Extensive colonization of apples by smut anamorphs causes a new postharvest disorder

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apple; basidiomycetes; Elstar; Entyloma; postharvest disorder; Tilletiopsis.

Abstract
Colonization of apples by ballistoconidium-forming fungi causes a new disorder, here named ‘white haze’. White haze may occur in mild form in the field, but only becomes problematic after Ultra-Low Oxygen storage, and, therefore, may be considered as a postharvest disorder. All isolates, obtained using the spore-fall method, were morphologically identified as anamorphs of smut fungi belonging to the genus Tilletiopsis. Sequence analysis of the D1/D2 and the ITS domains of the rDNA revealed nine novel taxa scattered among the Exobasidiomycetidae (Ustilaginales). Field experiments confirmed the erratic incidence of white haze over the years, and the development of the disorder seems to be enhanced at lower temperatures and a high relative humidity. Several scab fungicide treatments showed diminishing effects on the incidence of white haze.

Introduction
The most-widely planted apple cultivar in the Netherlands is ‘Elstar’. To provide the market for a longer period with this high-quality variety, the apples are usually stored under Ultra-Low Oxygen (ULO) conditions. In recent years, apple growers, auctions and extension workers have been complaining about a new disorder occurring on the apples, hereafter called ‘white haze’ (WH). Microscopical observations of the diseased apples showed that the disorder manifests itself by the occurrence of a thin whitish to pale-grey layer of fungal growth on the apple surface. WH may be present on apples while still in the orchard, but extensive overgrowth of apples occurs only after storage under ULO conditions and, therefore, it may be considered as a postharvest disease. Colonization may result in contamination of the whole bulk of apples in ULO storage rooms. Apples of the cultivar ‘Elstar’ are especially prone to the disorder, but WH manifests itself irregularly, as the symptoms do not occur every year with the same severity. The presence of WH on the apples reduces marketability and profitability for the apple growers. As the origin of the problem was unknown, we investigated its cause. In this paper, we describe the organisms involved in WH and their phylogenetic relationships. In addition, we studied the development of the disorder in the field and under ULO conditions, as well as the influence of orchard spraying schemes on its incidence.

Materials and methods
Apple samples
Apples showing distinct signs of WH (Fig. 1) were obtained from ULO storage rooms and orchards, and were investigated macroscopically and microscopically. In 1994, a selection of apples of the cultivar ‘Elstar’ showing signs of WH was obtained through the Plant Protection Service [Plantenziektenkundige Dienst (PD)] in Wageningen, the Netherlands, and in 1998 apples showing the symptoms were collected from orchards and ULO storage rooms in different regions of the Netherlands. Russetted apples and apples without any visible disorder were used as a control (Table 1, see http://www.cbs.knaw.nl/publications/whitehaze/). As
the WH isolates turned out to be phylogenetically related to the smut genus *Entyloma*, we included 22 reference specimens of this genus, as well as two additional *Tilletiopsis* cultures that were isolated from leaves infected with smut and rust fungi (Table 2).

**Isolation, morphology and physiology**

Direct morphological examination using light microscopy and scanning electron microscopy of the WH patches showed that the apples were covered by a thin mat of ballistosporous filamentous fungi. This was confirmed in various years, by different investigators and using apples from different geographic origins (Table 1). These observations suggested that WH is caused by ballistosporous fungi and, therefore, this mode of reproduction was used to isolate selectively the WH-related fungi by the spore-fall method as follows. Pieces of infected apple skin were fixed to the inside of the lid of a Petri dish, allowing the fungi to shoot conidia onto plates containing 1% yeast extract, 0.5% peptone and 4% glucose agar (YPGA), supplemented with 20 000 U streptomycin and 6600 U penicillin G per plate to inhibit bacterial growth. To prevent overgrowth of the slowly growing target fungi by quickly expanding moulds, the time for ballistoconidium discharge was limited to approximately 4 h per plate. The plates were incubated at 25 °C and examined daily.

For direct morphological observations by scanning electron microscopy, pieces of apple skin carrying WH were fixed in 3% glutaraldehyde, postfixed using 1% osmium tetroxide, and critical-point-dried after dehydration. Electron micrographs were taken with a field emission scanning electron microscope (XL30, Philips, Eindhoven, the Netherlands) at an acceleration voltage of 8 kV and a working distance of 7 mm.

The diversity of the isolated *Tilletiopsis*-like fungi from apples was assessed further using a selected panel of carbon compounds known to differentiate between *Tilletiopsis* and related *Entyloma* species (Boekhout, 1991). Cutinase, lipase, pectinase and protease activities were investigated using plate assays. Cutinase was detected according to the method described by Dantzig *et al.* (1986) with p-nitrophenyl butyraat (PNB, Sigma, St Louis, MO) as the substrate. Lipase was detected using Tween 40, Tween 60 and Tween 80 as substrates (Sierra, 1957; Hankin & Anagnostakis, 1975). Protease was detected according to the method described by Braga *et al.* (1998), using agar plates containing 0.75% casein. Pectinase was tested according to Biely & Sláviková (1994) using pectin plates and hexadecyltrimethylammonium as a clearance medium. Cellulase activity was tested by inoculating isolates on Peterson medium using cellulose azure as a substrate (Smith, 1977).

