fresh weight of roots was taken as the root biomass. Nematode biomass was then subtracted from infected root biomass to calculate net root biomass. The net root biomass of the infected plants was divided by the corresponding nematode biomass to obtain the nematode-to-root biomass ratio. This ratio was calculated from 15 rice plants and was averaged. All observations were rounded off to two and three digit after decimal. CD at 5% and SEM value were estimated statistically using STPR software for completely randomized design experiment.

RESULTS AND DISCUSSION

Effect of different tillage practices on development of root knot disease revealed that population of second stage juveniles of *M. graminicola* at initial stage was found higher in resource conservation practices (768-1030) as compared to conventional practices (65-400) and double zero tillage with sesbania (296) (Table 2). Chandel et al. (2002) found that the population density of the root-knot nematodes was higher in the non-puddled soils especially in unsubmerged conditions as compared to puddled and submerged soil. In the case of

conservation tillage practices such as reduced tillage or zero tillage, Pankaj et al. (2006) observed that zero-tillage fields had population densities of plant parasitic nematodes (*Tylenchorhynchus brevilineatus* and *Pratylenchus* spp.) higher than those of conventionally tilled fields because of ploughing that decreased nematode population densities significantly, irrespective of fertilizer application. After 30 days of sowing, two to three fold increases in population was found as compared to initial populations.

Decline in nematode population densities with intervene-tion of sesbania as brown manure with zero tillage rice-zero tillage wheat (double zero tillage) clearly showed that sesbania plays significant role in the management of populations of *M. graminicola.* Decomposition of organic residues results in the accumulation of specific compounds that may be nematicidal (Rodriguez-Kabana, 1986; Rodriguez-Kabana and Morgan-Jones, 1987). The green manure crops (leguminous crops) like *Sesbania rostrata* and *Aeschynomere afaraspera*, when grown in rotation have been shown to significantly increase yields of irrigated rice in the presence of rice root nematode (*Hirschmanniella oryzae)* by acting as trap crops of the nematodes (Germani et al., 1983).

Nematode biomass was much higher in RCTs as compared to double zero tillage with sesbania and conven-tional practices. Puddling of soil prior to planting paddy significantly reduces the population densities of root-knot nematode, *M. graminicola*, *Meloidogyne triticoryzae* and *Tylenchorhynchus mashoodi* (Gaur and Singh., 1993). However, increase in nematode biomass was also found associated with initial population of second stage juvenile of *M. graminicola.* Nematode to root biomass ratios was found narrow (1:17 to 1:65) in RCTs as compared to con-ventional practices which showed broad nematode to root biomass ratio (1:86 to 1:101). Double zero tillage with sesbania also shows broad nematode-to-root biomass ratio (1:100). Nematode-to-root biomass ratio was found inversely proportional to population densities of nematode. Development of root knot nematode in relation to different cultivation practices can also be understood from root knot index which was 4.0 to 5.0 (more than 50% roots formed gall) in the case of RCTs (Option No.3-9) over double ZT with sesbania and conventional methods which showed less than 3.0 root knot index (less than 25% roots formed gall). It was also found that those tillage practices where residues were left on the field itself (with residues) has high population densities, root knot index and narrow nematode to root biomass ratio as compared to practices where crops were harvested from ground level without leaving residues in the field.

The study revealed that those plots which got the intervention of resource conservation practices showed high population densities, root knot index and narrownematode to root biomass ratio of *M. graminicola* as compared to conventional ones. Contrarily, zero till rice- zero till wheat + sesbania sp. showed low population densities, root knot index and wide nematode to root biomass ratio which may serve as a better option for the management of *M. graminicola*. With the recent develop-ments of genetic tools in *M. graminicola*, it may also be of interest to follow the population dynamic of nematodes under different treatments in order to test more precisely the effects of agricultural practices on the biology of *M. graminicola* (Besnard et al., 2014).

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Yeasts in traditional Moroccan goat cheese

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Nine samples of goat's cheese were collected. A total of 68 yeasts were isolated and grouped according to their M13 PCR-fingerprints. Representative isolates of each fingerprint group were identified using rRNA and/or protein-coding gene sequencing leading to the identification of 18 yeast species. The dominant species were *Kluyveromyces lactis* (19.1%), *Saccharomyces cerevisiae* (11.7%), *Yarrowia lipolytica* (10.3%), *Candida parapsilosis* (10.3%), *Kazachstania unispora* (8.0%), *Kluyveromyces marxianus* (7.4%) and *Pichia fermentans* (5.9%). The yeast diversity of Moroccan goat's cheese was established using genotypic techniques which proved to be a straightforward approach for the identification of all isolates. This work yielded a well-characterized collection of yeasts from traditional and semi-industrial Moroccan goat cheeses which will be a resource of strains with specific properties.

Key words: Yeasts, goat cheese, identification, genotypic approach.

INTRODUCTION

For centuries, humans have used goats for their milk, meat and skin. Goat's milk cheeses are produced in many Mediterranean countries such as Spain. In Morocco, goat cheese is mainly prepared in a traditional and semi-industrial way. Brands of goat cheese on sale vary from one locality to another but all supermarkets offer goat cheeses (Chriqui et al., 2006). Due to the widely appreciated organoleptic and dietary characteristics, the production of goat's milk cheese has been stimulated. Goat's milk contains more easily digestible fat with higher proportions of medium-chain fatty acids and protein with higher levels of essential amino acids than cow's milk and has a higher vitamin and mineral content (Haenlein, 2001). Cheese is a very complex microbial ecosystem in which functional interactions of different partners such as lactic acid bacteria and yeasts play a major role in the ripening process (Viljoen, 2001; Mounier et al., 2008). Selection of suitable starter strains enables the cheese maker to control or modify flavour development (Beresford et al., 2001).

The traditional Moroccan goat cheese jben is a fresh cheese consisting of a salty curd obtained by spontaneous

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fermentation at ambient temperature which may be acelerated by addition of animal or vegetable rennet (Beresford et al., 2001). The cheese is sold locally and usually consumed within a few days, but may be preserved by salting, brining and sun-drying (Rubino et al., 2004). The traditional procedure for jben making begins with the collection of raw milk in an earthenware vessel and its spontaneous fermentation at ambient temperature until coagulation which may take up to 24-72 h depending on the temperature during the summer and winter seasons, respectively. To separate the liquid whey from the curd, the curdled milk is drained in a cloth bag for two to three days or up to 10 days depending on the desired cheese consistency. The cheese can then be partitioned, salted and left to drain and ripen further. To speed up this process and to improve safety and shelf life of the product in urban settings for household use or small scale comercial manufacturing, changes to the process are made that cause differences in sensory qualities of the product. Such a change is for example the use of small plastic moulds for draining and ripening instead of performing this step in larger batches with the help of reusable cloth bags (Benkerroum and Tamime, 2004).

To help safeguarding the traditional qualities of Moroccan goat cheese, the microbial consortia of remaining traditional cheese-making need to be studied. Goat cheeses harbour bacteria and fungi in complex microbial ecosystems (Bonetta et al., 2008). The role of yeasts is not simple to define. They may play a beneficial or detrimental role by influencing the sensory characteristics through the synthesis of aromatic compounds, desired or excessive gas production, colour and texture changes, as well as surface growth and may play a role as inhibitors of undesired bacteria(Viljoen, 2001; Fröhlich-Wyder, 2003).Similar to other dairy products, a selection of yeasts is expected to be present in goat's cheeses based on shared characteristics such as fermentation or assimilation of lactose, a high proteolytic and lipolytic activity, utilisation of lactic acid, tolerance of low pH (Fleet, 1990; Jakobsen and Narvhus, 1996). The yeast species detected in raw goat milk: *Candida* spp., *Cryptococcus* spp., *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces marxianus*, *K. lactis*, *Pichia fermentans*, *Rhodotorula* spp., and *Trichosporon beigelii* could very well form the origin of some of the yeast diversity in goat milk cheeses (Callon et al., 2007; Corbo et al., 2001). Analysis of Italian soft goat's cheeses showed the presence of *Geotrichum* spp. and *K. lactis* in almost all samples, while species such as *Candida* spp., *Clavispora lusitaniae*, *Kazachstania exiguus*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* were detected less frequently in traditionally produced cheeses (Foschino et al., 2006; Bonetta et al., 2008).

While the occurrence of bacteria in goat's cheeses has been widely investigated (Requena et al., 1992; Nikolic et al., 2008; Serhan et al., 2009), the information on the yeasts in these cheeses is limited. The objective of this work is to isolate and identify, using phenotypic and genotypic techniques, yeasts occurring in traditional and semi industrial goat's cheese produced in Morocco.

MATERIALS AND METHODS

Isolation of cultures

Nine goat's cheeses samples produced traditionally (3 samples) or incooperatives (6 samples) were collected in March 2007 (Table 1). The samples were immediately cooled, brought to the laboratory and analysed upon arrival. One gram of cheese was homogenized with 9 ml of sterile physiological water (0.9% NaCl) to obtain a 1:10 dilution. Serial dilutions up to 10^{-7}were made. One hundred microliter of each dilution were surface plated on Sabouraud Agar (HiMedia, Mumbai, India) supplemented with chloramphenicol (30 µg/ml) and on Dextrose Yeast extract Peptone Agar (HiMedia, Mumbai, India) supplemented with chloramphenicol (30 µg/ml). The plates were incubated under aerobic conditions for 48 to 72 h at 30°C. The Colony Forming Unit (CFU) for both media ranged from 5.15×10^4 to 1.00×10^8. A total of 68 colonies were randomly picked from plates with 30-300 colonies. Pure cultures of yeasts were stored at -80 °C in Sabouraud broth or Dextrose Yeast extract Peptone broth with 20% (v/v) glycerol (Akabandaa et al., 2013)**.** All strains were deposited in the CCMM/Yeast Collection under the numbers L75 to L142. A representative selection of yeast isolates has been preserved in the BCCM/MUCL collection under the numbers MUCL 52254 to MUCL 52269 and MUCL 52756 to MUCL 52761.

Physiological characterization and identification

All yeast isolates were studied using API ID 32C (BioMérieux, Marcy-L'Etoile, France) following the instructions given by the manufacturer. The software API Web (BioMérieux) was used for identification.

M 13 PCR-fingerprinting

Extraction of high-molecular weight DNA was performed by a combination of the QiagenDNeasy protocol and the Invisorb Spin Plant Mini Kit (Invitek, Germany) with modifications as described by Fidalgo-Jiménez et al. (2008). PCR fingerprinting was performed using the minisatellite specific oligonucleotide derived from the core sequence of the bacteriophage M13 with the sequence 5'-GAG GGT GGC GGT TCT-3'. PCR amplifications were performed as described by Fidalgo-Jiménez et al. (2008). The PCR fingerprint profiles were analysed using the BioNumerics software 6.1 (Applied Maths, Sint Martens Latem, Belgium). A Pearson product-moment correlation based dendrogram was generated using the UPGMA method.

Ribosomal and protein-coding gene sequence analysis

A selection of the D1/D2 region of the large subunit (LSU)rRNA, the internal transcribed spacer (ITS)rRNA sequences 1 and 2 including the 5.8S *rRNA*gene, and partial *ACT1* and *COX2* gene sequence were generated as needed to identify each isolate to the species level as described by Daniel et al. (2009) using the primers listed in Belloch et al. (2000) and Daniel and Meyer, (2003). Only sequences that showed differences to those of taxonomic reference

Table 1. Yeast species composition of Jben (n=9) from five regions of Morocco.

Jben sample	Location	Species	Number of isolates
F1, traditional	Chefchaouen	*Kluyveromyces lactis*	5
		Yarrowia lipolytica	4
F2, semi-industrial	El Gharb	*Candida inconspicua*	1
		Clavispora lusitaniae	1
		Kluyveromyces lactis	2
		Pichia fermentans	1
		Saccharomyces cerevisiae	1
F3, semi-industrial	El Gharb	*Candida inconspicua*	1
		Clavispora lusitaniae	1
		Kluyveromyces lactis	2
		Saccharomyces cerevisiae	1
F4, semi-industrial	Boufekrane	*Kluyveromyces lactis*	1
		Kluyveromyces marxianus	1
F5, traditional	Marrakech	*Candida zeylanoides*	1
		Filobasidium uniguttulatum	1
		Pichia fermentans	1
		Saccharomyces cerevisiae	3
		Torulaspora delbrueckii	1
		Yarrowia lipolytica	1
F6, semi-industrial	Rabat	*Kazachstania unispora*	4
		Kluyveromyces lactis	1
		Kluyveromyces marxianus	2
		Naumovozyma castellanii	1
F7, semi-industrial	Rabat	*Barnettozyma californica*	1
		Kasachstania unispora	2
		Kluyveromyces lactis	2
		Kluyveromyces marxianus	2
		Saccharomyces cerevisiae	3
F8, semi-industrial	Marrakech	*Candida anglica*	2
		Candida parapsilosis	2
		Candida spp.	1
		Candida zeylanoides	1
		Kazachstania servazii	1
		Pichia fermentans	2
		Yarrowia lipolytica	2
F9, traditional	Boufekrane	*Candida parapsilosis*	4
		Candida zeylanoides	1
		Filobasidium uniguttulatum	1
		Rhodotorula mucilaginosa	3

strains were deposited in public sequence databases. This were the D1/D2 LSU of isolate L124, accession number HF545834, the *ACT1* of isolate L97 accession number HF545835 and COX2 of isolate L94 accession number HF545836.

RESULTS

Physiological characterization and identification

Preliminary identification results obtained using the API ID 32Cmicrotest system were compared with those from molecular methods in Table 2. Thirty four isolates were correctly identified by API ID32C, while 14 isolates were incorrectly identified and 20 isolates could not be identified by API ID32C.

Isolates identified as *K. lactis* and *K. marxianus*, but none of the other species were able to utilize lactose as a carbon source (Table 2). In contrast, all isolates assimilated glucose and most isolates, but not *Y. lipolytica, P. fermentans, C. zeylanoides, C. inconspicua, Filobasidium uniguttulatum,* and *C. anglica* isolates, assimilated galactose. In general, the physiological profiles of different isolates within each of the species examined were similar except for those belonging to *K. lactis* and *C. parapsilosis* (Table 2).

PCR-fingerprinting

The 68 yeast isolates were typed by M13-PCR fingerprinting. Cluster analysis of the M13-PCR fingerprints (Figure 1) resulted in 14 clusters and 7ungrouped isolates (L121, L124, L129, L116, L104, L117 and L142).

