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Published in:
Fungal Biology

Link to article, DOI:
10.1016/j.funbio.2017.12.012

Publication date:
2018

Document Version
Peer reviewed version

Accepted Manuscript

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PII: S1878-6146(18)30001-1

DOI: 10.1016/j.funbio.2017.12.012

Reference: FUNBIO 885

To appear in: *Fungal Biology*

Received Date: 14 November 2017

Revised Date: 18 December 2017

Accepted Date: 21 December 2017

Please cite this article as: Kromphardt Olsen, K.J., Rossman, A., Andersen, B., Metabolite production by species of *Stemphylium*, *Fungal Biology* (2018), doi: 10.1016/j.funbio.2017.12.012.

Metabolite production by species of *Stemphylium*

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Abstract

Morphology and phylogeny has been used to distinguish members of the plant pathogenic fungal genus *Stemphylium*. A third method for distinguishing species is by chemotaxonomy. The main goal of the present study was to investigate the chemical potential of *Stemphylium* via HPLC-UV-MS analysis, while also exploring the potential of chemotaxonomy as a robust identification method for *Stemphylium.* Several species were found to have species-specific metabolites, while other species were distinguishable by a broader metabolic profile rather than specific metabolites. Many previously described metabolites were found to be important for distinguishing species, while some unknown metabolites were also found to have important roles in distinguishing species of *Stemphylium*. This study is the first of its kind to investigate the chemical potential of *Stemphylium* across the whole genus.

Keywords:

Antibacterial metabolites, chemotaxonomy, host specific toxins, morphology, orobol, phytotoxins

1. Introduction

The fungal genus *Stemphylium* Wallr. consists of species that are pathogenic especially to members of the legume family (*Fabaceae*) (Bradley et al. 2003), but also to asparagus, onion, garlic, parsley, pear, sugar beet and tomato in various plant families (Gálvez et al. 2016; Graf et al. 2016; Hanse et al. 2015; Köhl et al. 2009; Koike et al. 2013; Tanahashi et al. 2017). Some pathogenic fungal species have a narrow host range, like *S. loti* on *Lotus corniculatus* or *S. trifolii* on *Trifolium repens*, while others have

a broad range, such as *S. vesicarium,* which causes purple spot of asparagus and brown spot of pear but is also able to live as a saprobe on plant debris (Graf et al. 2016; Köhl et al. 2009; Puig et al. 2015). Some species, like *S. botryosum*, *S. eturmiunum* and *S. vesicarium*, can also occur on food products such as beans, pulses, tomato, apple, pear and cereal grain (Pitt and Hocking 2009; Samson et al. 2010; Snowdon 1990). Though *Stemphylium* metabolites have been detected in mouldy tomatoes (Andersen and Frisvad 2004), no mycotoxins *sensu stricto* have been associated with *Stemphylium* food spoilage.

Morphologically, *Stemphylium* is easy to distinguish from its relatives, *Alternaria* Nees and *Ulocladium* Preuss, by its percurrent or annellidic proliferation often with a distinct terminal swelling (Simmons 1967). Phylogenetically, the genus is also easy to delimit from *Alternaria* and *Ulocladium* (Ariyawansa et al. 2015). Within *Stemphylium* some species such as *S. botryosum* and *S. globuliferum* or *S. eturmiunum* and *S. vesicarium* appear similar and may be mixed up and misidentified using morphology alone whereas some taxa previously recognized as distinct species such as *S. alfalfa*, *S. herbarum, S. vesicarium* and others, fall in the same phylogenetic clade (Câmara et al. 2002; Inderbitzin et al. 2009) and are now based on molecular data synonymized as *S. vesicarium* (Woudenberg et al. 2017).

Chemically, individual *Stemphylium* strains have been shown to produce a broad variety of secondary metabolites, of which many probably play a role during host plant infection as phytotoxins or host-specific toxins (Trigos et al. 2011). Culture extracts of different strains of *S. vesicarium* have, for

instance, been shown to be pathogenic to either European pear cultivars or Japanese pear cultivars, but never both (Singh et al. 1999). The extracts contained host-specific toxins (SV-toxins I and II), compounds that have not been structurally elucidated (Tanahashi et al. 2017). Other research has shown that two endophytic strains of *S. globuliferum* produced alterporriols H and K, altersolanol L, stemphypyrone (Debbab et al. 2009) and alterporriols D and E, altersolanol A (= stemphylin), altersolanols B and C, and macrosporin (Liu et al. 2015), while an endophytic strain of *S. botryosum* produced altersolanol A (= stemphylin), curvularin, dehydrocurvularin, macrosporin and stemphyperylenol (Aly et al. 2010). Another study has shown a strain of *S. herbarum* (later identified as *Stemphylium* sp. by Kurose et al. 2015) that produced alterporriols D-G and altersolanol A (Kanamaru et al. 2012). Recently, it has also been shown that *Stemphylium* metabolites have biological activities, such as cytotoxic and antibacterial effects (Debbab et al. 2009; Liu et al. 2015) that may be of interest to the pharmaceutical industry.