**Molecular identification and phylogenetic analyses**

DNA was isolated from cultures using the CTAB method as described in O’Donnell *et al.* (1997) and from herbarium specimens following Lutz *et al.* (2004). The isolates were identified by sequence comparison of the D1/D2 domains of the 26S rDNA, and the ITS region, spanning the ITS 1 and 2 spacers and the 5.8S rRNA gene. A DNA fragment containing these two regions of the WH isolates was amplified using primers V9 ($5'$-TGC GTT GAT TAC GTC CCT GC) and Lr6 ($5'$-CGC CAG TTT TTC TGG TTA CC) in 100 μL reaction volumes. The PCR mix contained 0.8 μL primers V9 and Lr6 (5 pmol μL⁻¹), 10 μL 5 × dNTP (0.1 mM), 10 μL 10 ×
PCR buffer containing 1.5 mM MgCl₂, 0.2 μL Taq polymerase (0.125 U), 2.0 μL DNA (5 ng μL⁻¹), and 76.2 μL double-distilled water. PCR conditions were as follows: 20 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C, followed by storing at 4°C. The ITS and D1/D2 domains of the herbarium specimens were amplified as described in Begerow et al. (2002a). Cycle sequencing of the D1/D2 domains was performed using the primers NL1 (5'-TGC GTT GAT TAC GTC CCT GC) and RLR3R (5'-GGT CCG TGT TTC AAG AC) in 10 μL reaction volumes, containing 4 μL BigDye terminator ready reaction mix, 1 μL primer (4 pmol μL⁻¹), 15–45 ng DNA, and MilliQ water to make 10 μL. For ITS cycle sequencing the following primers were used: ITS1 (5'-TCC GTA GGT GAA CCT GCG G) for cultures, ITS1F (5'-CTT GGT CAT TTA GAG GAA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-TCC GGT TAC TTA GAG GAA GTA A) for herbarium specimens and ITS4 (5'-
4 min at 60 °C. Purification of the final sequencing products was performed by using the MultiScreen™ Filtration System (Millipore, Etten-Leur, the Netherlands) in combination with SephadexTM G-50 Super fine (Amersham Biosciences, Roosendaal, the Netherlands). Sequences were obtained with the ABI 3700 capillary sequencer (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). The sequences of both strands were combined and proof-read with the help of Sequencer™ 4.1 software (Gene Codes Corp., Ann Arbor, MI). DNA sequences determined for this study were deposited in GenBank. Accession numbers are given in Tables 1 and 2.

Sequences were compared with those available in GenBank. Pairwise distances were calculated with PAUP* version 4.0b10 under default settings (Swofford, 2001). To obtain a hypothesis on the phylogenetical position of the WH isolates, we compared two datasets. First, LSU sequences of all WH isolates together with sequences of all available Entyloma species covering all teleomorphic orders of taceae were analysed together with sequences of all available WH isolates belonging to the Exobasidiomycetidae family. The LSU alignments were used to characterize Tilletiopsis species and an assortment of species to analyse the major topology. Second, ITS sequences of all WH isolates were compared with those of the species to analyse the major topology. First, LSU sequences of all WH isolates together with sequences of all available Entyloma species covering all teleomorphic orders of taceae were analysed together with sequences of all available WH isolates belonging to the Exobasidiomycetidae family. The LSU alignments were used to characterize Tilletiopsis species and an assortment of species to analyse the major topology. Second, ITS sequences of all WH isolates were compared with those of the Entyloma-taceae were analysed together with sequences of all available

Table 2. List of studied species not obtained from apples, GenBank accession numbers, and reference materials