Ribosomal and protein-coding gene sequence analysis

Species identification using DNA sequences was performed for 22 isolates which were selected to represent the 14 clusters and 7 single isolates found by M13-PCR fingerprinting (Figure 1). One to three of the genetic markers D1/D2 LSU rRNA, ITS rRNA, partial *ACT1* and *COX2* genes were used to identify each representative isolate unequivocally. The D1/D2 LSU region was sequenced for all 22 isolates. In addition, the ITS region was used for *C. zeylanoides*, the *ACT1* gene for *C. parapsilosis*, *S. cerevisiae*, *K. marxianus*, *K. lactis* and the *COX2* gene for *S. cerevisiae*. The highly variable *COX2* gene sequences were used to confirm strain divergence indicated by M13 fingerprints. They differed in 21 nucleotide positions from each other. While isolate L97 showed a COX2 sequence identical to MUCL 51236 (FN394076), the sequence of isolate L94 was most similar to strain MUCL 51208 (FN394075) with seven substitutions and to the neotype strain sequences AY244992, AJ295248, AF442206 with nine substitutions. According to the currently most often detected intraspecies sequence variability of 0-3 nucleotide differences in the D1/D2 LSU rRNA region, 0-4 differences in the ITS region, and 0-11 differences in the *ACT1* gene (Daniel et al., 2009 and references therein) all investigated isolates, except strain L124, were identified to the species level as reported in Figure 1 and Table 1. Strain L124 was identified as a yet undescribed *Candida* species that is related to species in the *Pichia* clade. BLAST program (Altschul et al., 1997) searches detected two highly similar sequences (two substitutions over 555 bp of the D1/D2 LSU) labelled as *Candida* spp. and isolated from an industrial malting environment in Finland (VTT C-04532, DQ377644) (Laitila et al., 2006) and from a not specified substrate in Tibet (AS 2.3080, DQ451012) (Wu and Bai, unpublished).

Yeast species diversity

Nine jben samples led to the isolation of 16 ascomycetous and 2 basidiomycetous yeast species. Each sample yielded between two and seven species (Table 1). *Kluyveromyces lactis* was present in six samples from four regions; *S. cerevisiae* in four samples of two regions; *Y. lipolytica, C. zeylanoides, K. marxianus* and *P. fermentans*in three samples of two regions; *C. parapsilosis* and *F. uniguttulatum* in two samples of two regions; *C. inconspicua, Cl. lusitaniae,* and *K. unispora* in two samples of the same region. The remaining seven species were present in single samples. There was no clear correlation between the nature of cheese analyzed (traditional or semi-industrial) and the yeast species diversity recovered. The three most frequently obtained species in terms of occurrence in different samples and number of isolates were *K. lactis* (12 isolates from six samples, 19%), *S. cerevisiae* (eight isolates from four samples, 12%) and *Y. lipolytica* (seven isolates from 3 samples, 10%).

Concerning less frequently isolated species, some samples obtained from the same location lead to the isolation of the same species: samples F2, F3 from ElGharb: *C. inconspicua*, *Cl. lusitaniae*; F5, F8 from Marrakech: *P. fermentans*, *C. zeylanoides*; F6, F7 from Rabat: *Kaz. unispora*, *K. marxianus*. Other less frequently encountered species were observed from different sample origins: *C. parapsilosis* in F8 from Marrakech and F9 from Boufekrane; *P. fermentans* in F2 from ElGharb and F5/F8 from Marrakech; *C. zeylanoides* in F5/F8 from Marrakech and F9 from Boufekrane; *K. marxianus* in F4 from Boufekrane and F6/F7 from Rabat, *F. uniguttulatum* in F5 from Marrakech and in F9 from Boufekrane.

DISCUSSION

The present study characterised physiologically and identified genotypically 68 yeast isolates originating from nine goat's cheese samples. The comparison of physiological and genotypic techniques showed that 34 isolates were misidentified when using the API ID 32C microtest system. It was noted that even species that are regularly encountered in a clinical setting, for which the API ID32

Table 2. physiological and molecular identification of yeast isolates and carbone sources assimilated.

Identified species by molecular techniques (number of isolates, cluster numbers in Figure 1)	API ID32C identification (number of isolates)	Reference	Carbone sources assimilated by isolates of the same species
Kluyveromyces lactis **(13, VIII)**	*Kluyveromyces lactis* (8)	L85, L86, L87, L82, L84, L89, L83, L90	Lactose, saccharose, galactose L84, L85, L86: lactic acid, maltose, arabinose, N-acetylglucosamine, trehalose, mannitol, sorbose L89, L90: cellobiose, raffinose, maltose, trehalose, methyl-D-glucopyranoside, sorbitol, glycerol, palatinose, melezitose, mannitol L82, L83, L87, L88, L130, L131,L85: sorbitol, mannitol
	Kluyveromyces marxianus (5)	L130, L131, L132, L130, L88	
Saccharomyces cerevisiae **(8, II and XII)**	*Saccharomyces cerevisiae* (7)	L97, L98, L141, L99, L95, L96, L94	Galactose, saccharose, lactic acid, raffinose, maltose
	KLuyveromyces lactis (1)	L93	
Yarrowia lipolytica **(7, VI)**	*Yarrowia lipolytica* (7)	L75, L79, L78, L76, L80, L77, L81	N-Acetylglucosamine, lactic acid, glycerol, erythritol, potassium gluconate
Candida parapsilosis **(6, XIV)**	*Cryptococcus humicola* (4)	L108, L110, L107, L109	All isolates except L111: galactose, saccharose, levulinic acid, N-acetylglucosamine, arabinose, maltose, trehalose, potassium 2-ketogluconate, methyl-D-glucopyranoside, mannitol, sorbitol, xylose, glycerol, palatinose, melezitose, potassium gluconate, mannitol
	Unidentified (2)	L106, L111	
Kasachstania unispora **(6, XIII)**	Unidentified (6, L104 ungrouped)	L101, L102, L103, L105, L100, L104	Galactose
Kluyveromyces marxianus **(5, IX)**	*KLuyveromyces marxianus* (5)	L134, L135, L137, L138, L136	Galactose, saccharose, lactic acid, raffinose, lactose
Pichia fermentans **(4, VII)**	Unidentified (4)	L125, L126, L127, L128	N-Acetylglucosamine, lactic acid, xylose, sorbose, glucosamine
Rhodotorula mucilaginosa **(3, I)**	*Rhodotorula glutinis* (2)	L119, L120	Galactose, saccharose, raffinose, maltose, trehalose, mannitol, ribose, glycerol, palatinose, melezitose
	Rhodotorula mucilaginosa (1)	L118	
Candida zeylanoides **(3, XI)**	*Candida zeylanoides* (2, L116 ungrouped)	L114, L116	N-Acetylglucosamine, glucosamine, potassium 2-ketogluconate, mannitol, sorbitol, glycerol, sorbose
	Unidentified (1)	L115	
Candida inconspicua **(2, IV)**	*Candida inconspicua* (2)	L112, L113	Lactic acid, glycerol, glucose
Filobasidium uniguttulatum **(2, III)**	*Candida rugosa* (1)	L122	Sorbitol, xylose, mannitol
	Filobasidium uniguttulatum (1)	L123	

Table 2. Contd.

Candida anglica **(2, X)**	Unidentified (2)	L139, L140	Glycerol
Clavispora lusitaniae **(2, V)**	Unidentified (2)	L91, L92	Galactose, saccharose, maltose, trehalose, glucosamine N-acetylglucosamine, potassium 2-ketogluconate, sorbitol, methyl-D-glucopyranoside, xylose, glycerol, rhamnose, palatinose, melezitose, potassium gluconate, mannitol, sorbose
Torulaspora delbrueckii **(1)**	*Torulaspora delbrueckii* (1)	L117	
Naumovozyma castellii **(1)**	*Zygosaccharomyces* spp (1)	L129	
Barnettozyma californica **(1)**	Unidentified (1)	L121	
Kazachstania servazii **(1)**	Unidentified (1)	L142	
Candida spp **(1)**	Unidentified (1)	L124	

Profiles of species recovered once were not shown. All isolates assimilate glucose.

has been developed, that is, *Cl. lusitaniae* and *C. parapsilosis*, were not identified. A possible explanation may be that the strains have adapted to the isolation substrate (goat cheese) and did not show the typical assimilation profiles on which the API ID32C system is based. Progress in molecular biology has opened up possibilities for characterizing yeasts at the genomic level. The application of M13 PCR-fingerprinting coupled with the sequencing of ribosomal regions and protein-coding genes proved very efficient for the identification and classification of yeasts (Kurtzman and Robnett, 1998; Daniel et al., 2009; Vrancken et al., 2010). Two M13 fingerprint types were revealed in *S. cerevisiae* (clusters II and XII), and *C. zeylanoides* (cluster XI and one ungrouped isolate) an indication of genetic heterogeneity in these species (Figure 1). This genetic heterogeneity was confirmed for *S. cerevisiae* by divergent COX2 gene sequences (Vrancken et al., 2010). One isolate of Kaz. unispora was not grouped with isolates of *Kaz. unispora* (cluster XIII). This could be a methodical artefact since the visual inspection of the its profile reveals high similarity to those in cluster XIII.

The 68 isolates represent 12 genera and 18 species (Table 1). The large species diversity recovered from the nine samples examined suggests that the genuine diversity was undersampled. However, the recurrence of the three most frequently obtained species *K. lactis*, *S. cerevisiae*, and *Y. lipolytica* among species regularly reported from cheeses (Fröhlich-Wyder, 2003) indicates the validity of the present study to determine the most frequently occurring yeasts. Italian fresh goat cheeses showed *K. lactis* in cheeses of all sampled seven producers, *G. geotrichum* with six and *Y. lipolytica* with four producers (Foschino et al., 2006) and the dominance of *Geotrichum* spp. and *K. lactis*, along with *Y. lipolytica*, *Saccharomyces* spp., *Kazachstania exiguus* and *Candida* spp. (Bonetta et al., 2008). The obtained results are comparable to the mentioned studies with the exception that no *Geotrichum* isolates were found. However, Andrighetto et al. (2000) reported only *Y. lipolytica* and *K. lactis* from Italian goat cheeses. This study did not mention sample numbers and is difficult to judge in terms of representativeness.

The basidiomycetous yeasts are non-fermentative and can be assumed not to contribute substantially to the cheese-making process. Those ascomycetous yeast species that were detected in one or two samples or with low isolate numbers only are considered as fortuitous colonisers in this study. Given the low sample and isolate number analysed here, their contribution to unique qualities cannot be excluded.

Kluyveromyces marxianus (asexual form *C. kefyr*) is one of the most frequently isolated yeast from cheese (Fröhlich-Wyder, 2003), outnumbered only by *Debaryomyces hansenii*, which has not yet been found in goat milk cheeses. The distinction of *K. marxianus* and *K. lactis* (synonym *K. marxianus* var. *lactis*, asexual form *C. shaerica*) is difficult by physiology and by molecular methods. Therefore, the current study employed *ACT1* gene sequences to distinguish both species reliably.

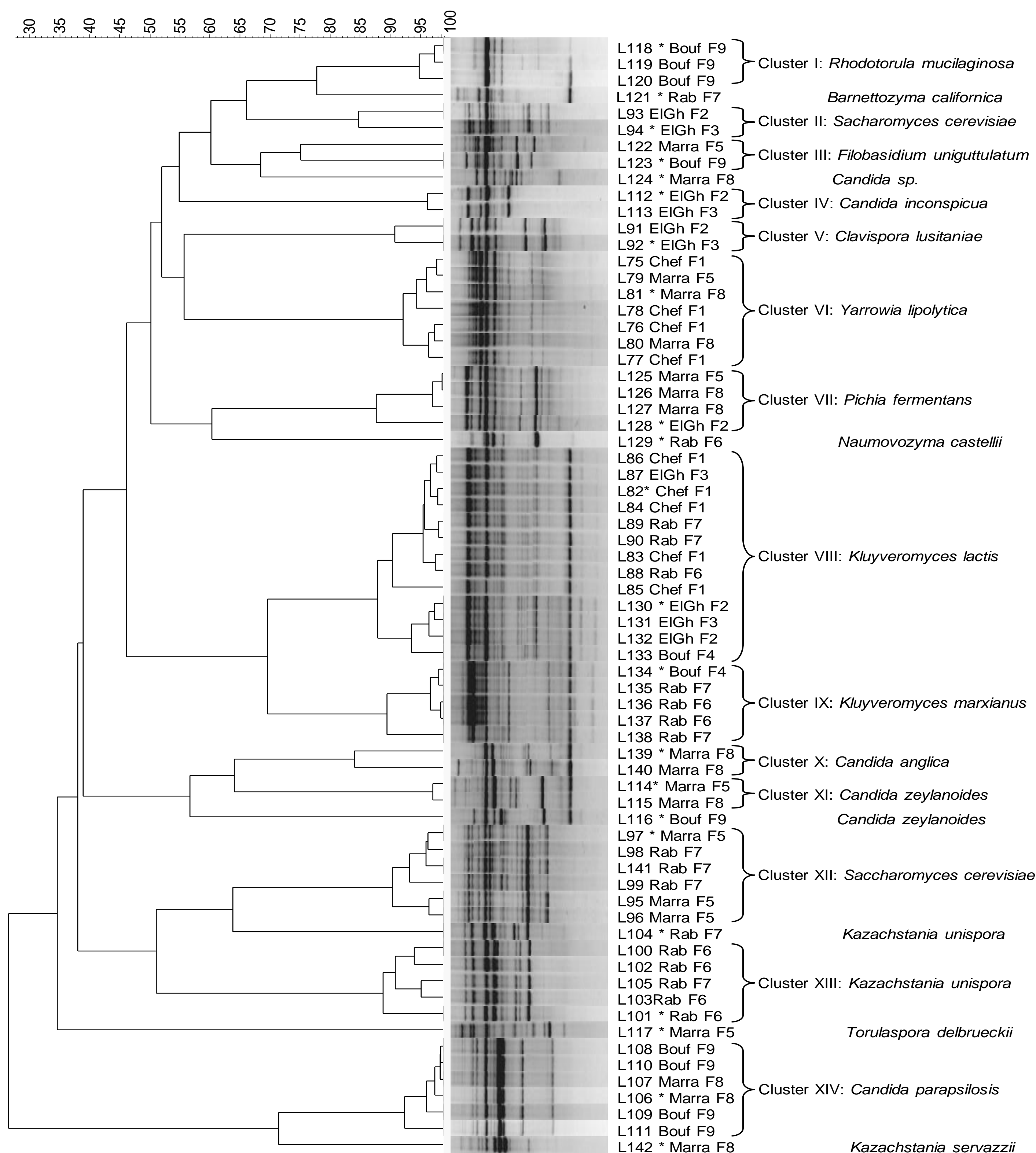

Figure 1. M13-PCR banding patterns of the yeasts isolated from Moroccan goat cheese and dendrogram based on UPGMA clustering of Pearson product-moment correlation coefficients. Isolate numbers are followed by a code for the location where the sample was obtained: Bouf = Boufekrane, Chef = Chefchaouen, ElGh = ElGharb, Marra = Marrakech, Rab = Rabat and the sample designation F1 to F9. Isolates with an asterisk were selected for sequencing analysis. Brackets designate clusters I to XIV and species identifications obtained using DNA sequence analysis are shown.