Chemotaxonomy as reviewed by Frisvad et al. (2008) has only been attempted in a few cases on *Stemphylium* (Andersen et al. 1995) and with little success. However, the study showed that *S. majusculum* and some strains of *S. botryosum* produced stemphol (Andersen et al. 1995). Chemotaxonomy has previously been useful in saprobic genera such as *Aspergillus* and *Penicillium* (Kim et al. 2012; Kozlovskii et al. 2017) and host-specific plant pathogenic *Alternaria* (Andersen et al. 2008; Brun et al. 2013), but less successful in saprobic or non-pathogenic species of *Alternaria* (Andersen et al. 2009) and *Fusarium* (de Kuppler et al. 2011). One purpose of this study was to examine if profiles of secondary metabolites are species-specific according to the latest phylogeny

(Woudenberg et al. 2017) and thereby would distinguish phylogenetically and/or morphologically similar species. Another purpose was to examine if individual metabolites are associated with specific host plants across species.

2. Materials and methods

2.1 Fungal strains

Eighty-seven *Stemphylium* strains were used in this study. Table 1 gives the identification numbers, original and new identity, host and origin of these strains. The strains were selected to include as many different species and habitats as possible and as many strains as possible that had been investigated in previous studies (Câmara et al. 2002; Inderbitzin et al. 2009; Woudenberg et al. 2017). An extended version of table 1 is available in supporting material table S1 giving strain numbers in other collections and other papers.

2.2 Micro- and macro-morphological examination

All 87 strains were inoculated in 3 points on Potato Carrot Agar (PCA (Simmons 2007)), V8 juice agar (V8 (Samson et al. 2010)), Potato Dextrose agar (PDA (Samson et al. 2010)) and Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES (Samson et al. 2010)) and grown under standardized conditions (Andersen et al. 2005; Simmons 2007). Selected strains were also inoculated on Spezieller Nährstoffarmer Agar (SNA, Samson et al. 2010). The unsealed PCA, SNA and V8 plates (9 cm diameter, plastic) were incubated in one layer for 7 days at 23°C under an alternating light/day cycle consisting of 8 h cool-white fluorescent daylight and 16 h darkness. The lamps (TLD, 36W/95o, Philips,

Amsterdam, Holland) were placed 40 cm from the plates. The DRYES and PDA plates (9 cm diameter, plastic) were placed in perforated plastic bags and incubated for 14 days in the dark at 25 °C. The micro-morphological characteristics of the strains were observed from PCA and V8 plates after 7 days of growth. Recording of primary conidiophore length, conidial size and shape (L/W ratio), colour and ornamentation were done at X200 magnification using slide preparations made in Shear's mounting liquid with clear Scotch tape as described in Samson et al. (2010). The PCA plates were then stored in the dark at 7 °C and checked for ascomata after 6 months. Colony characteristics (e.g. colour, texture and diameter) were recorded from DRYES plates after 7 days of growth. The morphological characteristics of each strain were registered and compared to reference strains.

2.3 Chemical extraction

The metabolite profiling was done on the 14-day-old DRYES and PDA cultures using a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al. 2005). Five agar plugs (6 mm ID) were cut from the two media and placed in a 2 ml screw top vial. Then 1.0 ml ethyl acetate/dichloromethane/methanol (3:2:1, vol/vol/vol) containing formic acid (1:100, vol/vol) was added to each vial and the plugs were extracted by ultra-sonication for 60 min. The extract was transferred to a clean 2 ml vial, evaporated to dryness in a gentle stream of N_2 and re-dissolved in 400 µl methanol. The methanol extract was filtered through a 0.45 µm filter into a clean 2 ml vial and kept at -18 °C prior to HPLC analysis.

2.4 Chemical analyses

Analyses were performed using ultra-high-performance liquid chromatography (UPHLC) with a diode array detector (DAD) and high-resolution maXis 3G QTOF mass spectrometer (MS) (Bruker Daltonics, Bremen, Germany), equipped with an ESI source and connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Kinetex 2.6-µm C18, 100 mm × 2.1mm column (Phenomenex, Torrance, CA, USA) (Klitgaard et al. 2014). A linear water-acetonitrile gradient was used (buffered with 20 mM formic acid) starting from 15% (vol/vol) acetonitrile and increased to 100% in 10 min, maintained for 3 min before returning to the starting conditions. MS was performed in ESI+ in the scan range m/z 100–1250, with a mass accuracy < 1.5 ppm (Klitgaard et al. 2014). The mass spectrum of sodium formate was used for calibration at the beginning (0.3-0.4 min) of each chromatogram by injection with a divert valve. UV/VIS spectra were collected at wavelengths from 200 to 700 nm. Data processing was performed using DataAnalysis 4.0 and Target Analysis 1.2 (Bruker Daltonics, Bremen, Germany) by the aggressive dereplication approach (Klitgaard et al. 2014), using a database of 297 known and putative *Alternaria* and *Stemphylium* compounds, tentatively identifying them based on accurate mass (deviation < 1.5 ppm) (Klitgaard et al. 2014) and if applicable an UV/VIS spectrum. All major peaks observed in the base peak chromatograms, not tentatively identified by this approach, were added to the search list of unknown compounds for mapping. All major peaks (known and unknown) for the 87 extracts were subsequently ordered in a data matrix.