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession no.</th>
<th>Reference material*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entyloma arnicale Ellis &amp; Everh.</td>
<td>AY854964</td>
<td>Germany, Bayern, Sonthofen on Arnica montana L., 27. 9. 2001. (TUB 012093)</td>
</tr>
<tr>
<td>Entyloma arnicale Ellis &amp; Everh.</td>
<td>AY854965</td>
<td>Germany, Bayern, Sonthofen on Arnica montana L., 15. 7. 2003. (TUB 012092)</td>
</tr>
<tr>
<td>Entyloma bidentis Henn.</td>
<td>AY854963</td>
<td>El Salvador, Departamento San Salvador on Bidens pilosa L., 12. 8. 1995. (M. Piepenbring 1686)</td>
</tr>
<tr>
<td>Entyloma calendulae (Oudem.) de Bary</td>
<td>AY854961</td>
<td>France, Cévennes, Le Vigan on Calendula officinalis L., 18. 05. 1998. (TUB 012094)</td>
</tr>
<tr>
<td>Entyloma chrysosplenii (Berk. &amp; Broome) Plowr.</td>
<td>AY854960</td>
<td>Germany, Baden-Württemberg, Lenningen on Chrysosplenium alternifolium L., 1. 5. 2002. (TUB 012095)</td>
</tr>
<tr>
<td>Entyloma compositarum</td>
<td>AY854959</td>
<td>Mexico, Veracruz, Xico on Parthenium hysterophorus L., 11. 1. 2001. (XAL, A. Romero 563)</td>
</tr>
<tr>
<td>Entyloma corydalis de Bary</td>
<td>AY860053</td>
<td>Germany, Bayern, Dillingen on Corydalis bulbosa (L.) DC., 1. 5. 1987. (Vánky Ust. Ex. 670)</td>
</tr>
<tr>
<td>Entyloma doebeleri M. Piepenbr.</td>
<td>AY854973</td>
<td>Costa Rica, San José on Dahlia imperialis Roetzl., 3. 11. 1992. (M. Piepenbring 584)</td>
</tr>
<tr>
<td>Entyloma erynigi (Corda) de Bary</td>
<td>AY854972</td>
<td>Germany, Sachsen-Anhalt, Halle on Eryngium campestre L., 24. 9. 2001. (TUB 012096)</td>
</tr>
<tr>
<td>Entyloma fergussonii (Berk. &amp; Broome) Plowr.</td>
<td>AY854970</td>
<td>Austria, Kärnten, Mallnitz on Myosotis sp., 28. 5. 2002. (TUB 012098)</td>
</tr>
<tr>
<td>Entyloma fergussonii (Berk. &amp; Broome) Plowr.</td>
<td>AY854971</td>
<td>Germany, Bayern, Grün tensee on Myosotis palustris (L.) Hill, 26. 9. 2001. (TUB 012097)</td>
</tr>
<tr>
<td>Entyloma gailhardianum Vánky ex Cif.</td>
<td>AY854968</td>
<td>Germany, Berlin on Gaillardia sp., 13. 6. 2003. (TUB 012099)</td>
</tr>
<tr>
<td>Entyloma hieracii Syd. &amp; P. Syd. ex Cif.</td>
<td>AY854967</td>
<td>Germany, Baden-Württemberg, Donaust auf on Hieracium lachenalii Gmel., 9. 5. 1999. (TUB 012100)</td>
</tr>
<tr>
<td>Entyloma matricariae Rostr. in Thülm.</td>
<td>AY854979</td>
<td>Sweden, Göteborg on Tripleurospermum perforatum (Mér.) Wag., 17. 8. 2002. (TUB 012101)</td>
</tr>
<tr>
<td>Entyloma microsporum (Unger) J. Schröt. in Rabenh.</td>
<td>AY854978</td>
<td>Germany, Bayern, Sonthofen on Ranunculus repens L., 10. 10. 1998. (TUB 012102)</td>
</tr>
<tr>
<td>Entyloma ranuncul-repentis Sternon</td>
<td>AY854980</td>
<td>Germany, Bayern, Bad Reichenhall on Ranunculus nemorosus DC., 22. 7. 1992. (M. Piepenbring 246)</td>
</tr>
<tr>
<td>Entyloma ranuncul-repentis Sternon</td>
<td>AY860052</td>
<td>Germany, Baden-Württemberg, Tübingen on Ranunculus repens L., 3. 10. 1998. (TUB 012105)</td>
</tr>
<tr>
<td>Tilletiopsis sp. G</td>
<td>AY854976</td>
<td>Isolated from leaves of Anemone ranunculoides L. infected with Tilletiopsis pruni-spinosa (Pers.) Dietel collected in Germany, Baden-Württemberg, Tübingen, 15. 4. 2001 (M. Lutz 1558). (TUB 012103)</td>
</tr>
<tr>
<td>Tilletiopsis sp. G</td>
<td>AY854977</td>
<td>Isolated from leaves of Myosotis palustris (L.) Hill infected with Entyloma fergussonii (Berk. &amp; Broome) Plowr. collected in Germany, Bayern, Grün tensee, 26. 9. 2001 (TUB 012097). (TUB 012104)</td>
</tr>
</tbody>
</table>