Strains of both species are able to metabolise a wide range of compounds including lactose, galactose and lactate (Kurtzman et al., 2011). Based on their similar physiology *K. marxianus* and *K. lactis* occupy very similar substrates. Notable differences are the growth of *K. lactis* on maltose, trehalose, methyl-α-glucoside and up to maximal 37°C, while *K. marxianus* is able to grow up to 45°C, assimilates inuline, has a higher growth rate, but does not grow on the before mentioned carbon sources (Kurtzman et al., 2011).

Saccharomyces cerevisiae is among the four most frequently reported yeasts from cheese (Fröhlich-Wyder, 2003). This species is frequently reported together with *K. marxianus* and *K. lactis* from pasta filata cheeses, which are marked by mild aromas (Romano et al., 2001). The generally lactose-negative *S. cerevisiae* (Kurtzman et al., 2011), proved to show strain variation in this ability, as for example a cheese isolate was shown to assimilate lactose and to degrade casein (Hansen and Jacobsen, 2001; Hansen et al., 2001). A positive growth stimulation of the main starter *Penicillium roqueforti* by *S. cerevisiae* was suggested by the same studies through a faster accumulation of aroma effective compounds and a more favourable sensory analysis of the cheeses produced with the combined starter relative to single starter conventional production. In general, the growth of *S. cerevisiae* in dairy product is assumed to be supported by utilisation of galactose.

Yarrowia lipolytica has been reported from cheese with a similar frequency as *S. cerevisiae* (Fröhlich-Wyder, 2003) and has been detected in goat milk cheeses repeatedly (Tornadijo et al., 1998; Foschino et al., 2006; Bonetta et al., 2008). The species is best known for extracellular enzyme production, in particular of lipolytic and proteolytic activities that lead to an either beneficial or spoilage role depending on the type of cheese (Suzzi et al., 2001). Considerable strain variations in enzymatic activities were ascribed to the strain diversity, and may, in view of recently evidenced species-level genetic variation, partly also be due to cryptic species existing in *Y. lipolytica* (Knutsen et al., 2007). Growth of *Y. lipolytica* in dairy products is thought to be supported in some strains by galactose and generally by lactic acid (Kurtzman et al., 2011), present in cheese through the action of lactic acid bacteria.

Typical cheese-associated yeasts are known to produce characteristic flavour-effective volatile sulphur compounds, free fatty acids, short-chain ketones and others. Comparisons of the major cheese-associated yeast species showed different amounts and compositions of species and strains (Sorensen et al., 2011; Kagkli et al., 2006). These results underline the importance to reproduce a species diversity in potential starter cultures that resembles the diversity found in typical traditional products as it may be impossible to imitate the regionally as typical perceived flavours by single-culture starters. The typical cheese yeasts diversity originated from unpasteurised goat milk or from the environment production (Boutrou and Guéguen, 2005). With the standardisation of food production and increasing hygiene requirements both sources may no longer contain the appropriate organisms and the application of wild starter cultures gains importance.

The results of the present study represent the first molecular analysis of the yeast microbiota of traditional and semi-industrial Moroccan goat cheese and revealed a considerable diversity. Because of the complex microbiota, and the interactions between different microorganisms with the cheese environment, species and strain selection for flavour improvement is not straightforward. Further evaluation of the metabolic properties of strains isolated in this study would improve the understanding of the Moroccan goat's cheese microbial ecosystem and support the selection of adequate starters for the industrial production of goat's cheese.

The present study is the first report on the molecular identification and physiological characterization of the dominant yeast species occurring in traditional and semi-industrial goat's cheeses produced in Morocco.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Establishing cause effect of multistressor environments on *Oreochromis niloticus* and its parasite communities

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This study investigated the composition and structure of the parasitic communities in *Oreochromis niloticus* with respect to levels of water quality in three different ecosystems. A total of 366 *O. niloticus* were examined from three water courses: CLAR ponds, (unpolluted), Abbassa privet ponds (slightly polluted) and Manzalla ponds (polluted). Trematodes, monogeneans, cestodes and acanthocephalans were found in all the sample sites. Trematodes dominate the parasite population. *Clinostomum tilapiae*, *Euclinostomum heterostomum, Diplostomum spathaceum*, *Posthodiplostomum minimum*, *Mesostephanus appendiculatus, Haplorchoides cahirinus*, *Cichlidogyrus aegypticus*, *Acanthosentis tilapiae* and larval stages of *Polyonchobothrium clariass* were found in all the sampled sites, but the distribution of these parasitic species varied between the three ponds. Meanwhile, *P. clarias*, *E. heterostomum*; and *A. tilapiae* were completely absent in the severely polluted site. The observed composition and structural changes among the sites were studied; the variability of the calculated infection indices (prevalence, mean abundance and mean intensity); and the degree of interactivity among parasites, as well as parameters of species richness and diversity suggest that the structure of parasite communities are affected by the water contamination levels.

Key word: *Oreochromis niloticus, Polyonchobothrium clarias, Euclinostomum heterostomum, Acanthosentis tilapiae,* multistressor environments.

INTRODUCTION

Parasites occur in nearly every population, they often interact in complex ways with other stressors. In some cases, the interaction may lead to a disproportionately negative effect on the host population. In other cases, the stressor may ameliorate the effects of parasitism (Lafferty and Kuris, 1999). In an aquatic environment, there is a profound and inverse relationship between environment quality and disease status of fish and parasites are one of

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the most serious limiting factors in aquaculture (Gupta et al., 2012). Most of reliable technologies for detection of pollutants and policies regulatory framework for managing the aquatic environment are expensive and time consuming. The use of parasites of fish and macro-invertebrates are considered one of the cheapest and reliable ways of tracking environmental perturbation in the aquatic system (Orzell and Platt, 2008). Aquatic pollution is still a problem in many fresh and marine environments since it has negative effects on the health of the respective organisms (Fent, 2007). However, the effect of environmental pollutants on fish parasite varies depending on the particular parasite and pollutant that interact (Lafferty and Kuris, 1999). Pollutants may affect the immune system of the fish either directly or by changing the water quality; that in-turn may reduce the fish immunity to parasitism (Poulin, 1992). Fish parasites are used as bio-indicators for monitoring environmental perturbations in aquatic ecosystems. Specific species of parasites or even the parasitic community as whole are utilized as indicators for pollution, as any change in the prevalence and intensity of infection in certain parasite taxa and the composition of parasitic communities are used to assess pollution impact on the aquatic environment (Nachey and Sures, 2009). Man-made pollutants and/or intensification of fish culture resulted in an increase of environmental changes, which may be stressful to fish (Lio-Po and Lim, 2002). Reviews have clearly demonstrated that many chemical contaminants modulate immune functions and affect disease resistance. (Galloway and Depledge, 2001). Anthropogenic stressors are a pervasive problem in freshwaters because these ecosystems can act as conduits or sinks that accumulate and concentrate contaminants (Relyea, 2009). Understanding how aquatic systems respond to stressors, which is fundamental to their management, is not straight forward because often multiple stressors are in operation (Culp and Baird, 2006). Stressors can have both lethal and non-lethal effects that impact on individuals and populations, and these may be additive antagonistic or synergistic factors (Hu et al., 2009). Hosts may become more susceptible to the morbidity effects of disease without any change in per capita infection because contaminants can induce cortisol production and suppress blood leukocytes (Forson and Storfer, 2006). Moreover, the diversity of fish parasites in contaminated water probably reflects two points: (1) disturbance of the entire parasite community and/or (2) a reduction in the immunological response of fish that might facilitate infection by parasite species with a relatively low epidemiological potential. Parasites are indicative of many biological aspects of their hosts, including diet, migration, recruitment and phylogeny (Williams et al., 1992). They may also be useful direct indicators of environmental quality status (Marcogliese and Cone, 1997). Parasites can thus be considered complementary to chemical analysis or traditional biological surveys as indicators of dysfunction at the ecosystem level. These findings suggest that parasites are characterized as fingerprint at community level according to the quality level of the water body in which they complete their life cycles.

Oreochromis niloticus was chosen to study the impact of pollution in the three locations due to its wide range of food selection, and its tolerance to chemicals and physical pollutant factors. The present study aimed to highlight the relationship between composition and structure of parasite communities hosted by *O. niloticus* in the three different located sites with varying levels of pollution and water quality. The results of this study may contribute to the understanding of changes in communities of metazoan parasites of chub due to pollution in freshwater ecosystems. And it demonstrate the eco-toxicological relevance of the biodiversity of metazoan communities fractionated according to deterrent criteria and demonstrate the suitability of a logical set of biodiversity measures which integrates standard diversity indices and a novel approach to the quantitative analysis of cumulative species.

MATERIALS AND METHODS

O. niloticus were sampled in three different ecosystems that differed according to their water quality.

Location of sampling ponds

The first site is CLAR ponds (central laboratory for Aqua-culture research in Abbassa), which is supplied with water from the Ismalia canal by way of the El Wadi El Quadim supply canal that originates from the Nile River. Water enters the CLAR ponds through sedimentation pond. The second site is Abbassa privet ponds for fish aquaculture, that is supplied with water from the Ismalia canal. The third water body is Manzalla ponds which is a brackish lake, sometimes called a lagoon, in northeastern Egypt, and served as a significant source of inexpensive fish for human consumption, but pollution and lake drainage have reduced the lake's productivity.

Water quality

The water quality, assessed by means of Woodwiss's Extended Biotic Index according to Ghetti (1986), and some microbiological (total coliforms and fecal coliform) and chemical pollution (lead, nitrate and phosphrous) indicators were estimated. A total of 366 fish were caught from April 2012 to August 2013.

Fish examination

The number of fish analyzed per sampling site is given as follows: 145 from CLAR ponds; 130 from Abbassa privet ponds; 91 from Manzalla ponds. Eyes, gills and gut of the fish were examined for external parasites and internal parasites in the laboratory and each fish was measured and weighed to estimate the coefficient of condition K (derived from the formula: $K = W\ 105 / L3$, where W and L, respectively, represent the individual weight in grams and the standard length in centimetres (Lévêque et al., 1988).

Table 1. Water quality data evaluated in the three sites.

Water quality parameter	CLAR ponds	Abbassa privet ponds	Manzalla ponds
	Non polluted	Signs of pollution detected	Polluted
Total coliforms (/100 ml)	2300	6100	31 000
Faecal coliforms (/100 ml)	240	1500	18 000
Conductibility (µs/cm)	241	276	1.9430
Nitrite (mg/L)	<1	<1	>1
Nitrate (mg/L)	0.03	0.05	0.07
Total phosphorous (mg/L)	<0.03	<0.03	0.09
Lead (µg/L)	<1	1	7

Table 2. Host condition as a constraint for parasites in the three ponds.

Parameter	Measurements	CLAR ponds	Abbassa privet ponds	Manzalla ponds
Fish length (cm)	Min-max	15.5-19.5	17-23	20.5-33
	Mean	17.5	20	26.5
	Standard deviation	3.21	3.9	3.53
Fish weight (g)	Min-max	50-330	120-250	155-340
	Mean	241	185	247.5
	Standard deviation	65.6	117	71.8
Coefficient of condition (*k*)	Min-max	1.2-3.6	0.7-2.1	0.9-2.9
	Mean	2.0	1.8	2.1
	Standard deviation	0.3	0.21	0.42

Parasitological studies

The level of parasite infection was quantified by using: prevalence (P), intensity (I), mean intensity and mean abundance (A) according to Bush et al. (1997). Species richness, dominance and diversity indices were used to compare parasite community structures. In addition to the application of standard indices of richness, that is, Margale f index, diversity, Shannon-Wiener, and dominance Simpson and Berger-Parker (Magurran, 1983), differences in parasite community structures among the sampled sites were also evaluated.

Statistical analysis

One way ANOVA was applied to test the significant differences between the mean values of intensity, abundance and prevalence in the three ponds.

RESULTS

The water quality, assessed by means of Woodwiss's Extended Biotic Index according to Ghetti (1986), and some microbiological and chemical pollution indicators are shown in Table 1. The total coliform, lead, nitrate and phosphrous estimated in a high values in Manzalla ponds (polluted) while the same Parameters were less in Abbassa privet ponds (slightly polluted).

Nine parasite species were recorded from the examined 366 *O. niloticus*, 300 *O. niloticus* were infected with one or more species of parasites. Trematodes were the predominant taxa comprising six species, *Clinostomum tilapiae*, *Euclinostomum heterostomum, Diplostomum spathaceum*, *Posthodiplostomum minimum*, *Mesostephanus appendiculatus, Haplorchoides cahirinus* (metacercaria); monogeneans were represented by one species *Cichlidogyrus aegypticus*, while cestodes were represented by larval stages of *Polyonchobothrium clarias*, and acanthocephalans were represented by one species, *Acanthosentis tilapiae.*

Fish size, weight and the coefficient of condition are shown in Table 2, the mean fish total length ranged between 17.5 and 26.5 cm, while the mean fish total weight ranged from 185 to 247.5 g. The condition factor did not differ significantly in the three localities (as estimated by *k*) and seem to be slightly higher at Manzalla ponds, and allow the exclusion of any consistent effect of the fish health status on the variability of parasite community.

The values of prevalence, intensity, mean abundance for each parasite are given in Table 3. *C. tilapiae*, *E. heterostomum, C. aegypticus*, *D. spathaceum*, *P. minimum*, *M. appendiculatus, H. cahirinus*, *A. tilapiae,* and larval stages of *P. clarias* were found in the three localities (unpolluted, slightly polluted and heavily polluted).

Table 3. Metazoan parasite species with their main eco-parasitological characteristics.

Parasite	CLAR ponds			Abbassa privet ponds			Manzalla ponds		
	I	A	P (%)	I	A	P (%)	I	A	P (%)
Clinostomum tilapiae	2.3 (1-6)	1.0	41.0	7.1 (1-28)	3.6	51.0	4.8 (1-18)	2.8	59.0
Euclinostomum heterostomum	14.7 (1-58)	13.4	94.0	8.7 (1-24)	0.7	8.0	0.0	0.0	0.0
Cichlidogyrus aegypticus	5.4 (1-24)	2.6	48.5	1.6 (1-3)	0.4	23.0	17.3 (1-80)	11.0	63.4
Diplostomum spathaceum	1.7 (1-3)	0.2	11.4	1.5 (1-2)	0.1	10.2	1.6 (1-5)	0.5	34.1
Posthodiplostomum minimum	8.4 (1-47)	4.3	51.4	30.0 (2-93)	28.7	94.8	5.5 (1-18)	4.1	75.6
Mesostephanus appendiculatus	12.2 (3-31)	1.7	11.4	25.6 (1-98)	13.8	53.8	18.0 (1-83)	6.6	36.6
Haplorchoides cahirinus	2.4 (1-5)	0.3	14.0	10.0 (1-67)	5.6	56.0	1.7 (1-7)	0.4	24.0
Polyonchobothrium clarias	0.0	0.0	0.0	1.00 (1)	0.03	3.0	0.0	0.0	0.0
Acanthosentis tilapiae	14.7 (1-58)	13.4	94.0	8.7 (1-24)	0.7	8.0	0.0	0.0	0.0

I: Intensity, A: abundance, and P: prevalence.