2.5 Data treatment and clustering

A binary matrix was constructed based on 87 strains and their production of 219 metabolites with both known and unknown chemical structures. The presence or absence of a particular metabolite

137 was scored as 1 or 0, respectively, for each strain. The matrices were subjected to cluster analysis in
138 NTSYS-pc version 2.11N (Exeter software, Setauket, NY, USA). The binary metabolite matrix consisted
139 of no standardization, using Yule, Jaccard and Simple Matching similarity coefficients and Unweighted
140 Pair Group Method with Arithmetic mean (UPGMA) clustering method.
141
142 **3. Results**
143 *3.1 Taxonomy/Nomenclature and Morphology*
144 The 87 *Stemphylium* strains used in this study were obtained from different fungal collections and the
145 original identification is given in Table 1 together with information on host and origin. Table 1 also
146 gives the new identification of individual strains based on our overall findings using morphology,
147 chemistry and names/synonyms proposed by Woudenberg et al. (2017). A supplementary table gives
148 all known identification numbers for each strain according to Câmara et al. (2002), Inderbitzin et al.
149 (2009) and Woudenberg et al. (2017). Sixteen species of *Stemphylium* are represented in this study.
150
151 Conidial measurements of selected *Stemphylium* cultures were conducted on strains grown on PCA,
152 SNA and V8 plates. The results show that conidial sizes in general were smallest on SNA and largest on
153 V8. Comparisons between SNA and PCA of three cultures show that conidia appeared paler in colour,
154 smoother and more ellipsoidal on SNA than on PCA (Fig. 1). Comparisons of PCA and V8 show that
155 most strains produced conidia that were darker and larger (5.9 µm on average, 4.1 to 25.0 µm) and
156 wider (1.5 µm on average, 3.7 to 5.9 µm) on V8 compared to PCA. However, there was no pattern or
157 system concerning which species produced larger or smaller conidia. The L/W ratio also changed and

most conidial shapes became more elongated on V8 compared to PCA, however, *S. globuliferum*, *S. loti* and *S. sarciniforme*, maintained their L/W ratio best. Conidial size and L/W ratio varied within the same culture and therefore the following conidial sizes are the maximum sizes on PCA. Conidial measurements for all strains, except the two *S. majusculum*, were within the limits of the respective species descriptions given in the literature (Câmara et al. 2002; Pei et al. 2011; Simmons 1969, 1985, 1989).

Common characteristics for *S. callistephi, S. lancipes, S. lycopersici, S. majusculum* and *S. solani* were their pointed conidia, production of ascomata and L/W ratio (> 1.9). Conidial size varied greatly from 81 x 25 µm (*S. lancipes*), over 64 x 24 µm (*S. callistephi*) and 50 x 21 µm (*S. solani*) to 40 x 18 µm (*S. lycopersici*). *Stemphylium majusculum* had a conidial size of 40-42 × 21-22 µm, an L/W ratio of 1.9 and the presence of ascomata. *Stemphylium trifolii* also had pointed conidia and an L/W ratio of 2.0, but much smaller (25-28 x 12-14 µm) and production of ascomata. Colony diameter on DRYES also varied from 31-33 mm (*S. majusculum*), over 27 mm (*S. callistephi*) and 26 mm (*S. lycopersici*) to 21-16 mm (*S. solani*), 16-22 mm (*S. trifolii*) and 10-12 mm (*S. lancipes*).

Stemphylium loti and *S. sarciniforme* had similar conidial size (29-30 x 22-23 µm and 26-31 x 21-25 µm, respectively), similar L/W ratio (1.3-1.4 and 1.1-1.3, respectively), lack of ascomata in culture and grew slowly on DRYES (6-16 mm). *Stemphylium globuliferum* and *S. gracilariae* had conidial sizes of 20-27 x 15-19 µm and 21-28 x 13-16 µm, respectively. Both species produced ascomata and had the same L/W ratio (1.4-1.7) and diameter on DRYES (14-26 mm).

With a few exceptions, the rest of the strains (62 in all) identified as *S. astragali, S. beticola, S. botryosum, S. eturmiunum, S. simmonsii, S. vesicarium* (including former *S. alfalfae* and *S. herbarum*) and strains with no species identification were more or less similar. Common for all of them was the production of ascomata, conidial size of 24-45 × 13-23 µm (average: 31 x 17 µm), L/W ratios between 1.3 and 2.5 (average: 1.8), but no clear species segregation was seen. Figure 2 shows the morphology of a selection of strains from this cluster. One of the exceptions was *S. vesicarium* # 25 (ex-type culture of *S. herbarum* (CBS 191.86)). It did not produce ascomata, produced only a few conidia and was very restricted in its growth on DRYES.