*Source acronyms: B, Herbarium of Botanischer Garten und Botanisches Museum Berlin-Dahlem, Freie Universität Berlin, Germany; TUB, Herbarium of Spezielle Botanik/Mykologie, Eberhard-Karls-Universität Tübingen, Germany; XAL, Herbarium of Instituto de Ecología, Veracruz, Mexico.
Entyloma species, three representatives of the Tilletiopsis
washingtonensis group and sequences of two Tilletiopsis
isolates from leaves infected with smut and rust fungi,
respectively, to elucidate the inner phylogeny of Entyloma-
taceae. To align sequences, we used MAFFT 3.85 (Katoh
et al., 2002) and to estimate phylogenetic relationships, we
applied a Bayesian approach of phylogenetic inference using
a Markov chain Monte Carlo (MCMC) technique as im-
plemented in the computer program MrBayes 3.0B4 (Huel-
senbeck & Ronquist, 2001) to each of the two alignments
independently. For Bayesian analysis, each alignment was
first analysed with MrModeltest 1.0b (Posada & Crandall,
1998) (J. A. A. Nylander, Uppsala University, Sweden) to
find the most appropriate model of DNA substitution. The
hierarchical likelihood ratio test proposed the DNA sub-
stitution model GTR+I+G for the LSU alignment and
HKY+I+G for the ITS alignment (see Swofford et al., 1996
for a survey of DNA substitution models). Thus, for each
alignment, four incrementally heated simultaneous Markov
chains were run over 2 000 000 generations using random
starting trees and default starting parameters of the respec-
tive DNA substitution model (Huelsenbeck & Ronquist,
2001). Trees were sampled every 100th generation, resulting
in an overall sampling of 20 001 trees for each alignment.
From these, the first 1001 trees were discarded (burnin = 1001). The trees sampled after the process that
had reached stationarity (19 000 trees) were used to com-
pute a 50%-majority rule consensus tree to obtain estimates
for the a posteriori probabilities of groups of species. This
Bayesian approach of phylogenetic analysis was repeated five
times for each alignment to test the independence of the
results from topological priors (Huelsenbeck et al., 2002).

The genetic diversity within the WH-related species was
further assessed by random amplified polymorphic DNA
(RAPD), PCR-fingerprinting and amplified fragment length
polymorphism (AFLP) analysis. RAPD was performed using
the OPA-05 (5'-AGG GGT CCT G) primer, and PCR-
fingerprinting used the following primers: (ATG)5, (GTG)5,
ERIC-IR (5'-ATG TAA CCT GGT GAG T) and, and
ERIC-2 (5'-AAG TAA GTG ACT GGG GTG ACG).
Reactions were performed in 50 μL reaction volumes con-
taining 2 μL primer (5 pmol μL⁻¹), 5 μL 5 × dNTP
(0.1 mM dNTP), 5 μL 10 × PCR buffer with 1.5 mM MgCl₂,
0.1 μL Taq polymerase (0.125 U), 5 μL DNA (5 ng μL⁻¹) and
32.9 μL double-distilled water. For the primers the following
PCR conditions were applied: (ATG)5, 40 cycles of 1 min
denaturation at 94 °C, 1 min annealing at 48 °C, 2 min
extension at 72 °C, followed by storing at 4 °C; (GTG)5, 40
cycles of 2 min denaturation at 94 °C, 1 min annealing at
60 °C, 2 min extension at 72 °C, followed by storing at 4 °C;
OPA-05, 40 cycles of 1 min denaturation at 94 °C, 1 min
annealing at 34 °C, 2 min extension at 72 °C, followed by
storing at 4 °C; primer Eric IR, 40 cycles of 1 min denatura-
tion at 94 °C, 1 min annealing at 55 °C, 2 min extension at
72 °C, followed by storing at 4 °C. The PCR products were
investigated after agarose gel electrophoresis. Banding pat-
terns were compared manually, and assigned a different
letter if they differed in more than two bands. AFLP was
performed according to (Boekhout et al., 2001).

Field experiments
The effect of scab fungicide regimens on the incidence of
WH after ULO storage was investigated using captan
(1.25 kg ha⁻¹), dithianon (0.38 kg ha⁻¹), tolylfluanid (0.75
kg ha⁻¹), dodine (0.59 kg ha⁻¹) kresoxim-methyl (0.10 kg
ha⁻¹), pyrimethanil (0.30 kg ha⁻¹) and an untreated con-
trol, and was tested at Randwijks experimental station in
the Netherlands in 1999 and 2000. The experimental design was
a randomized complete block design with 10 replicates.
Every replicate consisted of one row of Elstar, with six
experimental trees per fungicide treatment, flanked by a
buffer tree on both sides.

Trees were sprayed with a hand-held spraying gun till
runoff, with 0.4 L per tree corresponding with a full field
routinely with 1000 L. Sprayings were executed roughly
every 10 days, depending on Welte scab warning system data
(Batzer et al., 2000), until the end of the scab hazard period
(27 and 21 July for 1999 and 2000, respectively) resulting in
13 spray dates in both years.
The experiments were harvested on 3 and 5 September in
1999 and 2000, respectively (Fig. 2). A random sample of
60–70 apples was harvested from the lower branches of the
trees, where WH manifests itself primarily. Immediately
after harvest the apple samples were stored under ULO
conditions. The amount of CO₂ was maintained between
2% and 3%, and the oxygen level was kept at 1.2%. Temperature in the storage room was 1–2 °C and the relative
humidity approximately 99%.