Table 4. Comparison between the total mean values of intensity, abundance and prevalence in the three ponds using one way ANOVA.

Parameter	Different locations			P value	$F_{2,26}$
	CLAR ponds	Abbassa privet ponds	Manzalla ponds		
Intensity (I)	6.87	10.47	5.43*	P<0.01	8.55
Abundance (A)	4.1	5.96	2.8*	P<0.01	5.25
Prevalence (P)	40.63%	34.2%	9.7%*	P<0.01	7.99

*Significant differences between locations using one way ANOVA, P<0.01.

Table 5. Values of diversity, richness of species, equitability and dominance.

Site	Diversity	Richness of species	Equitability	Dominance	Dominance
CLAR ponds	1.64 (± 0.11)	2.1 (± 0.18)	0.68 (± 0.04)	0.29 (± 0.04)	0.547 (± 0.44)
Abbassa privet ponds	1.55 (± 0.10)	1.81 (± 0.15)	0.62 (± 0.01)	0.27 (± 0.03)	0.446 (± 0.35)
Manzalla ponds	1.52 (± 0.06)	1.6 (± 0.10)	0.66 (± 0.03)	0.28 (± 0.02)	0.500 (± 0.40)

In the heavily polluted site (Manzalla ponds), the prevalence was the highest in *P. minimum*, *C. aegypticus*, and *C. tilapiae* (75.6, 63.4 and 59%, respectively) and it was less in *H. cahirinus*, *D. spathaceum* and *M. appendiculatus* (24, 34.1 and 36.6%, respectively), while prevalence was 0% *in* both *E. heterostomum*, *A. tilapiae* and *P. clarias*.

In the slightly polluted site, Abbassa privet ponds, the prevalence was high in both *P. minimum*, *H. cahirinus*, *M. appendiculatus* and *C. tilapiae* (94.8, 56, 53.8 and 51%, respectively), it was decreased (23%) in *C. aegypticus* where it was minimum in the other species.

In the unpolluted pond, the prevalence was the high in *A. tilapiae*, *E. heterostomum*, *P. minimum*, *C. aegypticus* and *C. tilapiae* while it was 0% in the case of *P. clarias*.

Table 4 shows the significant differences between the data obtained from the Manzalla ponds and the other studied locations concerning intensity, abundance and prevalence using one way ANOVA. The total intensity value of the parasitic infection in Manzalla ponds was significantly low (5.43) as compared to 6.87 and 10.47 in CLAR ponds and Abbassa privet ponds respectively. The same trend of significance was recorded in the total abundance and prevalence values of parasitic infection between the three selected ponds in this study

Values of Margalef, Shannon-Wiener, dominance, Simpson's and Berger-Parker indices are shown in Table 5 which show that, the mean values of parasite species richness and diversity, according to Margalef and to Shannon-Wiener indices, showed significant increase from the polluted Manzalla ponds (1.52) to the unpolluted site CLAR ponds (1.64) which present the highest dominance value Simpson dominance (0.547), for the Berger-Parker index, the values are not structured along the pollution gradient.

DISCUSSION

Aquatic pollution is still a problem in many fresh and marine environments, it has a negative effects on the health of the respective organisms (Fent, 2007). According to their effects, pollutants can either be lethal or sub-lethal. The effects may manifest immediately (acute toxicity) or after prolonged exposure to the pollutant (chronic toxicity) (Sures, 2008). The number of studies investigating effects of pollutants and occurring parasites is still relatively low (Sures, 2006, 2007). Environmental stress has been suggested to increase host susceptibility to infections and reduce host ability to resist parasite growth and reproduction, thus benefiting parasites. This prediction stems from expected costs of immune defense; hosts in poor condition should have less resource to be allocated to immune function.

As a contribution to the ongoing debate on the role of environmental parameters on parasite community structure, this study provides evidence on the effect of water quality both on the level of parasite species richness as well as on the degree of interactivity of the component communities (Lio-Po and Lim, 2002).. Each parasite can interact differently with each stresses (Lafferty, 1997). In the present study, the monogenean *C. aegypticus* shows a good resistance to pollution stresses affecting other more sensitive gill parasites, while the digeneans *E. heterostomum* and *P. clarias*, are absent in the severely polluted site, but show highest levels of infection indices in the moderately polluted site. These values could be attributed to the use of a common intermediate host, the gasteropod pulmonate *Lymnaea stagnalis*, that is sensitive to chemical contaminants but tolerating a high organic pollution (Girod et al., 1980). The pollution, in this case, influenced the prevalence and the abundance of these parasites through the abundance of their intermediate host (Kiely et al., 2004). Even the digenean *P. minimum*; *M. appendiculatus* and *C. tilapiae* appeared to prefer habitats with moderate water pollution (94.8, 56.0 and 51.0%, respectively of *O. niloticus* were infected by these species), so that both unpolluted and polluted sites were less suitable for these species. Moderate organic pollution (Abbassa privet ponds) could increase the number of macrobenthic species, probable intermediate parasite hosts, and therefore the numbers of parasites themselves. Clearly, due to the stressing effects of pollution, fish defense responses against pathogenic agents, including parasites, could be reduced, making digenean easier.

Sindermann (1979) summarized some of the recent supporting evidence that toxins have a deleterious effect on the immune response of fishes. This study of Biscayne Bay fishes suggests that, in the presence of sub-lethal quantities of pollutants in a natural marine environment, fish suffered from gill damage which produced stress, physiological and physical compensation, leading to weakening, reduced immunity, and heavy parasitic infestation. It is very important to mention that the statistical analysis using one way analysis of variance (ANOVA) of the total values of intensity, abundance and prevalence between the tree water bodies indicated that the significantly lower values of these parameters in Manzalla ponds reflects the strong stressful impact of pollution on the parasitic communities on *O. niloticus* as compared to the two other sites. According to Hoffman (1976), eutrophication and pollution probably affect helminthes parasites as well as the hosts. The acanthocephalan *A. tilapiae* was collected only in the unpolluted sites. The absence of *A. tilapiae* in polluted ponds can be attributed to the sensitivity of the intermediate host, the *Cyclop* and *Mesocyclop strenus* which is known to be highly affected by pollution.

According to Mojetta (1998), cyclops show a high density in the CLAR ponds and Abbassa privet ponds and are completely absent in the Manzalla ponds sampled sites. Species richness and diversity indices show that parasite communities are affected by the different status of environmental conditions. For instance, when comparing the values of the Shannon Index with total coliforms the Pearson correlation value is 0.98 when comparing the three sites, while this value decreased to 0.78. By excluding site 3, the value rises to 0.99. This finding indicates that the differences between sites 1, 2 and 3 are not as striking.

The present data suggest that, parasite sensitivity to water pollution can be considered as indicators of water quality. Parasites represent an ubiquitous component of animal communities and are more abundant than their hosts.

In addition, a large number of parasite species require, to complete their life cycle, many types of verte-brate and invertebrate organisms acting as intermediate or definitive hosts; therefore, changes in the structure of a parasitic community reflect differences in the composition of the aquatic species (phyto and zooplankton, and benthos) commonly used as indicators of water quality. Parasites move through the food web and are situated at its top, integrating the adverse effects of various contaminants. The pollution ecology of many parasites remains unknown and not all host fishes are good models for environmental research. On the other hand, parasite species that are directly or indirectly sensitive to pollution have been found to disappear as the pollution levels increase, and may be considered good indicators for early detection of adverse environmental effects. Despite these contrasting pieces of evidence, the analysis of freshwater fish parasites could offer a useful and reliable indication or monitor of environmental quality in our case.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and identification of secondary metabolites producer *Nocardia* spp. from Iraqi soil

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One hundred and sixty nine *Nocardia* isolates were recovered from 111 Iraqi soil samples on six cultivation media, using paraffin baiting and dilution techniques. The North of Iraq soil was the richest in *Nocardia.* Paraffin baiting technique was more efficient in the isolation of *Nocardia* than dilution technique. Glucose asparagine agar was more suitable for cultivating *Nocardia* isolates followed by Sabouraud dextrose agar. All the 169 presumptive Nocardia isolates recovered were subjected to primary screening of antibacterial activity, with 10-55 mm inhibition zones against standard *Staphylococcus aureus* (NCTC 6571) and 10-38 mm inhibition zones against standard *Escherichia coli* (ATCC 25922). Eleven isolates with the highest antibacterial activities were selected for chemo-taxonomic studies. These isolates exhibited high similar features of 87-89% with *Nocardia brasiliensis.* Therefore, it is suggested to give proposed names for these new taxa: *Nocardia* sp.1, *N.* sp. 2, *N.* sp. 3, *N.* sp. 4, *N.* sp. 5, *N.* sp. 6, *N.* sp. 7, *N.* sp. 8, *N.* sp. 9, *N.* sp. 10 and *N.* sp. 11.

Key words: Secondary metabolite, *Nocardia* sp., antimicrobial activity.

INTRODUCTION

Actinobacteria, especially when isolated from soil produce many important industrial and commercial antitumor agents, bioactive metabolites and enzymes (Demain and Lancini, 2006). Filamentous nocardiform bacteria represent continuous reservoir of pharmacological, medicinal and agricultural important compounds (Berdy, 2005). Among them, Transvalencin Z as a strong antibiotic against Gram positive bacteria, fungi and tumor cells was recovered from *Nocardia transvalensis* (Akira et al., 2006) and cepha-mycin C was produced by *Nocardia lactemdura* (Kagliwal et al., 2009). Also, Speitling et al. (1998) and Mukai et al. (2009) reported the moderate activities of the metabolites of *Nocardia pseudobrasiliensis* (new anthrax-cycline, dimethyl mutactimycins) against Gram positive bacteria and their cytotoxic activities against P388, L1210 and Hela tumor cells. They also reported novel thiopeptide antibiotic and nocardithiocin with strong activity against rifampicin resistance and sensitive *Mycobacterium tuberculosis* and *Gordonia* sp.

Sakagami et al. (2005) demonstrated that nocobactin

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NAa and NAb from *Nocardia farcinica had* antitumor potential against human tumor cells; whereas gastric adenocarcinoma, breast carcinoma and hepatocellular carcinoma were inhibited by nocardichelins from *Nocardia* strain Acta 3026, which was isolated from mangrove soil (Schneider et al., 2007). The aim of this study is to isolate and identify *Nocardia* spp. from Iraqi soil with high secondary metabolites production.

MATERIALS AND METHODS

Sample collection

One hundred and eleven soil samples used in this study were collected from April-November 2009 from different regions in Iraq. Temperature was measured at the same time of collection; calcium carbonate, salinity and pH of each sample were measured in Marine Science Centre, Basra University.

Isolation technique

One gram of dried sifted soil sample was suspended in 9 ml of saline solution by shaking vigorously for 5 min. Serial decimal dilutions of the supernatant were prepared (El-Nakeeb and LeChevalier, 1962), in which 0.1 ml from each dilution was spread over the surface of six types of media: Glucose asparagine agar (GAA), Sabouraud dextrose agar (SDA), Glycerol agar (GA), Nutrient agar (NA), Yeast extract agar (YEA), modified Czapeks agar (MCA). Antifungal agent, cyclohexamide (actidione) of 50 µg /ml and anti-bacterial agent, chloramphenicol of 50 µg/ml were added to the sterilized media at 46°C (Nazar et al*.,* 1986). Plates were then incubated at 28-30°C for 7-14 days.

Paraffin baiting technique

Twenty five gm of each soil sample was transferred into a sterile conical flask. Into each of these conical flasks, a paraffin-coated sterile glass rod was introduced and covered with cotton and aluminum foil. The conical flask was incubated at 37°C for 4 weeks with some modifications (Mishira and Randhawa, 1969). Paraffin rod was scraped by cotton swab and streaked on different isolation media (GAA, SDA, GA, NA, YEA, and MCA); plates were incubated at 28-30°C for 7-10 days. After incubation period, colonies were streaked on yeast-malt extract agar (YMA) for sub culturing. After that, colonies were stained with Gram staining and acid-fast staining using the method of Ziehl-Neelsen (Benson, 2002).

Morphological and colonial characteristics

For identification of *Nocardia*, the microscopical features of bacterial cells growing on YMA such as cells shape, arrangement and stain were recorded. Also, colonial characteristics of culture isolates in different media were noted.

Primary screening of bacterial isolates

Presumptive *Nocardia* isolates have been screened for production of bioactive metabolites against standard isolates of *Staphylococcus aureus* and *Escherichia coli* using paper disc agar plate diffusion method (Bauer et al., 1966).

Biochemical tests

To know the Nocardia isolates with high antimicrobial activities, motility test, gelatin hydrolysis test, catalase test (Alexander and Strete, 2001), growth tests, tyrosine hydrolysis test, testosterone hydrolysis test, antibiotic resistance test, utilization of organic compounds as carbon source and utilization of some compounds as nitrogen source (Williams et al., 1983), determination of meso-diamino pimelic acid (DAP) and whole sugars in cell wall and melanin production test (Cross, 1981),citrate utilization test, urea's test, nitrate reduction test, starch hydrolysis test, casein hydrolysis test and esculin hydrolysis test (Benson, 2002) and lysozyme resistance test (Forbes et al., 2007) were done. Molecular method used for identification, which is crucial for confirming strain identity was not used due to the unavailability of its tools. However, selected isolates will be sent abroad for sequencing.

RESULTS

Physical and chemical parameters of soil samples

Table 1 showed that soil samples collected for this study have temperatures of 21-49.6°C and pH of 6.8-8.1, respectively; calcium carbonate and salinity ranges were 0.65-12.22 mg/l and 1.03-53.27 ppt.

Isolation techniques

Paraffin baiting technique was more efficacious than dilution technique for isolation of *Nocardia* from soil sample. Paraffin baiting technique gave the highest isolation rate of 400%, while dilution technique gave the highest isolation rate of 100%. Therefore, all other samples were treated with paraffin baiting technique.

Cultivation and distribution of isolates

An isolation rate of the following deceasing order was noticed: GAA 41.42%> SDA 21.30%> NA 14.79%> GA 11.83%> MCA 7.6%9> YEA 2.95%. Results showed that S-N soil samples recorded the highest isolation rate of 244.4% for recovery of isolates and A-S soil samples recorded the lowest isolation rate of 20% for recovery of isolates.

Morphological identification of isolates

One hundred and sixty nine isolates collected in this study were identified as Gram positive filamentous *Nocardia* fragmented into rod to coccid cells with diameter 1.2 µm (Figure 1). The results of acid fast staining divided *Nocardia* into three groups depending on the reaction mode with acid fast stain: acid fast, partially acid fast and non- acid fast.

The results of culturing isolates on different cultivation media showed that *Nocardia* colonies had chalky, dried cerebroid, wrinkled or heaped, domed and smooth with regular or filamentous margin; consistency may be

Table 1. Physical and chemical parameters of soil samples.