3.2 Chemistry

The cluster analysis in Figure 3 is based on 219 secondary metabolites of both known and unknown structure and shows that *S. globuliferum, S. gracilariae, S. lancipes, S. loti, S. majusculum, S. sarciniforme, S. solani* and *S. trifolii* form their own distinct clusters based on the production of species-specific metabolites or unique combinations of metabolites. However, several species were not completely separated. Cluster 1 contains strains identified as *S. botryosum, S. eturmiunum, S. lycopersici* and *S. astragali*, while Cluster 2 contains strains identified as *S. callistephi, S. vesicarium* including strains originally identified as *S. alfalfae* and *S. herbarum*. *Stemphylium* strains in Cluster 2 and *S. trifolii* had the broadest metabolite profile producing between 72 and 93 detectable metabolites, while *S. lancipes* and *S. sarciniforme* produced between 25 and 30 metabolites.

Table 2 gives the production of the known metabolites by *Stemphylium* species with two or more stains together with selected species-specific metabolites of unknown structure. Table 3 gives the Mass [M+H], putative formula and retention time (RT) for each of the unknown metabolites in Table 2.

Stemphypyrone was the only known metabolite produced by all 87 strains, whereas only two of the known metabolites, orobol and solanapyrone A, were species specific for *S. trifolii* and *S. lancipes*, respectively. Stemphyperylenone A was specific to *S. beticola* and *S. simmonsii*. All known metabolites could be detected in one or more strains in Clusters 1 and 2 and only strains in Cluster 2 had one species/cluster specific metabolite of unknown structure (Uke23).

Four species, represented by only one strain each, are not shown in Table 2, but had the following metabolite profiles: *S. astragali* produced alterporriol G/H, altersolanol K/L, macrosporin, stemphylin, stemphyltoxins I to III and stemphyperylenol; *S. callistephi* produced altersolanol K/L, macrosporin, stemphol, stemphylin, stemphyltoxins I to III and stemphyperylenol; *S. lycopersici* produced macrosporin and stemphylin; and *S. simmonsii* produced GsS-1, stemphol, stemphyltoxins I to III and stemphyperylenol. *Stemphylium vesicarium* #25 (ex-type culture of *S. herbarum* CBS 191.86) is not included in Cluster 2 in Table 2, because it produced only half of the metabolites that other *S. vesicarium* and *Stemphylium* sp2 strains produced, which included alterporriol G/H, altersolanol K/L, dehydrocurvularin, GsS-1, macrosporin, stemphol, stemphone, stemphylin, stemphyloxin I/II, stemphyltoxins I to III and stemphyperylenol.

3.3 Host specificity

Comparison between *Stemphylium* species and host (Table 1) did not give any strong connection except between *S. trifolii* and *Trifolium* spp. In general *Stemphylium* species seem to be associated with the pea family *Fabaceae.* A host/metabolite analysis did not show any associations between particular metabolites (known as well as unknown) and host plant.

4. Discussion

4.1 Taxonomy/Nomenclature and Morphology

In recent years, several papers (Câmara et al. 2002; Inderbitzin et al. 2009; Köhl et al. 2009) have suggested that *S. alfalfae*, *S. herbarum* and *S. vesicarium* together with other taxa represent the same species based on molecular data. Our morphological and chemical results are in agreement. Woudenberg et al. (2017) synonymised these species under the oldest name *S. vesicarium* (see www.indexfungorum.org for all synonyms) and throughout the discussion *S. vesicarium* will also be used for strains originally identified as *S. alfalfae* and *S. herbarum*.

Conidial measurements alone have always been problematic to use for identification of *Stemphylium* species. Size and shape of the conidia can vary within the same culture depending on the age. Most young *Stemphylium* conidia are small, spherical/ovoid, with one or few transverse septa. These juvenile conidia become mature within a day or so, developing darker, multiseptate dictyoconidia and assume the shape and size characteristic of its species. The medium also has an influence on conidial size and shape. Our results show that growth on PCA, SNA and V8 yield quite different appearances

(Fig. 1), which might contribute to the uncertainty of morphological identifications. For comparison, it is important to use the same medium. In this study, we have used both PCA and V8 since both media have been used in past descriptions (Simmons 1969, 1989, 2001). However, since SNA is a well-defined medium compared to PCA and V8, experiments should be conducted to see if useful characteristics are preserved on SNA, thus replacing PCA and V8.

