RESEARCH PAPERS

Etiology of stipe necrosis of cultivated mushrooms (*Agaricus bisporus*) in Egypt

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Summary. Internal stipe necrosis of cultivated button mushrooms (*Agaricus bisporus*) is caused by the bacterium *Ewingella americana* (Enterobacteriaceae), which is part of the endogenous bacterial population in mushroom sporocarp tissues. Isolation of the causal agent of stipe necrosis led to the recovery of three bacterial morphotypes. *Ewingella americana* was isolated from 90% of mushroom samples showing mild stipe browning, while *Pseudomonas fluorescens* and *P. tolaasii* were also isolated. Inoculation with *E. americana* into button mushroom sporocarps yielded typical browning symptoms which were distinguishable from those of the bacterial soft rot. This bacterium was re-isolated and its identification was verified, thus fulfilling Koch's postulates. However, inoculations with *P. fluorescens* and *P. tolaasii* caused no stipe browning. The strain identities were verified by biochemical identification and through analysis of their 16S rRNA gene sequences. This study has outlined the etiology of stipe necrosis of cultivated button mushroom in Egypt, and is the first report of *E. americana* in this country.

Key words: stipe browning, bacterial diseases, pathogenicity, phylogenetic relationship.

Introduction

Button mushroom (*Agaricus bisporus*) is the most widely cultivated and consumed mushroom throughout the world, and includes about 40% of total world mushroom production (Giri and Prasad, 2007). Vízhányó and Felföldi (2000) stated that mushrooms have short postharvest shelf life and are highly susceptible to microbial attack and to discoloration during storage. Their high water content facilitates microbial spoilage, and their high tyrosinase content leads to the enzymatic browning (Brennan *et al.*, 2000). Inglis *et al.* (1996) showed that internal stipe necrosis (ISN) has been recognized as an emerging problem within the mushroom industry. According to Munsch *et al.* (2002) most studies of this disease have been focused on *Pseudomonas* spp. As the cause, since they are the predominant bacte-

rial group and some are pathogenic and/or cause superficial spoilage of their hosts e.g. *Pseudomonas tolaasii*, *P. reactans*, *P. gingeri*, *P. costantinii* and *P. agaric* (Felsenstein, 1993). However, Richardson (1993) confirmed that Koch's postulates could not be fulfilled for *P. fluorescens* and did not initiate ISN under farm conditions. *E. americana* is a bacterium belonging to the Enterobacteriaceae, which has been previously associated with stipe browning of *A. bisporus* (Roy Chowdhury *et al.*, 2007). Prevalence of *E. americana* and its chitinolytic activity helped in establishment of ISN symptoms (Inglis *et al.*, 1996). The ecological niche or natural source of *E. americana* is unknown, but it has also been isolated from animals. Inglis and Peberdy, (1996) isolated this enterobacterium from 93% of cultivated mushrooms (*A. bisporus*) with 'internal stipe necrosis'. Symptoms of this disease appear as browning in the center of mushroom stipes, which may extend as slight browning of internal cap tissues. As the mushrooms mature, affected tissues may collapse, in some instances leaving only brown

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pegs of dry tissue adhering to the inside of the caps. The disease caused by this bacterial pathogen was detected in the United Kingdom (Inglis and Peberdy, 1996) and later in New Zealand (Braithwaite *et al.*, 2005) and Korea (Chan-Jung *et al.*, 2009). Several authors have studied the prevalence or the association of *E. americana* in healthy mushrooms in order to clarify its ecology and importance as a mycopathogenic agent (Reyes *et al.*, 2004).

The aims of the present study are to report the occurrence of ISN of *A. bisporus* in mushroom farms in Egypt; to isolate the bacterial pathogen from cultivated button mushrooms showing symptoms of the disease; to confirm the isolate pathogenicity; to report the phenotypic characteristics of this isolate and to phylogenetically relate it to pathogens associated with *A. bisporous* in Egypt.

Material and methods

Samples

Forty samples of cultivated mushrooms (*A. bisporus*) showing stipe browning symptoms were selected and then purchased from mushroom farms and retail stores in Cairo (Egypt) over one year of study from May 2011. The samples were refrigerated and packaged in polystyrene trays covered with plastic film. All the samples were transported immediately at 7ºC, examined and analyzed.