Assessment of the WH incidence
The 1999 apple samples were taken from the ULO storage on
7 March 2000 and stored at 18 °C for 3 weeks before
assessment of the incidence of WH in order to increase the
visibility of the disorder. In 2000, WH incidence assessment
was done directly after a shorter ULO storage period on 22
and 23 November, and without prior storage of the samples
at room temperature, as the disorder was already visible at
harvest and developed quickly during ULO storage.

For the assessment of the incidence of WH, the appear-
ance of all individual apples within a sample was judged and
the fruits were divided into three classes: no WH, moderate
WH (disorder visible but mild) and high WH incidence
(more than 25% of the surface covered with WH or big
patches present with heavy whitish powdery deposits larger than 1 cm²).

A ‘white haze index’ (WHI) was calculated per sample as follows:

\[
\text{WHI} = \frac{[\text{no. of apples high } \times 3] + [\text{no. of apples moderate } \times 1]}{\text{total no. of apples}}
\]

This formula takes into consideration that the presence of highly infected apples has a larger effect on the visible quality of an amount of apples than that of moderately or lowly infected apples. The results of the assessment were analysed statistically by means of a one-way analysis of variance, followed by pair-wise comparison of averages through t-tests. As the WH assessment for 1999 and 2000 was not executed after the same storage time, data for both years were analysed separately.

**Results**

**Isolation and identification of the causative organism**

Direct macroscopical and light- and scanning electron-microscopical observations of WH-affected apples performed by the authors and an employee of the PD (Wageningen) on apples originating from a number of geographic localities in the Netherlands in various years demonstrated that the WH patches consisted of extensive growth of filamentous fungi reproducing with elongated, falcate to cylindrical ballistoconidia (data not shown). In total, 24 isolates of these fungi were obtained from WH patches (Figs 1 and 3, Table 1). Also russetted apples and those without any visible disorder were found to be colonized by similar fungi: from each of these apple categories, nine isolates, belonging to three and five taxa, respectively, were obtained. The WH-related fungi were morphologically identified as belonging to the anamorphic basidiomycetous genus *Tilletiopsis* (Boekhout, 1991). Morphologically they corresponded with those observed directly in the diseased patches on the apple surface. The hyphae lacked clamp connections, but had sterigmata on which the ballistoconidia were formed (Fig. 3).

All isolates originating from the apples showed lipase, pectinase, protease and cutinase activities. Cellulolytic activity was found to be variable among the isolates (for strain data see www.cbs.knaw.nl/publications/whitehaze/).

Using D1/D2 and ITS sequences we identified nine groups of apple isolates belonging to the *Exobasidiomycetidae* (*Basidiomycota*, *Ustilaginomycetes*), and each of these seem to represent individual species. Table 3 presents the results of BLAST searches of the D1/D2 and ITS sequences of all WH isolates. Only very few gave 100% similarity values, such as isolates CBS 111629, 111630, 111631, which were 100% identical with *Tilletiopsis minor* in the D1/D2 domain, CBS 111622 showing 100% similarity with *Tilletiopsis pallescens* in the D1/D2 domain, and CBS 111626, 111623 and 111624, which agreed both in the D1/D2 and the ITS domains with *T. pallescens*. As this finding is in accordance with our phylogenetic analyses, we assigned these two groups of isolates to *T. minor* and *T. pallescens*, respectively.

![Fig. 3. Scanning electron micrograph of white haze on apple. Distinct growth of filamentous hyphae without clamp connection is visible. Inset shows a sterigma (arrow). Scale bar, 10 μm.](image)
Table 3. Similarities between D1/D2 and ITS sequences of white haze and sequences present in GenBank

<table>
<thead>
<tr>
<th>CBS number</th>
<th>ITS</th>
<th>LSU</th>
<th>Decided identity</th>
</tr>
</thead>
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<tr>
<td>111629–111631</td>
<td>Tilletiopsis minor</td>
<td>Tilletiopsis minor</td>
<td>Tilletiopsis minor</td>
</tr>
<tr>
<td></td>
<td>AB025699 99 457/459</td>
<td>AB1235286 100 463/463</td>
<td>Tilletiopsis minor</td>
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<td></td>
<td>Tilletiopsis minor</td>
<td>'Exobasidium vexans'</td>
<td>Nyland</td>
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<td></td>
<td>AB025702 99 456/459</td>
<td>AB1235288 100 463/463</td>
<td>Tilletiopsis pallescens</td>
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<td>Tilletiopsis pallescens</td>
<td>Tilletiopsis pallescens</td>
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<td>AB1235329 100 464/464</td>
<td>Tilletiopsis pallescens</td>
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<td>'Exobasidium vexans'</td>
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Acc. no., accession number from GenBank; %, the percentage of similarity for each Blast result; n/n, the number of identical base pairs from the query and the hit from the total number of basepairs compared.
Sequences of all other groups of WH isolates differed from those of any known species. These isolates may either represent nondescribed species or species not yet represented in the D1/D2 or ITS rDNA databases of GenBank, and we labelled them tentatively as *Tilletiopsis* species A–G.