Morphologically, species with oblong pointy conidia can be somewhat difficult to distinguish based on measurements of conidia alone, but other characteristics make it possible to distinguish these species. Strains of *S. lancipes* can be distinguished by their lanceolate, irregular conidia with several transverse constrictions and often having secondary conidiophores that emerge from the apex of the conidia. *Stemphylium callistephi, S. lycopersici* and *S. solani* are similar in conidial shape and size, but other characteristics make them distinct. In this study, *S. callistephi* never produced secondary conidia, while *S. lycopersici* grew secondary conidiophores, but only from the apex of the conidia and *S. solani* produced secondary conidiophores from all cells of the conidial body. Also, *S. lycopersici* tend to have a rectangular base compared to the other two species.

Based on conidial size alone *S. trifolii* is similar to *S. eturmiunum*, but *S. trifolii* have smooth, pointy, regular dictyoconidia that are paler in colour, with one darker transverse septum and no prominent constriction. Likewise, *S. majusculum* has conidia appearing similar to *S. vesicarium*, but their larger size and slightly more rectangular shape make them distinguishable. The type strain of *S. majusculum* (# 36 = EGS 29-094) had smaller conidia (43 x 19 µm) in this study compared to the maxima (64 x 35

µm) given by Simmons (1969) in the original description, but similar dimensions to that (49 x 22 µm) reported by Câmara et al. (2002). We can offer no explanation for these findings.

As described by Graham (1953) *S. loti* can be distinguished from *S. sarciniforme* by the paler colour of the conidia and conidiophores. The conidial shape of *S. loti* is similar to that of *S. globuliferum*, but this species can be distinguished by the limited growth on PDA of *S. loti* (15-30 mm) compared to *S. globuliferum* (41-69 mm). The conidia of *S. beticola* and *S. simmonsii* are similar to those of *S. globuliferum* and *S. loti* and therefore other methods like phylogeny used by Woudenberg et al. (2017) or chemotaxonomy should be used for distinguishing these species. Juvenile conidia of *S. gracilariae* are often ellipsoidal compared with the subglobose juvenile conidia of *S. globuliferum* and can be used to distinguish between the two species.

With the above described species *S. vesicarium*, *S. botryosum*, *S. eturmiunum* and other small-spored *Stemphylium* remain to be given significant distinguishable morphological traits. This requires intense expert knowledge, and therefore the distinguishing of these species should be done by other methods than morphology, such as multi-locus phylogeny as described by Câmara et al. (2002), Inderbitzin et al. (2009) and Woudenberg et al. (2017).

4.2 Chemotaxonomy

The results from this study show that metabolites alone are able distinguish most *Stemphylium* species with the exception of *S. botryosum* and *S. eturmiunum* in Cluster 1. Species that are only

284 represented by one strain such as *S. astragali, S. callistephi* and *S. lycopersici* must be studied further
285 with at least one other strain in order to find species-specific metabolites.
286
287 Our results show a distinct *S. globuliferum* cluster, containing the five strains (#15 (CBS 716.68 =
288 EGS17-151), #16 (FIP 108 = EGS 48-099), #17 (FIP186), #18 (FIP191), and #19 (FIP220). However, the
289 phylogenetical results of Woudenberg et al (2017) placed strains originally identified as *S.*
290 *globuliferum* with *S. simmonsii*, since these strains did not form their own cluster. Two of those strains
291 (#15 and #70 (FIP 227 = EGS 38-115 = CBS 133894)), which have been renamed *S. simmonsii* by
292 Woudenberg et al (2017), are also included in this study. One strain, #15, clusters with four other *S.*
293 *globuliferum* strains, whereas #70 clusters next to two *S. beticola* strains in our chemotaxonomy. This
294 discrepancy suggests that *S. beticola, S. globuliferum* and *S. simmonsii* are closely related, both
295 morphologically and molecularly, but not chemically. Strains of *S. globuliferum* produce stemphylin
296 and macrosporin, which neither *S. beticola* nor *S. simmonsii* do. Further molecular and chemical
297 analyses of the same material are needed in order to determine the true identity of these strains.
298
299 The metabolic profiles of *Stemphylium* seem to be more related to some of the large-spored, plant
300 pathogenic *Alternaria* species like *A. porri* and *A. solani* (Andersen et al. 2008) and *Ulocladium*
301 (Andersen and Hollensted 2008), than with the small-spored, saprobic *Alternaria*, such as *A. alternata*
302 (Polizzotto et al. 2012) and *A. infectoria* (Christensen et al. 2005). None of the *Stemphylium* strains
303 produced alternariols, altenuenes, tenuazonic acid or infectopyrones. Stemphypyrone is produced by
304 all strains as mentioned previously. It has only been isolated from one other genus of fungi, namely