Isolation of bacteria from diseased mushroom tissues

Using methods of Chan-Jung *et al*. (2009), approximately 1 g internal tissues of infected mushroom stipe was crushed in sterilized 1.5 mL Eppendorf tubes containing 1 mL of sterile distilled water. The crushed mushroom suspension was diluted in series, plated onto King's B agar (King *et al.*, 1954) and then incubated at 28ºC for 3 d. Bacterial colonies were then maintained at -70ºC in Luria-Bertani medium (LB) (Bertani, 1952). The percent incidence of bacteria was estimated as the number of mushroom samples (out of 40) in which the bacteria were observed.

Biochemical identification of bacterial isolates

Colony morphology was assessed on King's B agar. Gram staining, oxidase activity, nitrate reduction and indole production were also tested.

Molecular identification of the isolates

Molecular identification of the isolates was carried out through sequencing of their 16S rRNA gene using PCR gene amplification. DNA was extracted from 24 h cultures using Gen Elute TM Bacterial Genomic DNA kit from Sigma as in manufacture instructions. PCR reaction mixes were made up to 50 μL with 100 ng DNA, 30 nM each primer, 25 μL of Dreem Taq master mix from Fermentas as in manufacture instructions. Primers for amplification of partial 16S rDNA of 500 bp. including the V3 region were used. The forward primer was F1 5-'AGA GTTTGATCCTGGCTCAG 3' and the reverse primer was 517 R 5'- GTATTACCGC-GGCTGCTGGC 3' (Furushita *et al.*, 2003).

Thermocycling was performed on an Applied Biosystem thermocycler 2720. The cycling protocol was as follows: 5 min denaturing step followed by 30 cycles of 1 min at 95°C, 45 sec at 55°C and 45 sec at 72°C, followed by a final extension step for 7 min at 72°C. PCR products were run on 1% agarose gels.

DNA sequencing and phylogenetic analysis

At least three independent PCR reaction products were used for sequencing. Approximately 100 ng of PCR products were used for the DNA sequencing reactions. These were carried out by cycle sequencing with an Applied Biosystems Sequencer in the Animal Health Research Institute, Agriculture Research Center, Giza, Egypt. Nucleotide sequences were analysed in BLAST (Altschul *et al.*, 1997) (http://www.ncbi.nlm.nih.gov/blastn) and aligned in ClustalW (Thompson *et al.*, 1994) (http://www.ebi.ac.uk/clustalW). Sequences were copied into the BioEdit version 5.0.6 (Hall, 1999), which generates a consensus sequence of the gene. Phylogenetic analysis was performed in PHYLIP (Felsenstein, 1993). A neighbour-joining tree was performed. Confidence of the grouping was calculated by running the data in SEQBOOT, analyzing the data set 100 times in each of the previous programs. A consensus tree was constructed with the bootstrap values written on the nodes. The significant values of bootstraps were presented on the distance matrix based tree. Trees were viewed in Tree View version 1.5.2 (Page, 1996).

Pathogenicity test

Pathogenicity to the host was performed by injecting 1 mL of a buffer-washed bacterium whose

concentration was adjusted to 8×10^6 cfu mL^{-1}, directly into the stipes of white hybrid button mushrooms freshly harvested from mushroom farms (eight mushrooms for each bacterial isolate), using a sterile needle at each inoculation point (Inglis *et al.*, 1996). After inoculation, the mushrooms were incubated at ambient temperature (21–23°C), and then examined for symptom development (brown stipe lesions) after 24, 48 and 72 h incubation. Bacteria were then isolated from the eight inoculated mushrooms (as above), biochemical and molecular identification was repeated. The control treatments consisted of a batch of button mushrooms (eight sporocarps) injected with phosphate buffer solution. The experiment was repeated twice.