During both 1994 and 1998, *Tilletiopsis* sp. B was the most abundantly isolated species, as approximately 46% of the

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**Fig. 4.** Bayesian inference of phylogenetic relationships of white haze isolates and representatives of *Ustilaginomycetes*: Markov chain Monte Carlo analysis of an alignment of nuc-LSU rDNA sequences from the D1/D2 region using the GTR+I+G model of DNA substitution with gamma-distributed substitution rates, random starting trees and default starting parameters of the DNA substitution model. Majority rule consensus tree from 19,000 trees that were sampled after the process had reached stationarity. The topology was rooted with the representatives of the *Ustilaginales*. Numbers on branches are estimates for *a posteriori* probabilities. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. The taxonomical concept applied corresponds to Bauer et al. (Hislop & Cox, 1969; Bauer et al., 2001). *Tilletiopsis* isolates from apples are highlighted.
WH isolates belonged to this species. *Tilletiopsis* sp. A was not observed in 1998. Differences were also apparent between the isolates from the ULO storage and those isolated from the orchards in 1998. Only four species were isolated from the ULO storage, namely *Tilletiopsis* spp. B and D–F. Some species, namely *Tilletiopsis* sp. B, *Tilletiopsis* sp. D, and the majority of the isolates of *Tilletiopsis* sp. F, were isolated from russetted apples and *Tilletiopsis minor*,

![Bayesian inference of phylogenetic relationships of white-haze isolates and representatives of the Entylomatales: Markov chain Monte Carlo analysis of an alignment of base sequences from the ITS1/2 region of the nuc-rDNA including the 5.8S rDNA using the HKY+I+G model of DNA substitution with γ-distributed substitution rates, random starting trees and default starting parameters of the DNA substitution model. Majority rule consensus tree from 19 000 trees that were sampled after the process had reached stationarity. The topology was rooted with the specimens of the Tilletiopsis washingtonensis group. Numbers on branches are estimates for a posteriori probabilities. Branch lengths were estimated with PAUP* version 4.0b10 (Swofford, 2001) using maximum likelihood and the DNA substitution model proposed by MrModeltest 1.0b. They are scaled in terms of expected numbers of nucleotide substitutions per site. Tilletiopsis isolates from apples are highlighted.](image-url)
T. pallescens and Tilletiopsis sp. B occurred also on apples without signs of WH or russetting. Finally, Tilletiopsis sp. C and G were only isolated from apples without signs of WH or russetting (Table 1).

For both the D1/D2 and ITS alignment the different runs of Bayesian phylogenetic analyses yielded consistent topologies. The consensus tree of one run for each alignment illustrates the results (Figs 4 and 5). The phylogenetic hypothesis obtained by analyzing D1/D2 sequences of all apple isolates together with sequences of all known Tilletiopsis species and an assortment of species covering all teleomorphic orders of Exobasidiomycetidae and some Ustilago species as outgroup (Fig. 4) revealed all teleomorphic orders of the Exobasidiomycetidae (Bauer et al., 2001), the expected polyphyly of Tilletiopsis (Begerow et al., 2002b) and the split-up of the WH isolates in nine distinct groups. Apple isolates occurred scattered among the Exobasidiomycetidae. Two groups clustered within the Georgefischeriales: Tilletiopsis sp. A within the smut genus Jamesdicksonia in the Georgefischeriaceae, T. minor within the Gjaerumia ossifragi and some Tilletiopsis isolates from leaf surfaces. Five groups of isolates (Tilletiopsis spp. C–F) clustered within the smut genus Entyloma. The two remaining groups (Tilletiopsis sp. B and T. pallescens) formed a distinct cluster (T. pallescens group) within the Exobasidiomycetidae.

The phylogenetic hypothesis obtained by analysing ITS sequences of all apple isolates occurring scattered among Entyloma (Fig. 5) confirmed the polyphyly of the WH isolates. Although Entyloma ITS sequences differed relatively
Colonization of apples by smut anamorphs

In this study, we describe extensive postharvest colonization of apples by anamorphic smut fungi in the Netherlands. Light and scanning electron-microscopical observations of patches of WH on apples demonstrated that this is caused by a thin overgrowth of filamentous fungi with narrow, thin-walled, hyaline hyphae, which reproduce by ballistoconidia. We identified these fungi as belonging to the basidiomyceteous anamorph genus *Tilletiopsis* (Dex, 1930; Nyland, 1950; Brady, 1953; Boekhout, 1991). As some of the WH-related fungi occurred also on russetted apples and on apples without any visible disorder, it seems likely that the WH-fungi are part of the normal mycobiota occurring on apples. Due to some unknown environmental conditions they may develop into an overgrowth on the apples while still in the orchard or into a postharvest disorder during ULO storage.

*Tilletiopsis* is considered a typical phylloplane-inhabiting fungus and isolations from leaves of various plants using the spore-fall method resulted in many cultures of these fungi (Dex, 1930; Nyland, 1950; Boekhout, 1991). *Tilletiopsis* species were reported as part of the apple phylloplane during later stages of the growth season (Hislop & Cox, 1969; Pennycook & Newhook, 1978, 1981), and aerobiological studies demonstrated that *Tilletiopsis* spp. occur abundantly in air (Brown & Jackson, 1978).