Exserohilum sp. (Li et al. 2014), and thus stemphypyrone can be used as a chemical marker for the genus *Stemphylium*. Most of the known metabolites detected in this study (Table 2) have previously been found in strains of *Stemphylium*. Our results show that the production of known metabolites is not consistent in all stains of the same species (e.g. *S. gracilariae*) and often occurs in more than one species (e.g. macrosporin). On the other hand, all species in Table 2 were able to produce species-specific metabolites of unknown structure that could distinguish them from other species. Several novel connections have been made. All four strains of *S. loti* produced pyrenophorin and pyrenophorol, which are also produced by *Phoma* sp. and have antimicrobial activities (Zhang et al. 2008). All five strains of *S. trifolii* produced orobol, an isoflavone produced in red clover (*Trifolium pratense* (Klejdus et al. 2001)), which is interesting, since all five strains were isolated from clover. *Stemphylium trifolii* seems to be particularly adapted to *Trifolium* spp. in that both fungus and plant produce orobol. Other species, like *S. globuliferum* and *S. simmonsii*, also isolated from *Trifolium* spp., did not produce orobol. Two metabolites (Ukn185 and Ukn212) of unknown structure, but with recognizable UV-spectra, mass and RT (Table 3), were produced in large quantities by *S. beticola* and *S. simmonsii*. These two metabolites have previously been detected in species of *Chalastospora* as metabolites 1010 and 1120, respectively (Andersen et al. 2009). Unknown metabolites with phytotoxic activity have been reported from *Stemphylium*, such as SV- and SS-toxins (Zheng et al. 2010; Tanahashi et al. 2017), but no molecular information has been given, so direct comparison is not possible.

Metabolite profiling can be a powerful tool in fungal identification, but it has its limitations when it comes to strains that have been maintained and re-cultured for many years in culture collections. Our strain of the ex-type culture of *S. herbarum*, #25 (EGS 36-138 = CBS191.86), now *S. vesicarium*, has stopped sporulating and is also losing its ability to produce metabolites. The same phenomenon has been observed in *Alternaria* (Andersen et al. 2008). Only strains that can be unequivocally identified morphologically should be used in the selection process of species-specific metabolites or chemotaxonomic markers.

4.3 Host specificity

No connections were made between individual species and host plants. Some *Stemphylium* species, such as *S. globuliferum*, *S. sarciniforme* and *S. trifolii*, were isolated from species of alfalfa, clover, lentils, and pea (Table 1). Other species/taxa, like *S. eturmiunum*, *S. vesicarium* and *Stemphylium* sp. 2, have a broader host range comprising *Amaryllidaceae*, *Apiaceae*, *Brassicaceae*, *Poaceae*, *Rosaceae* and *Solanaceae* (Table 1). A search in U.S. National Fungus Collections shows that the species *S. vesicarium* (including *S. alfalfae* and *S. herbarum*) will have an extremely broad host range (Farr and Rossman 2017). One reason that a species can have such a broad host range could be that all strains produce that same non-host-specific metabolites. Trigos et al. (2011) proposed that macrosporin is a non-host specific toxin that plays a role in leaf necrosis due to its photosensitizing ability. Since macrosporin is a non-species-specific metabolite produced by 58 (67 %) of the tested strains, this metabolite might be a contributing factor to the broad host range of *Stemphylium,* especially among *S. botryosum* and *S. vesicarium*. It may also explain why one strain can be pathogenic to several, very

346 different host plants. Neergaard (1945) tested the pathogenicity of several strains of *S. botryosum* and
347 found that they had a broad host range attacking cabbage, carrot, lettuce, onion, pea, tomato,
348 *Dianthus* and *Godetia*, but neither wheat nor cucumber. Similarly, strains of *S. lycopersici* have shown
349 to have a broad host range (Nasehi et al. 2014) being pathogenic to tomato, eggplant, pepper and
350 lettuce, regardless of original host. However, none of the *S. lycopersici* strains were pathogenic to
351 cabbage (Nasehi et al. 2014).
352
353 **5. Conclusion**
354 The chemical potential of the genus *Stemphylium* is broad as numerous unknown compounds have
355 been found in this study. The chemotaxonomic investigation of the whole genus revealed
356 distinguishable characteristics for most of the included species, while a subset of the investigated
357 strains produced similar metabolic profiles. Our chemotaxonomic study supports the phylogenetically
358 based findings by Woudenberg et al. (2017) who proposed to synonymize *S. alfalfae, S. herbarum, S.*
359 *vesicarium* and others into *S. vesicarium*. The results from this study show that at least two to four
360 strains of a species are necessary to give diverging branches in the chemotaxonomy. Therefore, future
361 chemotaxonomic investigations should include more species and more strains from some of the
362 investigated species, such as *S. astragali, S. callistephi* and *S. lycopersici.* Also, as presented here, a
363 solid group of a single species can identify species-specific metabolites, which can be used for
364 identification. Furthermore, investigation and comparison of conidial morphology showed differences
365 in conidial size from the same strain, when comparing conidia from different media. Thus, the
366 cultivation conditions have implications when comparing results to described reference strains.

367

368 **6. Acknowledgement**

369 We would like to thank Kristian Fog Nielsen for technical and analytical assistance while conducting

370 HPLC-UV-MS analysis.

371

372 **References**

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504 **Table 1.** *Stemphylium* strains used in this study with original and new name, host and country of
505 origin.