Results

Isolation and identification of the stipe necrosis causal agent

Isolation of the causal agent of the bacterial stipe necrosis from infected button mushroom samples led to the recovery of only three bacterial colony morphotypes in the isolation plates after 2 days of incubation on King's B agar at 30ºC. One isolate of each morphotype was identified later biochemically and by sequencing of their 16S rRNA. The most common colony type was recovered from about 36 of 40 (90%) diseased mushroom samples. These colonies were round; brown; non-fluorescent and 1.5~2.5mm in diameter. This isolate was a Gram negative rod, was oxidase negative, nitrate reducing and did not produce indole. The second colony type was fast growing, and the colonies were rounded; yellowish white in colour showing bright green fluorescence under ultraviolet light. The third colony type had colonies that were circular, domed and yellowish white in color with a creamy consistency and produced yellowish-green pigment. PCR amplification and sequencing of the resultant 16S rRNA was compared with the 16S rRNA GenBank. The isolate of the first colony type (designated Mush 10) showed 99% sequence similarity with *E. americana*. Sequencing of 16S rRNA of isolates of the other two colony types (Mush 48 and Mush 17) respectively showed high similarity of 98% and 100% with *P. fluorescens* and *P. tolaasii.* A neighbour joining tree was constructed using 500 bp sequences of 16S rRNA gene recovered from the isolates and compared with a selection of sequences already in GenBank (Figure 2). The isolate Mush 10 was grouped with *E. americana* strains with 100% bootstrap value, confirming the identification. The *Pseudomonas* isolates were divided into two groups; the first included Mush 17 with *P. tolaasii* while the second isolate Mush 48 was grouped with *P. fluorescens*. These groups were separated with high bootstrap values. The 16S rRNA gene sequences were deposited in the GenBank database and the accession numbers of the three isolates are KC432609 (Mush 10), KC432610 (Mush 17) and KC432611 (Mush 48).

Pathogenicity test

The isolate identified as *E. americana* inoculated into stipe tissue of button mushroom at a concentration of 8×10^6 cfu mL^{-1} did not cause symptoms of stipe browning or necrosis after 24 h incubation (Figure 1a), whereas a clear internal browning of the stipe tissues was observed after 48 h incubation (Figure 1b). More pronounced browning disorders and necrosis along the length of the inoculation point (with black small masses of necrotic cells) were obtained after incubation for 72 h (Figure 1c), compared with the buffer-inoculated control samples (Figure 1d). The eight mushrooms and the two replications of the experiment exhibited the same patterns of disease development. Following these successful inoculations, *E. americana* was re-isolated from all inoculated mushrooms, and its identification was confirmed biochemically and with 16S rRNA gene sequencing in all the cases (verifying Koch's postulates). This confirms that the isolated bacterium (*E. americana*) has mycopathogenic potential. On the other hand, both *P. fluorescens* or *P. tolaasii* failed to cause browning of the stipes into which they were inoculated (Figure 1e and f).

Discussion

Little is known about the prevalence of Enterobacteriaceae in cultivated mushrooms. Most studies have focused on pseudomonads since some are either pathogenic agents or may promote the induction and growth of fruit bodies (Cho *et al.*, 2003). The present study represents the first report of stipe necrosis of cultivated mushroom in Egypt. From 90% of diseased mushrooms, *E. americana* was prevalently isolated, and this bacterium re-

