Postharvest rot and mummification of strawberry fruits caused by *Neofusicoccum parvum* and *N. kwambonambiense* in Brazil

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**ABSTRACT**

In addition to the rots that are commonly found on strawberries, a new disease was found in 7% of stored fruits during a survey of strawberry diseases at the postharvest stage. Koch’s postulates were satisfied, and the fungi were identified as *Neofusicoccum kwambonambiense* and *N. parvum* based on morphology and phylogenetic analysis of the internal transcribed spacers, β-tubulin, RNA polymerase subunit II and transcription elongation factor 1-α regions. This is the first report of *Neofusicoccum kwambonambiense* in Brazil and the first report of *Neofusicoccum* spp. causing mummification and postharvest rot of strawberry.

**Key words:** Fragaria *x* ananassa, Botryosphaeriales, Dothideomycetes, postharvest pathology, stored fruit, tropical fungi.

A major problem in the strawberry production chain is the occurrence of fruit rot caused by fungi, especially at the postharvest stage. In Brazil, the main fungal species that cause postharvest rot in strawberry are *Botrytis cinerea* Pers., *Rhizopus stolonifer* (Ehrenb.) Vuill., *Pilidium concavum* (Desm.) Höhn., *Geotrichum candidum* Link. and *Colletotrichum* spp. Others fungi associated to rotting of strawberry fruit at postharvest stage are *Rhizoctonia solani* Kühn, *Phytophthora* spp., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Pestalotia longisetula* Guba., *Gnomonia comari* Karst. and *Alternaria* spp. (Costa et al., 2003; Tanaka et al., 2005; Lopes, 2011).

The occurrence of diseases in strawberries varies according to the region, climate conditions and crop management (Maas, 1998). Moreover, when the symptoms are similar to those caused by other pathogens, diagnostic errors are common. In such cases, the damage caused by certain pathogens may be ignored or overestimated depending on the situation (Lopes et al., 2010; Lopes, 2011). Therefore, the knowledge regarding the epidemiology and management of the disease depends on its correct etiologic identification such that postharvest damage to strawberry fruits can be reduced.

From March 2009 to February 2010, a survey of diseases in strawberry fruits was performed in the mountainous region of the state of Espírito Santo, Brazil. Fruits from different commercial plantations were collected and stored at 25°C and 100% relative humidity for seven days. In addition to the rots that are commonly found on strawberries in postharvest, a new fruit rot was verified in 7% of 3,500 stored fruits. Because the symptoms of this postharvest rot have not been previously reported in strawberries, the aim of this research was to determine the etiology of this disease in Fragaria *x* ananassa based on morphological and molecular approaches.

The initial symptoms of rot were observed from the first to the fourth day of storage. Initially, the grayish-white mycelium grew, covering the entire fruit, which gradually became gray to black (Figure 1A, B). The fruits became mummified one week after the initial observation. The formation of droplets due to leakage of cellular liquid was commonly observed on the surface of affected fruits (Figure 1C). However, sporulation not was observed on the surface of infected fruits.

The fungi were isolated on potato dextrose agar (PDA) by removing the tip of the hyphae from infected fruits. Three isolates were transferred to 2% water agar medium containing sterilized corn straw to induce sporulation. Single spore isolates were obtained from the samples, and derived cultures were deposited at the “Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes”, Universidade Federal Rural de Pernambuco (UFRPE), Brazil (codes CMM 1842, CMM 1845 and CMM 1846). When the fungi were grown at 25°C in petri dishes (9 cm in diameter) containing PDA medium, colonies of the CMM1842 covered the medium in the plates in three days whereas it took four days for CMM1845 and CMM 1846 to reach the plate borders. Although CMM 1842 exhibited a higher growth rate, both isolates exhibited maximum growth between 25 and 30°C. Fungal structures were scraped, mounted in drops of lactophenol on microscope slides and examined under a light microscope. Biometric
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data were based on 30 measurements of various structures. The hyaline conidia were ellipsoidal to fusiform and aseptate with thin, smooth walls, measuring 7.5-20 × 2.5-5 μm. These morphological characteristics indicated that the pathogen belongs to the *Neofusicoccum parvum/N. ribis* complex (Pavlic et al., 2009a, 2009b). However, the species of this complex show conidial dimensions (length and width) that overlap among species, which cannot be distinguished based only on morphological characteristics (Pavlic et al., 2009b). Thus, a molecular characterization of the isolates was performed based on a combined analysis of the following sequences: internal transcribed spacer (ITS) regions 1 and 2 (including the 5.8S rRNA gene and 28S rDNA), β-tubulin, transcription elongation factor 1-α (TEF1-α) and RNA polymerase subunit II (RPB2) (Pavlic et al., 2009b).