Soil type	Collection site	Symbol	Number of samples	Temperature (°C)	pH	$CaCO_3$ (mg/l)	Salinity (ppt)
Agriculture	Garden	A-G	41	41	21.3	12.22	4.28
	Missan sugar cane field	A-S	5	5	37.2	3.76	4.11
River margin	Garmat-Ali	R-G	7	7	21.1	7.54	1.03
	Al- Ashar	R-AS	3	3	21	9.64	1.39
	Al-Shafi	R-SH	3	3	41.6	8.16	5.77
Sandy	Rumaila	S-R	10	10	24.5	8.94	5.24
Oil-contaminated	5-mile	O-M	15	15	49.6	0.65	6.50
Salty	Garmat-Ali	S-G	8	8	23.2	4.38	53.27
Manure	Garmat-Ali	M-G	10	10	28	2.24	13.18
Sulphur saturated	North of Iraq	S-N	9	33.3	7.9	4.3	3.31

Figure 1. Gram staining of *Nocardia.*

mucoid, pasty and cartilaginous or leathery with different diameters range from 2-8 mm. Colonies appeared with different colours as white, creamy, red, yellow, pink, orange, pale tan and purple. Soluble brown pigment was produced by some isolates on different media. Colonies had the ability to grow as convoluted fold on some media as yeast-malt extract agar (Figure 2).

Primary screening of isolates

Primary screening of the 169 isolates from the different soil samples showed antibacterial activities with inhibition zones against standard isolates of *S. aureus* NCTC 6571 (10-55 mm) and *E. coli* ATCC 25922 (10-38 mm). The primary screening enables us to select *Nocardia* isolates with secondary metabolites of high antimicrobial activity (Table 2) and finally subjected them to conventional biochemical tests to know (Table 3) their growth characteristics (Table 4).

All isolates were able to utilize glucose, sucrose, lactose, fructose, galactose, cellobiose, arabinose, xylose, rhammnose, raffinose, mannose, maltose, mannitol, glycerol, sodium acetate, sodium citrate, butanol, sorbitol, trehalose, inositol and adonitol as a sole source of carbon and potassium nitrate, lysine, threonine, serine, methionine, asparagines and phenylalanine as a sole source of nitrogen. The structure of *Nocardia* cell wall contains amino acid (meso-diaminopimelic acid with green spot and high R_f(0.9), sugars as arabinose with red brown spot and R_f (0.53) and galactose with brown spot and R_f (0.38).

According to principal taxonomic characters in Sykes and Skinner (1973), Cowan et al. (1974), Holt et al. (1994) , Kageyama et al. (2004) , Kageyama et al. (2005), Watanabe et al. (2006) and Forbes et al. (2007), these isolates showed a range of chemical properties consistent with their classification in the genus *Nocardia* and not corresponding with any published *Nocardia* sp. But these

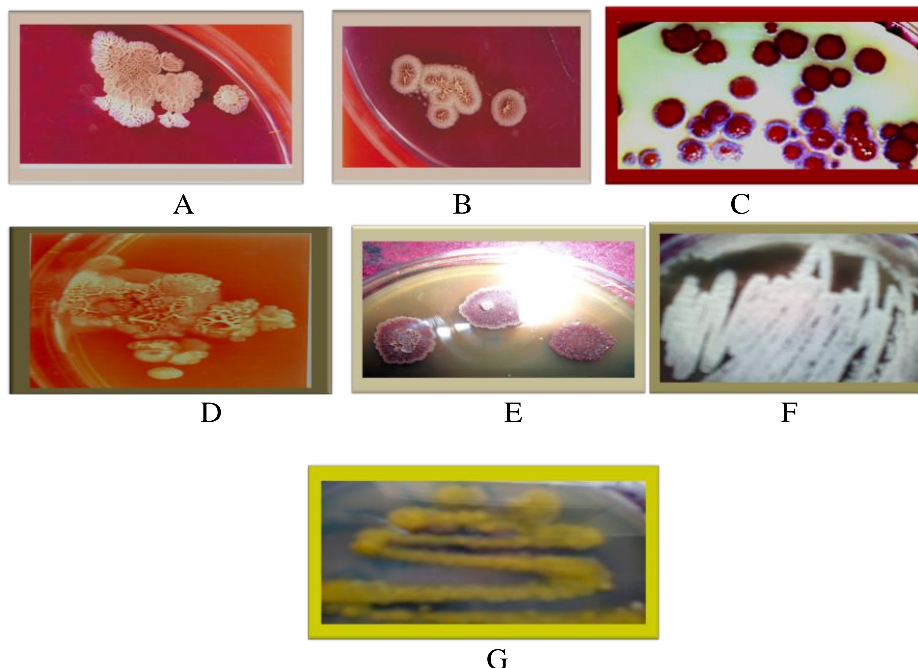

Figure 2. Colonies of *Nocardia.* (A) White yeast-malt extract agar with crystal violet, (B) pale tan, (C) red yeast-malt extract agar, (D) Creamy, (E) pink, (F) chalky growth on yeast-malt extract agar, (G) yellow.

Table 2. Designated *Nocardia* with high antibacterial activity against standard *S. aureus* and *E. coli.*

***Nocardia* isolate designate**	**Soil sample**	**Cultivation media**	**IZ (mm) of antibacterial activity against *S. aureus***	**IZ (mm) of antibacterial activity against *E. coli***
1	A-G	NA	21	30
2	A-G	YEA	40	30
3	A-G	YEA	24	38
4	A-G	GAA	55	10
5	R-G	GA	16	25
6	O-M	NA	10	20
7	A-G	SDA	13	15
8	S-N	GA	23	10
9	S-N	NA	13	14
10	O-M	SDA	30	10
11	S-N	GA	14	30

isolates exhibited similar character ranging from 87-89 % with *N. brasiliensis* (SPSS 2007). Therefore, it is suggested to give proposed names for these taxa including *Nocardia* sp.1, *N.* sp. 2, *N.* sp. 3, *N.* sp. 4, *N.* sp. 5, *N.* sp. 6, *N.* sp. 7, *N.* sp. 8, *N.* sp. 9, *N.* sp. 10 and *N.* sp. 11.

DISCUSSION

Isolation of *Nocardia* from soil

Paraffin baiting technique led to an unequivocal isolation and identification of *Nocardia* from soil due to the ability of *Nocardia* enzymes to degrade paraffin and utilize it as a sole source of carbon Ayyar et al., 1992; Narang et al., 2004; Kaur and Oberoi, 2005). This conforms with Kurup et al. (1968) who confirmed that this technique was successful for the isolation of *Nocardia* from soil. Many *Nocardia* species were isolated from soil by paraffin baiting technique such as *N. brasiliensis, Nocardia asteroids and Nocardia caviae* from Argentina soil (Komaid et al., 1987) and Delhi soil (Kumar and Mohaptra, 1968).

Jayabarath et al. (2010) established that salty soil is a

Table 3. Biochemical characteristics for the identification of the 11 isolates of *Nocardia*.

Test \ Isolate	1	2	3	4	5	6	7	8	9	10	11
Catalase	+	+	+	+	+	+	+	+	+	+	+
Urea's	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
Melanin	+	+	W	+	+	-	+	+	+	-	-
Hydrolysis of Casein	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	-	-
Testosterone	-	-	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+	+	+	+
Liquification of gelatine	+	+	-	+	+	-	+	+	+	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+

+ = good growth, - = no growth, w = weak.

Table 4. Growth characteristics of the 11 isolates of *Nocardia.*

Test \ Isolate	1	2	3	4	5	6	7	8	9	10	11
Soluble pigments	Cr-G	Cr+R	P+R	Cr-G	Cr-G	R	Cr-G	Cr+R	Cr	Cr	R
Acid-fast	-	Par	+	-	+	+	Par	Par	-	Par	-
Motility	-	-	-	-	-	-	-	-	-	-	-
Growth at											
10°C	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	
45°C	+	+	+	+	+	+	+	+	+	+	+
Growth at PH											
5	w	w	w	w	w	w	w	w	w	w	w
7	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+
Growth at NaCl (%)											
4% NaCl	++	++	++	++	++	++	++	++	++	++	++
7% NaCl	++	++	++	++	++	++	++	++	++	++	++
10% NaCl	++	++	++	++	++	++	++	++	++	++	++
13% NaCl	++	++	++	++	++	++	++	++	++	++	++
Growth at the presence of											
Sodium azide 0.01%	++	++	*	++	++	++	++	++	++	++	+
Sodium azide 0.02%	w	w	W	w	w	+	w	w	+	+	+
Crystal violet 0.001%	++	+	++	++	++	+	++	+	++	+	++
Phenol 0.1%	w	-	+	w	w	W	-	w	W	-	-

++ = very good growth, + = good growth, - = no growth, w = weak, Cr = creamy, P = pink, G = green, R = red, par = partial.

good reservoir for *Nocardia amarae, Nocardia farcinica* and *Nocardia vaccini. Nocardia* was considered as one of the most common aerobic bacteria responsible for degradation of hydrocarbons in petroleum-contaminated soil (Pucci et al., 2000; Barathi and Vasudevan, 2001; Baek et al., 2004; Alquati et al., 2005; Wyszkowska and Kucharski,

2005; Chikere et al., 2009; Gomes et al., 2009; Nih-Cong et al., 2010) and in gasoline-contaminated aquifers (Rosenberg and Gutnick,1981). Cain (1958) isolated *Nocardia erythropolis* from manure heaps. His results supported that of manure samples. Sandy and agriculture soils (garden and sugar cane) represented a rich source of *Nocardia* (Orchard and Goodfellow, 1974; Padoley et al., 2009). Bredholdt et al. (2007) isolated *Nocardia* from shallow water sediments of the Trondheim fjord (Norway); all these explain the results of Figure 2.

Glucose asparagine agar exhibited a good growth and more numbers of *Nocardia* from marine sediment and Thailand soil, respectively, but less numbers of action-mycetes were recorded with Nutrient agar (Kokare et al., 2004; Srivibool and Sukchotiratana, 2006). Sabouraud dextrose agar containing cyclohexamide was used for isolating *Nocardia* species from Iran soil (Aghamirian and Ghiasian, 2009). Glycerol agar containing soil extract represented a perennial source of organic matter, carbon, nitrogen, minerals and vitamins and a natural medium for growth of many organisms and glycerol, which is important in the growth of actinomycetes (El-Nakeeb and Lechevalier, 1962). Therefore, Xu et al. (1996) isolated *Nocardia* from 4,200 China soil by glycerol asparagine agar medium.

Morphological identification of *Nocardia*

The results of microscopical and cultural tests of *Nocardia* have revealed that they are Gram positive filamentous rod or cocci cells (Figure 1). This is correlated with other studies conducted by Whitmore et al. (1961) and Hattori et al. (2003). *Nocardia* also showed different response to acid fast staining; isolates were divided into non- acid fast, partial acid fast and acid fast due to the average carbon numbers of the mycolic acids. Each species of *Nocardia* possesses a characteristic profile of mycolic acid composition (Yano et al., 1978). Zhang et al. (2004) and Xu et al. (2005) demonstrated that *Nocardia xishanensis* and *Nocardia polyresistens* respectively isolated from soil were partially through acid-fast staining. The colonies' texture varies among isolates (Figure 2) depending on the degree of mycelia development; and the colonies exhibited multiple shapes and colors due to carotenoid pigments (Cowan et al., 1974). Many studies agreed with this result such as that of Whitmore et al. (1961) who showed that *Nocardia* exhibited gray, red, yellow, white, smooth, domed colonies with rough powdery surface. Zhang et al. (2004) established *Nocardia* colonies as yellow to orange, convex to irregular with filamentous margin. Kurup and Schmitt (1970) noticed colonies of *Nocardia* on the paraffin bait as yellow, white, brown, pink. Hattori et al. (2003) isolated *N. africana* with orange wrinkled colonies.

Primary screening of *Nocardia* isolates

Primary screening was necessary for evaluating the antimicrobial activities of *Nocardia* isolates against microorganisms tested including *S. aureus* and *Candida albicans* (Anansiriwattana et al., 2006; Vengadesh et al., 2011; Vinhot et al., 2011) due to their ability to produce bioactive metabolites against them (Chandrashekhara, 2010). *Nocardia iowensis* isolated from garden soil of Osceola, Iowa, USA represented a source of antibiotic production (Lamm et al., 2009). The appropriate conditions characterized this soil as neutral pH and suitable temperature in the presence of calcium carbonate played a good role in the exuberance of *Nocardia* from this soil (Table 3).

Biochemical identification of *Nocardia*

As a result of primary screening, eleven of 169 isolates with high antibacterial activity were selected and identified (Tables 2 to 4). The biochemical features of the 11 isolates did not correspond with any *Nocardia* species but exhibited high similar features (87-89 %) with species of *N.brasiliensis*. Therefore, it was suggested to name them as *Nocardia* sp.1*, N.* sp. 2*, N.* sp. 3*, N.* sp. 4*, N.* sp. 5*, N.* sp. 6*, N.* sp. 7*, N.* sp. 8*, N.* sp. 9*, N.* sp. 10 and *N.* sp. 11. This was in accordance with other studies that recorded new species of *Nocardia* (Kageyama et al., 2004, 2005). The results were obtained by comparing the biochemical features of new isolates with published ones.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Incidence of kernel smut caused by *Tilletia barclayana* in Egyptian rice cultivars

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The study was conducted to determine the effects of cultivars susceptibility to infection with the pathogenic isolates on the pathogenicity of rice kernel smut fungus (*Tilletia barclayana*). Rice (*Oryza sativa* L.) cultivars vary in susceptibility to kernel smut disease. The susceptibility of 18 commercial rice cultivars to *T. barclayana* was evaluated. Moreover, pathogenicity of 46 isolates of *T. barclayana* which had been isolated from six governorates in Egypt was studied using Giza 178 rice cultivar and Hybrid 1. The activities of certain oxidative enzymes, e.g. peroxidase (POX) and polyphenoloxidase (PPO) and total protein were determined in healthy and inoculated cultivars. Intron-exon splice junctions (ISJ) protocol was used to study the relationship among rice cultivars differing in their susceptibility to infection with the pathogen. Results showed that Giza 178 rice cultivar and Hybrid 1 were the most susceptible cultivars for rice kernel smut disease. Isolate no. 35 from the pathogenic fungus was the most aggressive isolate. Additionally, an increment in the defense-related enzymes and total protein was observed in rice cultivars as a result of inoculation with *T. barclayana.* ISJ technique showed the relationship between the studied rice cultivars and lines upon susceptibility to *T. barclayana*. It can also differentiate between the isolated *T. barclayana* isolates upon virulence on the studied rice cultivars and lines.

Key words: Rice kernel smut disease, intron-exon splice junctions, rice, enzymes activity.