Figure 1. *Agaricus bisporus* stipe inoculated with *Ewingella americana* bacterium at a concentration of 8×10^6 cfu ml^{-1}, (a): after 24h incubation at 23°C; showing no apparent symptoms of stipe brown reaction or necrosis, (b): after 48h incubation; showing a clear internal browning of the stipe tissue, (c): after 72h incubation; showing more pronounced browning disorders and necrosis along the length of the inoculation point (with black small masses of necrotic cells), (d): control stipe inoculated with sterile buffer. *Agaricus bisporus* stipe inoculated with, (e): *Pseudomonas fluorescens*, (f): *Pseudomonas tolaasii* bacteria at a concentration of 8×10^6 cfu ml^{-1}, showing no apparent browning symptoms of the stipe along the inoculation point after 72h incubation at 23°C.
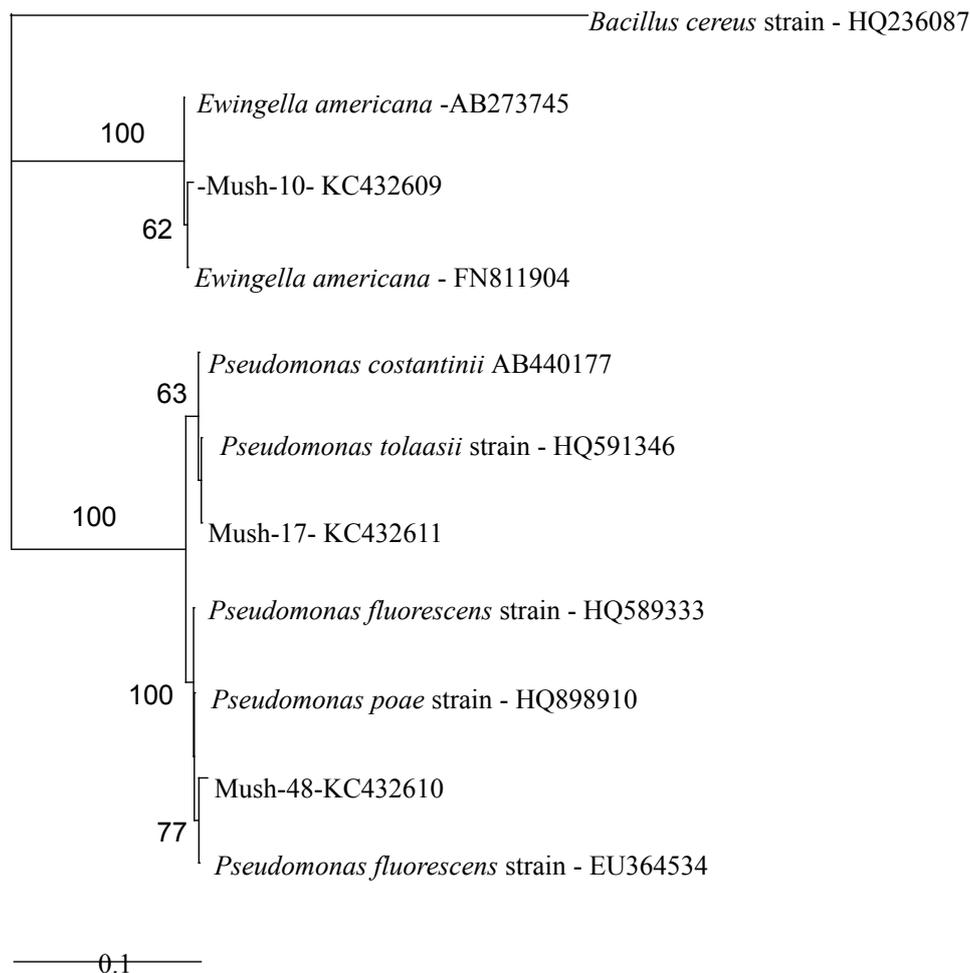
Figure 2. Neighbour joining tree comparing16S rRNA gene sequences of the three morphotypes isolated from diseased stipes of *A. bisporus* and sequences selected in Genbank. Where, Mush 10: *Ewingella americana*; Mush 17: *Pseudomonas tolaasii*; Mush 48: *Pseudomanas fluorescens*.

produced the disease symptoms in pathogenicity assays. On the contrary, *P. fluorescens* and *P. tolaasii* isolated from diseased mushrooms did not cause stipe necrosis symptoms. Previous studies have shown that both organisms caused browning of the caps (Abouzeid, 2012). Similarly, Inglis and Peberdy (1996) and Roy Chowdhury *et al.*, (2007) have reported the isolation of *E. americana* in cultivated mushrooms, specifically in button mushroom (*A. bisporus*) with ISN.

From the scientific literature, little information is available on the isolation of *E. americana*, its occurrence or its pathogenic potential in other species of cultivated mushrooms. According to the pathoge-

nicity test described here, the browning and ISN of *A. bisporus* occurred after 72 h incubation, confirming the findings of Inglis *et al*. (1996). These lesions have been attributed to the chitinolytic activity of *E. americana* in the stipes where the chitin of the hyphae is diffuse and not well crystallized, making it more available to enzyme action (Mol and Wessels, 1990; Inglis and Peberdy, 1997; Inglis *et al.*, 2000). According to Reyes *et al*. (2004) the Enterobacteriaceae family is not of great quantitative importance in the microbiology of cultivated mushrooms; nevertheless, the species of *E. americana* appears to be part of the normal flora of cultivated mushrooms, due to its frequency of occurrence.

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