*Tilletiopsis* species are well-known antagonists against powdery mildews (Hoch & Provvidenti, 1979; Hijwegen, 1986, 1988; Klecan et al., 1990; Knudsen & Skou, 1993; Urquhart et al., 1994). It may be that the WH isolates are able to reduce the growth of other fungi occurring on the apple surface and, if so, this may contribute to their success as apple colonizers. The extensive colonization of apples by *Tilletiopsis minor* and *Tilletiopsis pallescens* may, however, diminish the prospects for their commercial application as biocontrol agents, as registration as a biocontrol agent will be more complicated.

The fungal isolates were identified as nine distinct taxa, of which only two could be assigned to known *Tilletiopsis* species, namely *T. minor* and *T. pallescens*. Five groups of WH isolates (*Tilletiopsis* spp. C–F) clustered within the smut fungus *Entyloma*, but none could be assigned to a teleomorphic *Entyloma* species (Vánky, 1985; Begerow et al., 2002b). Therefore, these isolates may either represent anamorphs of *Entyloma* species not yet present in the D1/D2 and ITS rDNA databases of GenBank, or *Entyloma* species restricted to the *Tilletiopsis* stage. *Entyloma* species are well-known plant pathogens on a wide range of vascular plants (Vánky, 1985). The formation of ballistoconidia is well documented in these fungi (Hanna, 1938; Nyland, 1950; Brady, 1953; Boekhout, 1991), and asexual stages cannot be distinguished morphologically from those occurring in the genus *Tilletiopsis* (Dex, 1930; Boekhout, 1991). Until now, asexual stages of *Entyloma* have not been observed freely in nature, but are known to occur on the host plants only (Hanna, 1938; Vánky, 1985; Boekhout, 1991). This is the first report of *Tilletiopsis* species clustering within *Entyloma*, occurring on a host that obviously is not the host of the teleomorph. Interestingly, these species occur on a nonherbaceous host where they cause extensive colonization on fruit, whereas *Entyloma* species usually are found on

Field trials

The application of different scab control fungicides had a clear effect on the incidence of WH in 1999. All fungicide treatments, except pyrimethanil, diminished WH if compared to the water spray control treatment. Kresoxim-methyl was significantly less effective than dithianon and dodine. In 2000, however, data were not as convincing as in 1999. Only tolylfluanid significantly reduced WH if compared to the control treatment. Dodine treatment resulted in a lower WH index than both kresoxim-methyl and pyrimethanil treatments, but could not be distinguished significantly from the water-treated control. Both dithianon- and captan-treated trees showed a lower WH incidence than trees treated with kresoxim-methyl.

Discussion

In this study, we describe extensive postharvest colonization of apples by anamorphic smut fungi in the Netherlands. Light and scanning electron-microscopical observations of patches of WH on apples demonstrated that this is caused by a thin overgrowth of filamentous fungi with narrow, thin-walled, hyaline hyphae, which reproduce by ballistoconidia.

Intraspecific genetic diversity

Intraspecific variation in the sequences of the D1/D2 domains and the ITS regions was absent or very limited (Fig. 5). The genetic diversity within each species was assessed further by AFLP and RAPD analysis (Fig. 6, see www.cbs.knaw.nl/publications/whitehaze/). In general, good concordance was observed between the AFLP and RAPD patterns, and the D1/D2 and ITS clustering of the isolates. In some species, such as *Tilletiopsis* sp. F, *T. minor* and *T. pallescens*, different RAPD patterns occurred (see www.cbs.knaw.nl/publications/whitehaze/). The AFLP technique discriminated between two subtypes in *Tilletiopsis* sp. B, which contained two AFLP clusters 2 and 3 (Fig. 6), whereas the D1/D2 sequences were identical. The polymorphisms observed in the ITS sequences were not concordant with those observed using AFLP. Small AFLP polymorphisms were apparent among the isolates of most species present with more than one isolate.
herbaceous plants (Hanna, 1938; Vánky, 1985; Bauer et al., 2005). In addition, *Tilletiopsis* sp. G was isolated from healthy apples and leaves infected with rust and smut fungi, respectively. This species seems to have a broad ecological range, growing on both apples and leaves, and in the latter case possibly in association with microparasites. *Tilletiopsis* sp. A is the first record of a *Tilletiopsis* isolate within the *Georgescheriaceae*. The species clusters within the smut genus *Jamesdicksonia*, which is found exclusively on *Cyperaceae* and *Poaceae* (Bauer et al., 2001). *Tilletiopsis* sp. B forms a distinct cluster with *T. pallescens* (i.e. *T. pallescens* group), which cannot be assigned to any of the teleomorphic groups of the *Exobasidiomycetidae*. Whereas the hitherto unknown *Tilletiopsis* sp. B is only known from apples, *T. pallescens* is mostly isolated from fungi (Boekhout, 1991).