To obtain representative fungal DNA, a single spore-derived culture from three selected isolates was grown on PDA at 25ºC for one week. The genomic material was extracted from approximately 40 mg of fungal tissue using the Wizard Genomic DNA Purification Kit (Promega) following the protocol described by Pinho et al. (2012).

The target sequences of the ITS region, 28S rDNA, β-tubulin, TEF1-α and RPB2 were amplified using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) for ITS (White et al., 1990), LR0R (5′-ACC CTC AGT AGT GAC ACC TTC GC-3′) and LR5 (5′-ACC CTG ATG AGT ATT AGG CTT GAC-3′) for the partial β-tubulin (Glass & Donaldson, 1995; O’Donnell & Cigelnik, 1997), EF1F (5′-TGC GGT GGT ATC GAC AAG CGT-3′) and EF2R (5′-AGC ATG TTG TCG CCG TTG AAG-3′) for the partial TEF1-α (Jacobs et al., 2004) and 5F2 (5′-GGG GGG AYC AGA AGA AGG C-3′) and 7cR (5′-CCC ATR GCT TGY TTR CCC AT-3′) for the partial RPB2 (Sung et al., 2007; Liu et al., 1999) following the protocols of Pinho et al. (2013) and Sung et al. (2007).

Amplification of ITS, β-tubulin, TEF1-α and RPB2 produced sequences of approximately 550, 630, 680 and 860 bp, respectively. The nucleotide sequences were edited with BioEdit software (Hall, 2012), and the new sequences were deposited in GenBank. ITS, TEF1-α, β-tubulin and RPB2 sequences from additional species were retrieved from GenBank (Table 1). Consensus regions were compared with the GenBank database using the MegaBLAST program.