INTRODUCTION

Rice kernel smut, known as grain smut (Biswas, 2003), is caused by the pathogen *Tilletia barclayana* which causes a partial bunt that affects both yield and quality. This disease was recorded in Egypt in 1999 (Ismail, 2003). *T. barclayana* causes the endosperm of the rice grain to be replaced partially or completely by a black mass of smut

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spores. In general, long-grain rice cultivars, the predominant type grown in the United States, are the most susceptible cultivars to infection with *T. barclayana* (Biswas, 2003). Even a low incidence of infection can cause substantial economic losses in hybrid rice seed production. In India only a maximum of 0.5% infection is allowed in certified seed (Biswas, 2003). Templeton et al. (1960) indicated that 1 to 10 rice grains per panicle may show partial or complete smut infection. This range of infection likely represents from 1 to 15% direct grain yield losses. Rice kernel smut epidemics on recently released cultivars increased the concern of rice growers because of the undesirable effects of kernel smut on both grain quality and yield (Cartwright et al., 1999). In 1982, Murty and Singh evaluated 31 rice varieties for infection with *T. barclayana*. They found that seven varieties had no infection while 24 cultivars suffered very slight infection, ranging from 0.04 to 0.8%. Additionally, Muthusamy and Ahmed (1997) studied reactions of 10 early maturing cultivars and 9 later maturing ones to natural infection by *T. barclayana*. They reported that the cultivars with a shorter growing season were infected more than those with a longer season. Misra et al. (1994) conducted disease surveys for 144 rice seed samples collected from 7 different regions in the Philippines during dry and wet seasons using the standard blotter method, 39 fungal species belonging to 30 genera were isolated. The percentage of infestation by different species varied with location. *T. barclayana* was evenly distributed irrespective of the season. The incidence of kernel smut *T. barclayana* was observed on cytoplasmic genetic male-sterile (CMS) lines and their respective maintainers. The disease was generally more severe on the CMS lines and hybrids, when compared with their maintainers, restorers and inbred rice cultivars (Chahal et al., 1993; Zheng, 2005).

It was reported that plants defend themselves from the invader pathogens by either their structural barriers or antimicrobial compound which prevent colonization of the tissues. The induced defense responses include hypersensitive responses, the production of reactive oxygen species (ROS), pathogensis-related protein and ion fluxes across the plasma membrane (Zhao et al., 2005; Elsharkawy et al., 2012a, b; Elsharkawy et al., 2013; Hassan et al., 2014).

The use of molecular markers has proven its value for a variety of purposes in molecular biology. DNA fingerprinting, gene mapping and phylogenetic studies have tremendously benefited from polymerase chain reaction (PCR) technology. Random amplified polymorphic DNA (RAPD) markers generate DNA fingerprints with a single synthetic nucleotide primer (Williams et al., 1990) which could efficiently detect polymorphism based on comparison throughout the genome. Molecular markers technology provides novel tools for DNA fingerprinting of rice cultivars to assess cultivar seed purity. Semi-random PCR primers targeting intron-exon splice Junctions (ISJ) were used to analyze the rice genome with the aim of evaluating potential of these markers for identification and classification of rice cultivars (El-Moghazy, 2007). DNA-based markers are highly heritable, available in high numbers and exhibit enough polymorphism, hence they can be used to discriminate closely related genotypes of plant (Yashitola et al., 1997). For this reasons, DNA fingerprinting for cultivar or varietal identification has become an important tool for genetic identification in plant breeding and germplasm management (McGregor et al., 2000). Weining and Langridge (1991) reported that the targeting of the intron-exon splice junctions in conjunction with primers of random and defined sequences provides a source of extensive variation in PCR products. The ISJ's primers were able to differentiate between studied varieties and species. El-Moghazy (2007) studied the genetic diversity among eleven rice genotypes using three ISJ primers. A total of twenty-six DNA fragments were amplified and high degree of polymorphism was observed. The objectives of this study are to evaluate rice cultivars with infection of rice kernel smut disease and to differentiate between rice cultivars in response to infection by rice kernel smut disease at molecular levels.

MATERIALS AND METHODS

Isolation and identification of rice kernel smut disease in Nile Delta

Infected rice grains showing typical kernel smut disease symptoms were collected from six governorates, that are, Kafr El-Sheikh, Dakahlia, Gharbia, Damietta, Beheira and Sharkia of Egypt. All samples were collected during rice maturity stage. Teliospores were collected from these infected rice grains and germinated after soaking in a solution of NaOH 1% for two days and centrifuged for 30 min (4,000 x g) then teliospores were soaked in distilled water for two days and transferred onto a 2% water agar (home-made) (Anil and Singh, 1987). The plates were incubated at 25°C±1 for 12 days till the germination of teliospores. Primary basidiospores were transferred onto potato-sucrose agar (PSA) (home-made) (Trione, 1964). Identification of *T. barclayana* isolates was carried out according to the morphological, microscopic characteristics and type of teliospore germination in Plant Pathology Laboratory, Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt using the key given by Fischer and Holton (1957) and Duran (1973). Rice cultivars and lines were obtained from RRTC.

Pathogenicity tests

Pathogenicity test of the isolated 46 isolates of *T. barclayana* was carried out using Giza 178 rice cultivar and Hybrid 1 (highly susceptible cultivars for rice kernel smut disease) grown in 30 x 25 cm diameter pots under greenhouse condition at RRTC. Plants at flowering stage were sprayed with 5×10^7 secondary sporidia/ml of each isolate using electrical spray gun (atomizer) in four replicates. 2.5 g/L of gelatin were added to spore suspension inocula to enhance infection (Bastiaans, 1993). The inoculated plants were held in a moist chamber with at least 90% RH and 25-28°C for 24 h and then moved to the greenhouse for maturity stage after inoculation. Disease severity was recorded using number of infected grains and total number of grains per 50 panicles (Slaton et

al., 2004).

Evaluation of certain rice cultivars and lines with infection of the causal fungus

The response of eighteen rice cultivars and two hybrids to infection with the most aggressive isolate of the causal fungus (isolate no. 35) was recorded. This experiment was conducted as mentioned above in pathogenicity test.

Disease assessment

To estimate severity and percentage of infection of rice plants in 50 panicles at maturity stage, the plants were examined to record the infected plants and calculate the infection percentage according to the following equations (Slaton et al., 2004):

$$\text{Disease severity} = \frac{\text{No. of infected grains}}{\text{Total no. of rice grains}} \times 100$$

$$\text{Percentage of infection} = \frac{\text{No. of infected grains}}{\text{Total no. of rice panicles}} \times 100$$

Randomized complete block design in plastic pots (30 x 25 cm diameter) with four replicates was used. The pots were kept in the greenhouse at 30-35°C and fertilized one time with urea 46.50% N at 3 g/pot.

Enzyme activities and total protein assay of rice cultivars

The activities of certain oxidative enzymes, e.g. peroxidase (POX) and polyphenoloxidase (PPO) and total protein were determined in healthy and inoculated cultivars. Samples were collected from rice hills at 3, 6, 9, 12 and 15 days after flowering stage. The tested cultivars were Giza 171, Giza 175, Giza 177, Giza 178, Giza 181, Giza 182, Egyptian yasmine, Sakha 101, Sakha 102, Sakha 103, Sakha 104, Sakha 105, Hybrid 1, Hybrid 2, BL 1, Doular, Giza 159, Giza 176, Rieho and Sakha 106. The experiment was repeated three times with three replicates per treatment.

Enzymes extracts

Enzymes extracts were prepared according to the methods recommended by Maxwell and Bateman (1967). 500 mg fresh weight of rice leaf samples were ground in a mortar and pestle containing liquid nitrogen. The resulting powder was macerated for 30 s. and homogenized with 3 ml of sodium phosphate buffer pH 6.8 (0.01 M). Triturated tissues were strained through 4 layers of cheese cloth and filtrates were centrifuged for 15 min at 6.000 rpm in a refrigerated centrifuge. The clear supernatant was taken as the enzymes source.

Peroxidase (POX) assay

Peroxidase enzyme activity was determined according to the method described by Allam and Hollis (1972) and Srivastava (1987) by measuring the oxidation of pyrogallol to pyrogallin in presence of H_2O_2. Peroxidase activity was expressed as changes in absorbance (optical density per 1 min/0.5g sample, OD/min/0.5g). The absorbance was measured at 425 nm and recorded at 0, 1, 2, 3, 4 till 5 min intervals using spectrophotometer (Milton Roy, Spectronic, 1201 Digital).

Polyphenoloxidase (PPO) assay

PPO was determined according to the method adopted by Matta and Dimond (1963). Polyphenoloxidase activity was expressed as changes in absorbance (optical density/ min/0.5 g), the absorbance was measured at 495 nm and recorded at 0, 1, 2, 3, 4 till 5 min intervals using spectrophotometer (Milton Roy, Spectronic, 1201 Digital).

Total protein assay

Determination of total protein using coomassie brilliant blue G-250 is based on the observation that coomassie brilliant blue G-250 exists in two different color forms, red and blue. The red form is converted to the blue form upon binding the dye with protein. The protein-dye complex has a high extinction coefficient, thus leading to great sensitivity in measurement of the protein. Protein contents were determined according to Bradford (1976).

Relationship between the tested rice cultivars as well as isolates of the causal pathogen of rice kernel smut using intron-exon splice junctions (ISJ) protocol

ISJ protocol was used in this work to study the relationship among rice cultivars differing in their susceptibility to infection with the pathogen. The tested rice cultivars were Sakha 101, Sakha 105, Hybrid 1, Giza 178, Giza 181 and Egyptian yasmine. Also, ISJ technique was used to differentiate between 13 isolates of such pathogenic fungus differing in their virulence to rice cultivars. Four primers, ISJ-5 (5'-CAG GGT CCC ACC TGC-3'), ISJ-6 (5'-GAC CGC TTG CAG GTA AGT-3'), ISJ-7 (5'-TGC AGG TCA GGA CCC T-3') and ISJ-9 (5'-AGG TGA CCG ACC TGC A-3') of intron splicing junction (ISJ) were used.

DNA isolation and quantification

DNA of the 6 selected rice genotypes and 13 isolates of the fungus were isolated using CTAB (Cetyl- Tetramethyl Ammonium Bromide) method (Murray and Thompson, 1988). For DNA isolation, 100 mg of fresh seedling leaves as well as fungal growth mycelia of different isolates were homogenized in chilled mortar and pestle using liquid nitrogen. 700 µl of 2X CTAB extraction buffer were added and homogenized as well. The samples were transferred to Eppendorf tubes and incubated at 65°C for 30-60 min with occasional gentile swirling. 700 µl of chloroform isoamyle alcohol (24:1) were added and mixed by inverting the tube several times. The sample was centrifuged at 15000 rpm for 15 min at 4°C. The aqueous was transferred to a new centrifuge tube with a wide bore tips to avoid DNA shearing. 0.6 volume of chilled isopropanol was added and followed by quick and gentle inversion and incubated at -20°C for 30 min. DNA pellet was precipitated at 10000 rpm for 10 min at 4°C. Pellet was washed three times with 70% ethanol, well dried and dissolved in 100 µl TE. DNA was quantified using gel quantification method in which the samples were loaded on 0.8% agarose gel in 0.5X TAE running buffer and using known concentrations of λ uncut genomic DNA as standard. After some cycles of dilutions, the concentration of DNA was approximately adjusted to 15 ng/ µl which is suitable for PCR reaction. PCR conditions were: initial denaturation at 94°C for 3 min, 45 cycles of amplification under the following parameters: template denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min and extension at 72°C for 2.30 min by the end of the 45th cycle, final extension at

Table 1. Infection percent of Giza 178 rice cv. and Hybrid 1 by forty six isolates of *T. barclayana* under greenhouse conditions.

Governorate	Infection (%)							
	0-20		21-40		41-60		Total	
	Giza178	H 1	Giza178	H 1	Giza178	H 1	Giza178	H 1
Kafr El-heihk	10*	10	4	4	-	-	14	14
Beheira	-	-	1	1	-	-	1	1
Sharkia	-	-	1	1	-	-	1	1
Gharbia	3	6	4	1	-	-	7	7
Dakahlia	9	11	7	6	1	-	17	17
Damietta	3	4	3	2	-	-	6	6
Total	26	32	19	14	1	-	46	46

*= Number of isolates

72°C for 7 min followed by storage at 4°C.

Electrophoresis, staining and analysis

DNA amplified fragments were loaded in 1.2% agarose gel containing ethidium promide (2 µl/100 ml). The 0.5X TAE was used as a running buffer and 50 and 100 bp DNA ladders (0.5 µg/µl by fermentas) as molecular weight markers. Electrophoresis was conducted at 70 V for 3 h. Then, gels were photographed and analyzed using BioDoc Analysis software (Biometra, Germany).

Phylogenetic tree construction

The presence/absence of matrix for amplified DNA fragments of the four ISJ markers was used to study the phylogenic relationships among the studied genotypes. The statistical software NTSYS pc2.0 (Rohlf, 2000) was used to estimate the genetic relationships among the tested genotypes. Employing the computer package NTSYS pc2.0, Nei and Lei's similarity coefficients (Nei and Lei, 1979) were calculated and used to establish genetic relationships among the genotypes based on un-weighed pair group method of arithmetic means (UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA). The completely randomized design was applied in laboratory and greenhouse experiments. Completely randomized block and split-plot designs were adopted according to Gomez and Gomez (1984). The treatment means were compared using the least significant difference (LSD) at 5%.

RESULTS

Isolation, identification and pathogenicity test of *T. barclayana*

Forty six fungal isolates were isolated from rice kernels showing typical symptoms of rice kernel smut collected from all the surveyed governorates. However, seventeen isolates was obtained from Dakahlia gov. (Table 1). Identification of these isolates were performed at Plant Pathology Laboratory, Rice Research & Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt. These isolates were identified according to their morphological characteristics as *T. barclayana* (Bref.) Sacc. and P. Syd., 1899. Pathogenicity test was carried out using Giza 178 rice cultivar and Hybrid 1 (the most susceptible cultivars). Data presented in Tables 1 and 2 indicated that the tested isolates varied in their virulence. All isolates were divided into three categories of infection percent, that are, 0.0 - 20, 21 – 40 and 41 – 60%. However, isolates numbers 40, 20, 13, 24 and 35 isolated from Damietta, Gharbia, Kafr El-Sheikh and Dakahlia governorates, respectively, proved to be the most aggressive isolates on Giza 178 rice cv. and Hybrid 1. At the same time isolate no. 31 isolated from Dakahlia gov. was the least virulent one which recorded the least disease severity among all the tested isolates.

Evaluation of certain rice cultivars and lines towards infection with *T. barclayana*

An aggressive isolate of *T. barclayana* (isolate no. 35, isolated from Giza 178 cv. in Dakhlia gov.) was used for evaluating the susceptibility of eighteen commercial rice cvs. and Hybrids. Results indicated that all the tested rice cultivars varied in their susceptibility toward the infection with the tested isolate (Table 3). However, Giza 178 was the most susceptible rice cultivar followed by Hybrid 1 since they were severely infected. On the other hand, Sakha 101 and Sakha 105 were the least susceptible cultivars. Concerning the tested lines, disease severity of GZ 6903-1-2-2-1 and GZ 8566-6-1-1-4-1 ranged from 0.028 to 1.71, respectively (Table 4). However, it is clear from data presented in Tables 3 and 4 that number and germinated spores increased with increasing the disease severity.