Our results demonstrate that identification of WH-related isolates using the most up-to-date sequence databases is still complicated. However, in our opinion, it is the only reliable identification tool, as physiological and morphological characterization are even less accurate (T. Boekhout, personal observation), and infection structures on the proper host plants are not known. Genetic polymorphisms were demonstrated in almost all species investigated, which is in agreement with previous studies on *Tilletiopsis* isolates from British Colombia (Canada) (Urquhart et al., 1997).

It seems that these asexual stages of the exobasidiomycetous smuts occur more widely in nature than previously known. With our isolates, the polyphyley of *Tilletiopsis* is extended to eight lineages within the *Exobasidiomycetidae* (*Entylomataceae*, *Georgescheriaceae*, *Gjaeruaceae*, *Tilletiariaceae*, *Tilletiopsis albescens*, *T. pallescens* group, *Tilletiopsis* sp. and *T. washingtonensis* group), some of them comprising several *Tilletiopsis* species (Begerow et al., 2000, 2002b; Bauer et al., 2005). Four of them contain isolates from apples (*Entylomataceae*, *Georgescheriaceae*, *Gjaeruaceae* and *T. pallescens* group), indicating the phylogenetic diversity of the putative causal agents of WH.

Interestingly, a species not found in the present study, *Tilletiopsis washingtonensis*, has been found to utilize volatiles produced by ripe ‘Golden Delicious’ apples (Vishniac et al., 1997). We do not know if this is true for the *Tilletiopsis* isolates from WH, but it is tempting to speculate that the possible ability to assimilate apple volatiles may be an explanation for the abundant occurrence of these fungi on the apple surface. The capability of WH-related fungi to secrete a variety of enzymes, such as lipase, cutinase, pectinase and protease, may also contribute to their occurrence on the apple surface (Urquhart & Punja, 2002). The development of WH was particularly prominent in years with a high amount of rainfall during the apple harvesting season, namely 1994, 1998 and 2001 (B. Heijne, unpublished observation). This suggests that high humidity and lower temperatures favour the development of WH. It is known that various species of *Tilletiopsis* and *Tilletiopsis*-like anamorphs of *Entyloma* spp. are able to grow at 4°C and at high relative humidity (Boekhout, 1991; Hajlaoui & Bélanger, 1991; Bélanger & Labbé, 2002), and it has been suggested that the presence of condensed water on agar plates increases the number of colonies being formed (Kaiser, 1936).

In short, the WH-associated fungi seem to have a suite of characteristics allowing them to colonize efficiently the apple surface in the orchard and further develop under the specific conditions of ULO storage. These characteristics include the ability to grow at high humidity and lower temperatures, an efficient mechanism of dispersal by ballistococidia, production of lipolytic, pectinolytic and proteolytic enzymes, and the possible antagonism against other apple-inhabiting fungi. A sixth factor may be the ability to assimilate volatiles produced by the apples. These latter two factors, however, need further investigation.

As seen in our field research, WH showed a highly erratic pattern of occurrence, which is probably related to weather conditions occurring during the end of the apple growth season. In particular, a combination of high relative humidity and low temperatures seems to favour the development on the apple surface of WH-related fungi, which in ULO storage may develop rapidly into WH.

Scab control spraying schemes can diminish the incidence of WH. Dodine, tolylfluanid, dithianon and captan treatment had a better reducing effect than kresoxim-methyl and pyrimethanil. None of the spraying schemes could fully prevent the development of WH during later stages of the apple growth season and the results were not consistent over the two seasons. This lack of consistent results may be because the severity of WH was very different in the two years investigated. Moreover, the scab control spraying is discontinued long before the actual harvest, and this may give the fungi an opportunity to colonize the apple surface during the ripening stage. Probably, no large reduction of WH can be expected from adaptation of the scab spraying scheme. It could, however, be worth considering executing the last one or two scab control sprayings with one of the fungicides that significantly reduced the incidence of WH, namely tolylfluanide, captan, dodine or dithianon. Our results do not justify the introduction of an additional spraying to reduce the risk of WH. Targeting the end of the season and the causal fungi could improve the effect on WH, but introducing spraying close to harvest is unwanted due to consumers’ demands.

To eliminate the problem, other measures have to be considered. The adaptation of ULO storage conditions to discourage development of the fungi is a first option. Another solution may be the mechanical removal of the fungus by washing the apples after storage but just before auction. In practice, growers confronted with the problem...
have already experimented with the latter (Nieuwenhuize, 1994). A third option may be the introduction of antagonists to *Tilletiopsis* spp. before storing the apples. However, in all cases it is necessary to understand the ecology of the WH-causing fungi in more detail, in particular their dispersal and growth at low temperature and high humidity. These studies should include the whole range of taxonomic and genetic variation of the fungi involved.

**References**