### Table 1 - GenBank accession numbers of *Neofusicoccum* spp. DNA sequences used in the phylogenetic analysis.

| Species                  | Isolate | ITS          | BT           | EF           | RPB2        |
|--------------------------|---------|--------------|--------------|--------------|-------------|
| *N. parvum*              | CMM1846 | KC507812     | KC507806     | KC507809     | KC507803    |
| *N. kwambonambiense*     | CMM1842 | KC507813     | KC507807     | KC507810     | KC507804    |
| *N. parvum*              | CMM1845 | KC507814     | KC507808     | KC507811     | KC507805    |
| *N. parvum*              | CMW9071 | AY236938     | AY236909     | AY236880     | EU339571    |
| *N. parvum*              | MUCC676 | EU339545     | EU339482     | EU339519     | EU339568    |
| *N. parvum*              | CMW9080 | AY236942     | AY236916     | AY236887     | EU339572    |
| *N. parvum*              | MUCC211 | EU301017     | EU339480     | EU339517     | EU339566    |
| *N. parvum*              | MUCC673 | EU339553     | EU339483     | EU339520     | EU339570    |
| *N. kwambonambiense*     | MUCC210 | EU301016     | EU339478     | EU339515     | EU339564    |
| *N. kwambonambiense*     | MUCC157 | EU339522     | EU339479     | EU339516     | EU339522    |
| *N. cordaticola*         | CMW14123| EU821924     | EU821864     | EU821894     | EU821954    |
| *N. cordaticola*         | CMW14124| EU821925     | EU821865     | EU821895     | EU821955    |
| *N. cordaticola*         | CMW14056| EU821903     | EU821843     | EU821873     | EU821933    |
| *N. cordaticola*         | CMW14054| EU821906     | EU821846     | EU821876     | EU821936    |
| *N. batangarum*          | CMW28320| FJ900608     | FJ900635     | FJ900654     | FJ900616    |
| *N. batangarum*          | CMW28637| FJ900669     | FJ900636     | FJ900655     | FJ900617    |
| *N. umdonicola*          | CMW14079| EU821915     | EU821885     | EU821885     | EU821945    |
| *N. umdonicola*          | CMW14127| EU821896     | EU821896     | EU821956     | EU821956    |
| *N. umdonicola*          | CMW14096| EU821913     | EU821883     | EU821943     | EU821943    |
| *N. ribis*               | CMW7772 | AY236925     | AY236906     | AY236877     | EU339554    |
| *N. ribis*               | CMW7773 | AY236936     | AY236907     | AY236878     | EU339555    |
| *N. occultatum*          | MUCC322 | EU301031     | EU339473     | EU339510     | EU339559    |
| *N. occultatum*          | MUCC270 | EU339529     | EU339471     | EU339508     | EU339557    |
| *N. occultatum*          | MUCC296 | EU301034     | EU339475     | EU339512     | EU339561    |
| *N. karanda*             | MUCC247 | EU301028     | EU339476     | EU339513     | EU339562    |
| *N. karanda*             | MUCC125 | EU339525     | EU339477     | EU339514     | EU339563    |
| *N. australis*           | CMW6837 | AY339262     | AY339254     | AY339270     | EU339573    |
| *N. australis*           | CMW9072 | AY339260     | AY339252     | AY339268     | EU339573    |
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closest hit sequences were aligned using the multiple sequence alignment program MUSCLE (Edgar, 2004) and built in MEGA v.5 software (Tamura et al., 2011). All of the ambiguously aligned regions within the dataset were excluded from the analyses. Gaps (insertions/deletions) were treated as missing data. The resulting alignment was deposited in TreeBASE (http://www.treebase.org/) under accession number S13850. The manually adjusted alignment contained 28 strains and, of the 1781 characters used in the phylogenetic analysis, 113 were parsimony-informative, 125 were variable and 1655 were conserved. Although the 28S rDNA sequences were not used in the phylogenetic analyses, they were deposited in GenBank (accession nos. KC507815 and KC507816).

Bayesian inference (BI) concatenated analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and later with the concatenated sequences (ITS, TEF1-α, β-tubulin and RPB2). Before launching the BI, the best nucleotide substitution models were determined for each gene with MrMODELTEST 2.3 (Posada & Buckley, 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The HKY+I model of evolution was used for ITS and TEF1-α, whereas GTR+I was used for RPB2 and β-tubulin. The phylogenetic analysis of the concatenated alignment was performed on the CIPRES web portal (Miller et al., 2010) using MrBayes v.3.1.1 (Ronquist & Huelsenbeck, 2003). The remainder of the phylogenetic analysis was conducted as described by Pinho et al. (2012).

The BI analysis showed that the CMM 1842 isolate belongs to *N. kwambonambiense*, whereas CMM 1845 and CMM 1846 belong to *N. parvum* (Figure 2). *Neofusicoccum parvum* infects a broad range of hosts in a wide range of botanical families (Pavlic et al., 2009a, 2009b; Sakalidis et al., 2011; Farr & Rossman 2013), and in Brazil, it was recently reported as a pathogen in mango (Costa et al., 2010; Marques et al., 2013). *Neofusicoccum kwambonambiense* has been described in *Corymbia*

![Figure 2 - Multilocus phylogenetic tree inferred from Bayesian analysis using the ITS, β-tubulin, EF-1α and RPB2 regions. Bayesian posterior probability values are indicated at the nodes. Strain numbers are indicated after species names, and the specimens used in this study are highlighted in bold. *Neofusicoccum australe* represents the outgroup taxon.](image)
The presence of both species represents new threats to strawberries crop in tropical areas where the climate favors their development. Therefore, control strategies should be investigated, especially for *N. parvum*, which causes serious damage to others important crops (Pavlic et al., 2009a, 2009b; Sakalidis et al., 2011; Farr & Rossman, 2013).

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