Enzyme activities and total protein assay

Data presented in Tables 5, 6 and 7 showed gradual

Table 2. Pathogenicity test of isolates of T. barclayana, isolated from rice cultivars grown in different governorates using Giza 178 cv. and Hybrid rice 1 under greenhouse conditions.

Number of isolate	Governorate	Source of isolate	Infection (%)		Disease severity	
			Giza178	H 1	Giza178	H 1
1	Kafr EL-Sheikh	Giza178	14.6	13.7	4.00	2.00
2		Giza178	15.0	11.4	1.36	1.30
3		Giza177	19.7	10.7	4.50	4.10
4		Sakha 104	11.3	8.00	6.64	4.25
5		Hybrid 1	12.0	12.0	4.88	2.43
6		BL1	19.0	9.00	3.30	0.64
7		Hybrid 1	30.7	20.6	2.30	1.81
8		Sakha 104	19.4	19.3	2.00	1.77
9		Hybrid 2	16.7	16.6	4.40	1.20
10		Gz1368-55-4	24.4	24.3	3.50	3.33
11		Sakha 101	8.00	7.20	2.88	1.10
12		Sakha 105	22.7	22.6	2.65	2.3
13		Giza 181	28.0	27.4	6.70	5.67
14		Egyptian Yasmine	17.6	15.7	2.60	1.32
15	Gharbia	Giza177	20.7	20.0	0.52	0.56
16		Hybrid 3	22.4	15.0	3.33	1.04
17		Hybrid 1	23.0	22.0	4.00	0.65
18		Giza 178	19.7	19.6	3.90	1.89
19		Hybrid 4	28.0	18.0	3.20	1.69
20		Giza177	18.7	18.6	6.13	5.92
21		Giza177	18.7	8.70	3.43	0.86
22	Dakahlia	Giza 178	16.7	6.70	3.20	0.34
23		Giza 177	17.7	11.0	3.30	0.53
24		Giza 178	31.0	32.7	8.10	7.86
25		Giza 178	22.6	9.00	3.82	1.64
26		Reiho	19.0	9.00	3.77	0.88
27		Giza 178	16.3	11.4	3.00	1.32
28		BL1	37.4	31.4	4.00	1.85
29		Giza 178	26.0	26.0	5.50	4.31
30		Hybrid 2	26.3	25.7	5.45	4.89
31		Gz7576-10-3-2-1	17.0	13.7	0.87	0.54
32		BL1	12.3	12.4	3.63	1.93
33		Sakha 102	14.0	13.4	5.00	2.60
34		BL1	12.0	11.7	3.26	0.50
35		Giza 178	41.0	36.7	9.66	8.70
36		Hybrid 2	32.4	32.4	4.42	2.20
37		Sakha 103	14.7	14.7	3.63	1.24
38		Giza 177	21.4	14.6	5.35	2.71
39	Damietta	Giza 178	19.0	19.0	3.63	1.25
40		Giza 178	37.0	27.0	6.50	6.25
41		Hybrid 2	29.0	15.3	3.90	1.78
42		Giza 177	18.0	11.4	4.27	2.55
43		Giza 178	26.4	26.3	4.01	2.03
44		Giza 177	11.0	10.3	3.00	1.93
45	Sharkia	Hybrid 1	26.6	26.70	3.27	1.20
46	Beheira	Giza 178	34.0	30.5	3.70	3.17
L.S.D. 5 %			7.34	6.80	0.74	0.85

Table 3. Evaluation of certain commercial rice cultivars and two rice hybrids for infection with isolate no. 35 of *T. barclayana* under greenhouse conditions.

Cultivar	Inoculated		After milling/ inoculated	
	Disease severity	Infection (%)	No. of spores/ gm	Germinated spores
Giza 159	0.43	24	2000	33
Giza 171	0.9	33.4	1600	50
Giza 175	1.38	61.5	1200	143
Giza 176	0.48	28.5	2000	177
Giza 177	0.09	4.67	2000	85
Giza 178	4	70	1000	394
Giza 181	0.35	19.2	2000	175
Giza 182	0.7	29	2000	94.33
Egyptian Yasmine	0.16	16	4000	101
Reiho	0.34	18	4000	100
Hybrid 1	1.22	38.1	8000	355
Hybrid 2	1.58	65	6000	335
Sakha 101	0.002	2.48	2000	85
Sakha 102	0.05	4.67	2000	95
Sakha 103	0.05	3	2000	115
Sakha 104	0.33	17.5	2000	90
Sakha 105	0.07	6	2000	225
Sakha 106	0.58	28.57	2000	100
BL 1	0.7	29	2000	121
Doular	0.4	22.8	4000	257
L.S.D. 5 %	0.273	9.96	3.55	15.8

Table 4. Evaluation of some rice lines for infection with isolate no. 35 of *T. barclayana* under greenhouse conditions.

Rice lines	Inoculation		After milling/inoculated	
	Disease severity	Infection (%)	No. of spores x10^3/g	Germinated spore
GZ 6903-1-2-2-1	0.040	0.40	2.00	53.0
GZ 8544-18-3-1-1-1	1.450	7.17	4.00	130.0
GZ 8566-6-1-1-4-1	1.717	7.17	2.00	154.0
GZ 7764-38-1-3-3	0.175	0.67	4.00	34.0
GZ 7769-2-1-1-2	0.028	0.28	6.00	100.0
L.S.D. 5%	0.123	0.96	1.34	11.57

increase in POX and PPO activities as well as total protein contents in both healthy and inoculated plants. However, the increment in the activity of POX and PPO and total protein levels were higher in inoculated plants than in healthy ones. Additionally, it was clear from our data that maximum increase in POX and PPO activities and total protein contents was recorded after 6 and 9 days from inoculation and then enzyme activities started to decrease. Also, results showed that POX, PPO and total protein levels in resistant cultivars (Sakh 105, Sakha 101 and Giza 177) and moderately resistant cultivars (Sakha 104 and Giza 182) were higher than those in highly susceptible cultivars (Giza 178 and Hybrid 2).

Relationship between the tested rice cultivars and isolates of *T. barclayana* using Intron-Exon Splice Junctions (ISJ) protocol

Results illustrated in Figure 1A, B, C and D showed that the phenogram constructed based on the molecular data of ISJ banding patterns was generated by using primers ISJ-5, ISJ-6, ISJ-7 and ISJ-9. The highest similarity percentage was observed in the most similar genotypes. The used primers detected reasonable levels of polymorphism among the tested genotypes. Hence, the overall similarity levels generated by these primers were 72 to 88%.

Table 5. Peroxidase activity in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*, the causal fungus of rice kernel smut disease.

Cultivar	POX activity/ minute (as optical density)									
	Healthy					inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.86	0.91	1.10	1.00	0.95	0.88	0.92	1.20	1.12	0.98
Giza 171	0.45	0.54	0.9	0.80	0.77	0.59	0.64	1.18	0.87	0.85
Giza 175	0.69	0.81	1.10	0.90	0.85	0.79	0.91	1.20	1.02	0.95
Giza 176	0.33	0.64	0.90	0.85	0.67	0.43	0.73	1.05	0.93	0.75
Giza 177	1.00	1.25	1.26	1.26	1.10	1.14	1.38	1.39	1.38	1.20
Giza 178	0.35	0.53	0.75	0.73	0.57	0.38	0.63	0.86	0.84	0.62
Giza 181	0.86	1.15	1.16	1.16	1.10	0.95	1.17	1.28	1.20	1.05
Giza 182	0.86	1.00	1.25	1.20	1.15	0.96	1.25	1.26	1.27	1.10
Egyptian Yasmine	0.74	0.87	1.26	1.13	1.11	0.85	0.98	1.28	1.23	1.15
Reiho	0.47	0.56	0.55	0.64	0.42	0.57	0.64	0.70	0.65	0.64
Hybrid 1	0.45	0.54	0.62	0.58	0.57	0.55	0.65	0.65	0.68	0.59
Hybrid 2	0.37	0.57	0.95	0.83	0.56	0.45	0.68	1.05	0.93	0.67
Sakha 101	0.76	0.98	1.20	1.13	1.05	0.76	0.99	1.28	1.22	1.17
Sakha 102	0.78	0.96	1.15	1.03	0.92	0.88	1.06	1.18	1.17	0.98
Sakha 103	0.75	0.85	1.15	1.10	0.95	0.78	0.88	1.20	1.13	0.98
Sakha 104	0.65	0.87	1.00	0.90	0.80	0.69	0.88	1.10	0.95	0.97
Sakha 105	0.90	1.27	1.30	1.28	1.01	0.98	1.32	1.31	1.45	1.08
Sakha 106	0.53	0.60	0.82	0.8	0.65	0.54	0.68	0.83	0.84	0.73
BL 1	0.44	0.55	0.61	0.66	0.46	0.53	0.63	0.71	0.67	0.59
Doular	0.54	0.67	0.62	0.68	0.56	0.58	0.69	0.64	0.69	0.58
L.S.D. 5%	0.13	0.14	0.15	0.12	0.17	0.14	0.13	0.20	0.42	0.22

Table 6. Polyphenoloxidase activity in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*.

Cultivar	PPO activity/ minute (as optical density)									
	Healthy					inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.27	0.28	0.3	0.29	0.27	0.53	0.54	0.55	0.54	0.50
Giza 171	0.44	0.44	0.47	0.46	0.44	0.58	0.63	0.57	0.56	0.56
Giza 175	0.4	0.42	0.5	0.42	0.45	0.59	0.6	0.64	0.6	0.52
Giza 176	0.18	0.22	0.21	0.21	0.2	0.23	0.27	0.29	0.28	0.24
Giza 177	0.56	0.59	0.62	0.61	0.58	0.61	0.72	0.83	0.81	0.75
Giza 178	0.24	0.25	0.24	0.24	0.23	0.26	0.29	0.36	0.28	0.25
Giza 181	0.25	0.25	0.32	0.27	0.26	0.25	0.27	0.28	0.26	0.25
Giza 182	0.22	0.22	0.24	0.26	0.22	0.39	0.49	0.49	0.48	0.47
Egyptian Yasmine	0.26	0.29	0.32	0.30	0.25	0.45	0.48	0.52	0.50	0.44
Reiho	0.25	0.40	0.50	0.40	0.40	0.35	0.41	0.59	0.45	0.49
Hybrid 1	0.43	0.43	0.59	0.43	0.33	0.56	0.56	0.68	0.63	0.46
Hybrid 2	0.43	0.46	0.45	0.41	0.41	0.58	0.59	0.60	0.59	0.58
Sakha 101	0.51	0.51	0.56	0.51	0.42	0.67	0.68	0.68	0.67	0.66
Sakha 102	0.42	0.43	0.43	0.42	0.42	0.48	0.49	0.52	0.51	0.43
Sakha 103	0.13	0.12	0.12	0.13	0.12	0.23	0.23	0.24	0.23	0.24
Sakha 104	0.45	0.45	0.49	0.44	0.42	0.49	0.49	0.52	0.51	0.45

Table 6. Contd

Sakha 105	0.69	0.72	0.74	0.69	0.68	0.79	0.80	0.81	0.79	0.78
Sakha 106	0.41	0.42	0.43	0.43	0.41	0.52	0.54	0.55	0.53	0.53
BL 1	0.17	0.18	0.19	0.18	0.14	0.29	0.31	0.35	0.34	0.32
Doular	0.24	0.25	0.26	0.25	0.24	0.26	0.28	0.31	0.27	0.24
L.S.D. 5%	0.13	0.1	0.12	0.12	0.08	0.16	0.17	0.16	0.25	0.16

Table 7. Total protein contents in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*, the causal fungus of rice kernel smut disease.

Cultivar	Total protein (mg/g fresh weight)									
	Healthy					Inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.44	0.5	0.75	0.68	0.35	0.45	0.53	0.78	0.75	0.73
Giza 171	0.4	0.45	0.53	0.4	0.38	0.43	0.52	0.55	0.52	0.50
Giza 175	0.77	0.85	0.87	0.85	0.75	0.79	0.87	0.9	0.86	0.77
Giza 176	0.46	0.58	0.67	0.55	0.45	0.49	0.63	0.77	0.58	0.47
Giza 177	0.88	0.93	0.95	0.84	0.83	0.91	0.96	0.98	0.9	0.85
Giza 178	0.52	0.55	0.57	0.49	0.35	0.57	0.59	0.59	0.54	0.46
Giza 181	0.42	0.48	0.55	0.45	0.4	0.44	0.55	0.59	0.47	0.42
Giza 182	0.34	0.45	0.45	0.41	0.4	0.43	0.5	0.5	0.45	0.43
Egyptian Yasmine	0.41	0.46	0.47	0.42	0.32	0.49	0.47	0.49	0.49	0.36
Reiho	0.42	0.53	0.55	0.47	0.47	0.45	0.55	0.6	0.52	0.45
Hybrid 1	0.32	0.41	0.5	0.40	0.37	0.33	0.43	0.52	0.46	0.40
Hybrid 2	0.52	0.56	0.51	0.50	0.48	0.54	0.6	0.61	0.53	0.51
Sakha 101	0.89	0.98	1.17	1.20	0.95	0.9	1.02	1.21	1.21	0.99
Sakha 102	0.85	1.00	0.97	0.85	0.81	0.91	1.10	1.20	1.02	0.90
Sakha 103	0.94	1.10	1.10	0.94	0.85	0.98	1.12	1.17	0.95	0.88
Sakha 104	0.73	0.87	0.90	0.83	0.76	0.75	0.91	0.94	0.88	0.83
Sakha 105	1.01	1.17	1.19	1.03	0.93	1.15	1.23	1.24	1.14	0.97
Sakha 106	0.96	1.02	1.08	1.12	0.98	0.98	1.05	1.12	1.17	1.07
BL 1	0.25	0.35	0.42	0.50	0.17	0.27	0.36	0.46	0.53	0.42
Doular	0.79	0.93	0.93	0.85	0.78	0.87	0.94	0.96	0.85	0.82
L.S.D. 5%	0.1	0.17	0.12	0.14	0.15	0.10	0.28	0.39	0.2	0.16

Cluster analyses of six cultivars based on molecular data using the tested four primers resulted in two main groups (Figure 2). The first one included both Sakha 105 and Sakha 101 as moderately resistant cultivars, while the second one included four rice cultivars, namely, Hybrid 1, Giza 178, Giza 181 and Egyptian Yasmine as highly and moderately susceptible to rice kernel smut disease. However, the second group could be divided into another two subgroups according to similarity percentages. The first subgroup could be divided into another two sub-subgroups; namely, Giza 178 and Hybrid 1 as highly susceptible, and the second subgroup was Giza 181 as moderately susceptible, while the second one included Egyptian yasmine cv. as moderately susceptible.