Analysis #	ID #[a]	New names[b]	Original names[c]	Host	Origin
1	CBS 192.86*	*S. vesicarium*	***S. alfalfae* T**	*Medicago sativa*	Australia
2	FIP 151*	*S. vesicarium*	*S. alfalfae*	*Medicago sativa*	USA
3	FIP 152*	*S. vesicarium*	*S. alfalfae*	*Medicago sativa*	USA
4	FIP 149	*S. astragali*	*S. astragali*	*Astragalus sinicus*	Japan
5	CBS 714.68*	*S. botryosum*	*S. botryosum*	*Medicago sativa*	Canada
6	FIP 112	*S. botryosum*	*S. botryosum*	*Medicago sativa*	New Zealand
7	FIP 166	*S. callistephi*	*S. callistephi*	*Callistephus chinensis*	USA
8	FIP 080	*S. eturmiunum*	*Stemphylium* sp.	*Brassica oleracea*	USA
9	FIP 109	*S. eturmiunum*	*S. eturmiunum*	*Vicia sativa*	New Zealand
10	FIP 266	*S. eturmiunum*	*Stemphylium* sp.	-	India
11	IBT 8213	*S. eturmiunum*	*S. eturmiunum*	*Hordeum vulgare*	Denmark
12	IBT 8224	*S. eturmiunum*	*S. eturmiunum*	*Brassica napus*	Italy
13	IBT 8231*	*S. eturmiunum*	*S. eturmiunum*	*Solanum lycopersicum*	Greece
14	IBT 40618	*S. eturmiunum*	*S. eturmiunum*	*Capsicum annuum*	Denmark
15	CBS 716.68*	*S. globuliferum*	*S. globuliferum*	*Commelina* sp.	USA
16	FIP 108	*S. globuliferum*	*Stemphylium* sp.	*Medicago lupulina*	New Zealand
17	FIP 186	*S. globuliferum*	*S. botryosum*	*Medicago sativa*	USA
18	FIP 191	*S. globuliferum*	*Stemphylium* sp.	*Trifolium repens*	USA
19	FIP 220	*S. globuliferum*	*Stemphylium* sp.	*Trifolium repens*	USA
20	CBS 482.90*	*S. gracilariae*	***S. gracilariae* T**	*Gracilaria* sp.	Israel
21	FIP 001	*S. gracilariae*	*Stemphylium* sp.	-	USA
22	FIP 003	*S. gracilariae*	*Stemphylium* sp.	-	USA
23	FIP 084	*S. gracilariae*	*Stemphylium* sp.	*Brassica napus*	Italy
24	IBT 8227	*S. gracilariae*	*Stemphylium* sp.	*Brassica napus*	Italy
25	CBS 191.86*	*S. vesicarium*	***S. herbarum* T**	*Medicago sativa*	India
26	FIP 015	*Stemphylium* sp. 1	*Stemphylium* sp.	*Pisum sativum*	New Zealand
27	FIP 023	*Stemphylium* sp. 1	*Stemphylium* sp.	*Daucus carota*	New Zealand
28	FIP 184	*Stemphylium* sp. 2	*Stemphylium* sp.	*Medicago sativa*	New Zealand
29	CBS 101217*	*S. lancipes*	*S. lancipes*	*Aquilegia* sp.	New Zealand
30	FIP 153*	*S. lancipes*	***S. lancipes* T**	*Aquilegia* sp.	New Zealand
31	FIP 162	*S. loti*	*S. loti*	-	-
32	FIP 174	*S. loti*	*S. loti*	*Lotus corniculatus*	USA
33	FIP 175	*S. loti*	*S. loti*	*Lotus corniculatus*	USA
34	FIP 217	*S. loti*	*Stemphylium* sp.	-	-
35	FIP 156*	*S. lycopersici*	*S. lycopersici*	*Solanum lycopersicum*	Dominican Rep.
36	FIP 129*	*S. majusculum*	***S. majusculum* T**	*Lathyrus maritimus*	USA
37	IBT 8223	*S. majusculum*	*Stemphylium* sp.	*Lathyrus maritimus*	USA
38	FIP 170	*S. sarciniforme*	*S. loti*	*Lotus corniculatus*	USA
39	FIP 238*	*S. sarciniforme*	*Stemphylium* sp.	*Cicer arietinum*	Iran
40	IBT 8217*	*S. sarciniforme*	*S. sarciniforme*	*Cicer arietinum*	USA
41	IBT 8221	*S. sarciniforme*	*S. sarciniforme*	*Cicer arietinum*	Iran
42	CBS 408.54*	*S. solani*	*S. solani*	*Solanum lycopersicum*	USA
43	FIP 125	*S. solani*	*S. solani*	*Solanum lycopersicum*	USA