Similarly, ISJ molecular marker technique was used to determine the relationship between 13 isolates differing in their virulence. The aforementioned four primers, namely, ISJ-5, ISJ-6, ISJ-7 and ISJ-9 were used in this study. It is clear from Figure 3A, B, C and D that the isolates were classified into three distinct groups. The first group included the highly virulent isolates no. 35 (4) and 24 (6) isolated from Dakahlia gov. The second group included the moderately virulent isolates no. 45 (2), 7 (9), 46 (3), 29 (5), 3 (8), 40 (10), 20 (13) and 13 (11) isolated from Sharkia, Kafr El- Sheikh, Behira, Dakahlia, kafr El-Seikh,

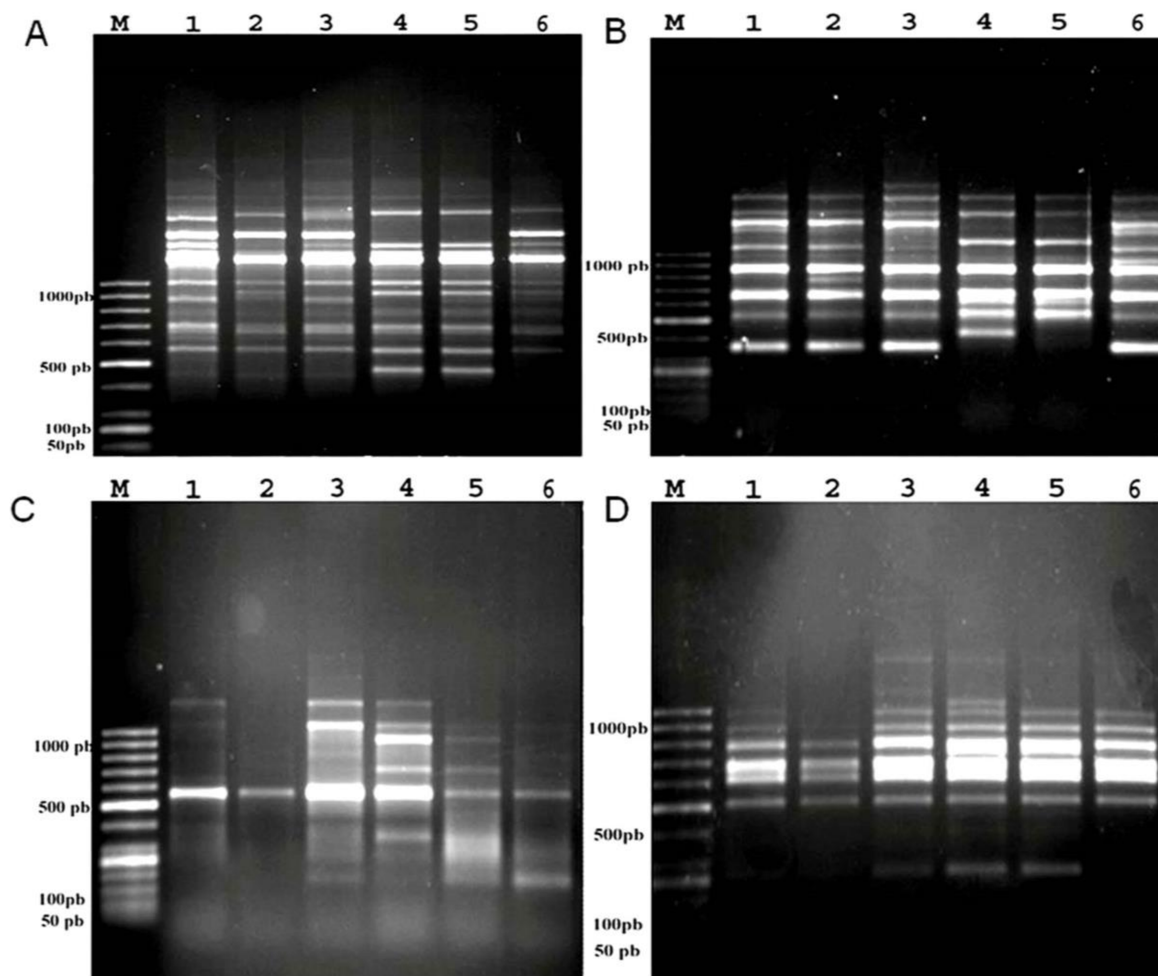

Figure 1. ISJ banding patterns of rice genotypes obtained by the primer, ISJ-5(A), ISJ-6(B), ISJ-7(C) and ISJ-9(D) electrophoresed gel. Lanes from left to right are Giza 178 (1), Egyptian Yasmine (2), Hybrid 1 (3), Sakha 105 (4), Sakha 101 (5) and Giza 181 (6). M = Molecular marker.

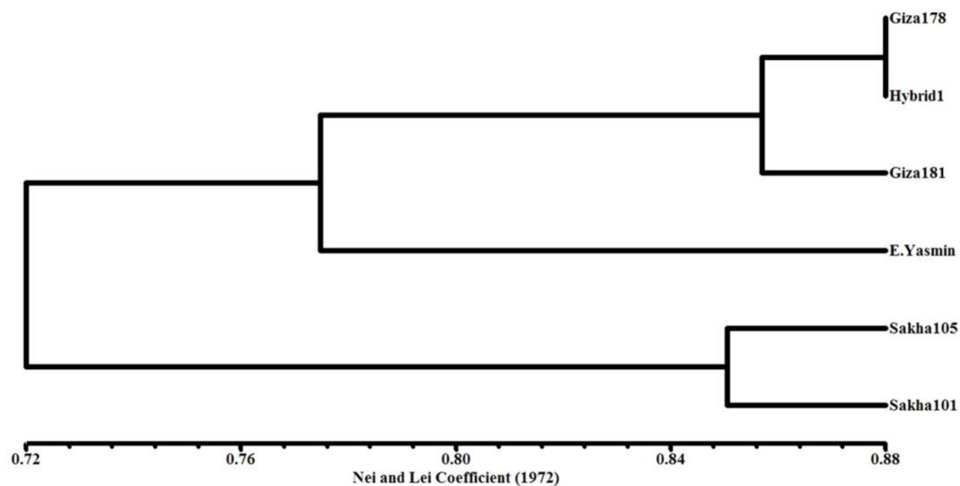

Figure 2. Phenogram based on molecular data of ISJ banding patterns of rice genotypes obtained with the four primers, ISJ-5, ISJ-6, ISJ-7 and ISJ-9.

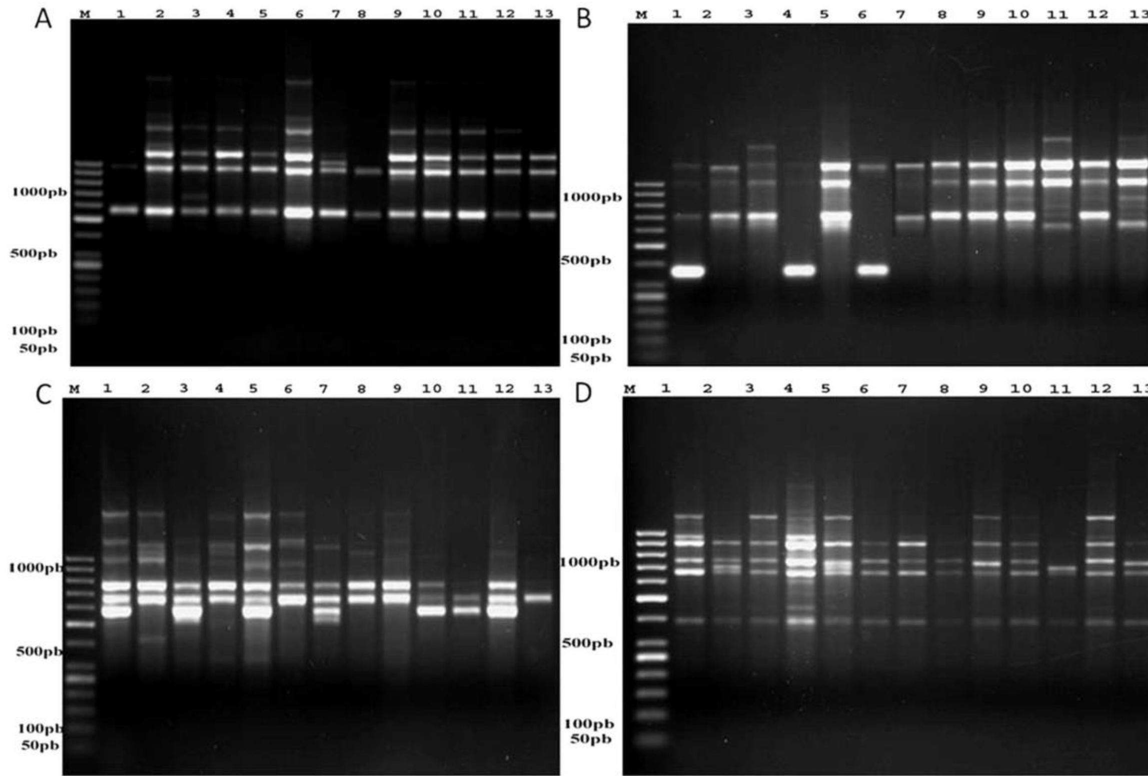

Figure 3. ISJ banding patterns of tested rice kernel smut isolates of *T. barclayana* obtained with the primer, ISJ-5(A), ISJ-6(B), ISJ-7(C) and ISJ-9(D) electrophoresed gel. Lanes from left to right are numbers of isolates 1 to 13. M = Molecular marker.

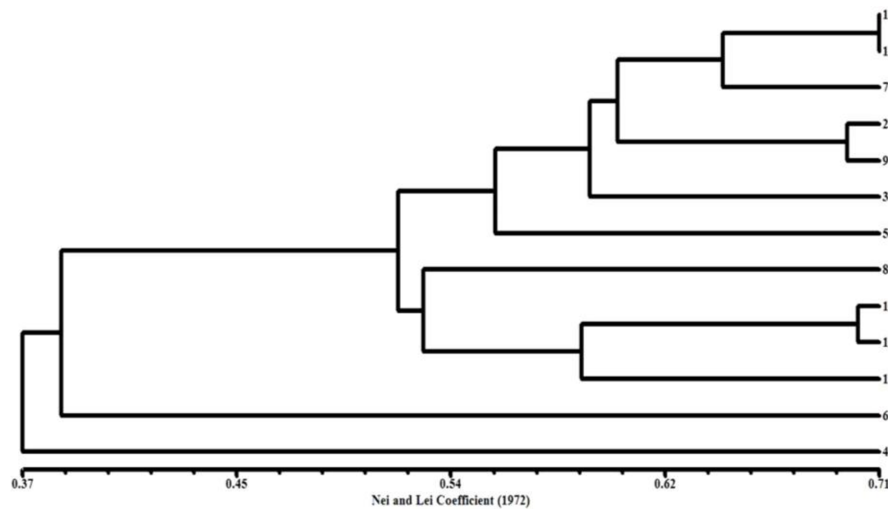

Figure 4. Phenogram based on molecular data of ISJ banding patterns of rice kernel smut isolates of *T. barclayana* obtained with the four primers, ISJ-5, ISJ-6, ISJ-7 and ISJ-9.

Damietta, Gharbia and Kafr El-Sheikh govs., respectively. The third group included the least virulent isolates no. 15 (1), 31 (12) and 2 (7) isolated from Gharbia, Dakahlia and Kafr El-Sheikh, respectively. In the present study, the isolates were screened inside the greenhouse under optimum conditions for disease development.

Clustering the thirteen isolates based on similarity of DNA fingerprint by using the aforementioned four primers resulted in two main groups (Figure 4). The first one included one isolate no. 35 (4) as highly virulent, while the

second one included twelve rice kernel smut isolates. However, the second group divided into other subgroups according to similarity percentages.

DISCUSSION

Rice kernel smut, caused by *T. barclayana*, causes a partial bunt that affects both yield and quality. Forty six fungal isolates of this fungus were isolated from diseased rice plants showing clear symptoms of black smut, purified and identified as *T. barclayana*. This fungus was previously reported to be the causal agent of rice kernel smut disease (Takahashi, 1896; Biswas, 2003). Pathogenecity tests revealed that these isolates were pathogenic to kernel rice with various degrees. Additionally, the evaluated rice cultivars and lines showed different degrees of susceptibility to *T. barclayana*. These findings were in agreement with the previous studies (Murty and Singh, 1982; Muthusamy and Ahmed, 1997). Since they reported that there were variations between rice cultivars in relation to infection with *T. barclayana*.

In the present study, an increment in the defense-related enzymes, that are, POX and PPO and total protein contents was observed in rice cultivars as a result of inoculation with *T. barclayana*. Among induced defense responses, production and accumulation of pathogensis related proteins are very important (Van Loon et al., 2006; Elsharkawy et al., 2012a, b; Elsharkawy et al., 2013; Hassan et al., 2014). Attempts have been made to exploit these anti-fungal proteins to develop disease resistant transgenic crop plants (Lin et al., 1995; Tabei et al., 1997). Peroxidases are involved in a broad range of physiological processes throughout the plant life cycle, probably due to the high number of enzymatic isoforms (isoenzymes) and to the versatility of their enzyme-catalyzed reactions (Passardi et al., 2005). Thus, plant peroxidases are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis and the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Gabr, 2010). Additionally, our results showed that total protein levels in resistant cultivars were higher than those in highly susceptible cultivars. Onifade and Agboola (2003) postulated that proliferation of microorganism synthesizes several enzyme proteins that cause rearrangement of nutritional composition of substrate due to formation of several degrading products thereby increasing its protein content.

Molecular markers technology provides novel tools for DNA fingerprinting of plant cultivars. Semi- random PCR primers targeting intron-exon splice junctions (ISJ) were used in this study to analyze the rice genome with the aim of evaluating potential of these markers for identification and classification of rice cultivars and also to study the relationship between isolates of *T. barclayana.* Results showed that ISJ technique can determine the relationship between studied rice cultivars and lines upon susceptibility to *T. barclayana*. Similarly, it differentiated the virulence between the isolated *T. barclayana* isolates upon virulence on studied rice cultivars and lines. Many authors used this technique and other techniques in this respect since they reported that conventional character-rization of cultivars based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics and influenced by environmental conditions. In contrast, DNA-based markers are highly heritable, available in high numbers, and exhibit adequate polymorphism, hence they can be used to discriminate closely related genotypes of a plant as well as microbes isolates (Yashitola et al., 1997; Gawel et al., 2002; El-Malky, 2004; El-Wahsh and Ammar, 2007).

In conclusion, our results indicate that Giza 178 rice cultivar and Hybrid 1 were highly susceptible to rice kernel smut disease. The isolate no. 35 from the pathogenic fungus (*T. barclayana*) was the most virulent isolate. Additionally, increased levels of defense-related enzymes and total protein were observed in rice cultivars after inoculation with *T. barclayana*. ISJ protocol is very useful technique to investigate the susceptibility of rice cultivars and also to differentiate virulence among isolates of *T. barclayana*.

Conflict of Interests

The authors have not declared any conflict of interests.

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