Analysis #	ID #[a]	New names[b]	Original names[c]	Host	Origin
44	FIP 137	*S. solani*	*S. solani*	*Coronilla* sp.	-
45	FIP 138	*S. solani*	*S. solani*	*Lupinus*	USA
46	BA 1399	*Stemphylium* sp. 2	*Stemphylium* sp.	*Quercus* sp.	Spain
47	BA 2319	*Stemphylium* sp. 2	*Stemphylium* sp.	*Malus* sp.	USA
48	BA 463	*Stemphylium* sp. 2	*Stemphylium* sp.	*Prunus avium*	Denmark
49	BA 516	*Stemphylium* sp. 2	*Stemphylium* sp.	*Prunus avium*	Denmark
50	BA 570	*Stemphylium* sp. 2	*Stemphylium* sp.	*Prunus avium*	Denmark
51	BA 608	*Stemphylium* sp. 2	*Stemphylium* sp.	*Prunus avium*	Denmark
52	FIP 026	*Stemphylium* sp. 1	*Stemphylium* sp.	*Daucus carota*	New Zealand
53	FIP 035	*S. beticola*	*Stemphylium* sp.	*Spinacia oleracea*	USA
54	FIP 066	*Stemphylium* sp. 1	*Stemphylium* sp.	*Pisum sativum*	New Zealand
55	FIP 083	*Stemphylium* sp. 2	*Stemphylium* sp.	*Allium cepa*	Mexico
56	FIP 107	*Stemphylium* sp. 1	*Stemphylium* sp.	*Medicago sativa*	New Zealand
57	FIP 110	*Stemphylium* sp. 2	*Stemphylium* sp.	*Trifolium pratense*	New Zealand
58	FIP 113	*Stemphylium* sp. 2	*Stemphylium* sp.	*Medicago sativa*	New Zealand
59	FIP 145	*Stemphylium* sp. 2	*Stemphylium* sp.	*Malus* sp.	New Zealand
60	FIP 157	*S. botryosum*	*S. botryosum*	*Medicago sativa*	USA
61	FIP 163	*S. botryosum*	*S. botryosum*	*Medicago sativa*	USA
62	FIP 165	*Stemphylium* sp. 2	*S. botryosum*	-	-
63	FIP 173	*S. botryosum*	*S. botryosum*	*Lupinus*	USA
64	FIP 178	*Stemphylium* sp. 2	*Stemphylium* sp.	*Petroselinum crispum*	USA
65	FIP 179	*Stemphylium* sp. 2	*Stemphylium* sp.	*Petroselinum crispum*	USA
66	FIP 180	*Stemphylium* sp. 2	*Stemphylium* sp.	*Petroselinum crispum*	USA
67	FIP 181	*Stemphylium* sp. 2	*Stemphylium* sp.	*Petroselinum crispum*	USA
68	FIP 182	*Stemphylium* sp. 2	*Stemphylium* sp.	*Petroselinum crispum*	USA
69	FIP 222*	*S. beticola*	*Stemphylium* sp.	*Lens culinaris*	USA
70	FIP 227*	*S. simmonsii*	*Stemphylium* sp.	*Trifolium pratense*	USA
71	FIP 230	*Stemphylium* sp. 2	*Stemphylium* sp.	*Lens culinaris*	USA
72	FIP 242	*Stemphylium* sp. 1	*Stemphylium* sp.	*Trifolium pratense*	-
73	FIP 289	*S. botryosum*	*S. botryosum*	*Allium fistulosum*	France
74	FIP 292	*S. botryosum*	*S. botryosum*	*Allium fistulosum*	France
75	IBT 10199	*Stemphylium* sp. 2	*Stemphylium* sp.	*Citrus maxima*	-
76	IBT 8214	*Stemphylium* sp. 2	*Stemphylium* sp.	*Trigonella foenum-graecum*	Egypt
77	IBT 8220	*Stemphylium* sp. 2	*Stemphylium* sp.	*Pyrus* sp.	Italy
78	IBT 9032	*Stemphylium* sp. 2	*Stemphylium* sp.	*Triticum aestivum*	Denmark
79	FIP 140	*S. trifolii*	*S. trifolii*	*Trifolium repens*	-
80	FIP 141	*S. trifolii*	*S. trifolii*	*Trifolium repens*	Canada
81	FIP 194	*S. trifolii*	*S. trifolii*	*Trifolium repens*	-
82	FIP 197	*S. trifolii*	*S. trifolii*	*Trifolium* sp.	-
83	FIP 241	*S. trifolii*	*Stemphylium* sp.	*Trifolium* sp.	-
84	CBS 715.68*	*S. vesicarium*	*S. vesicarium*	*Pisum sativum*	Canada
85	FIP 057*	*S. vesicarium*	*S. herbarum*	*Lathyrus odoratus*	Netherlands
86	IBT 7159	*S. vesicarium*	*Stemphylium* sp.	*Hordeum vulgare*	Denmark
87	IBT 7161	*S. vesicarium*	*Stemphylium* sp.	*Hordeum vulgare*	Denmark

506

[a] Culture collections from where the strain originated. BA: Collection of Birgitte Andersen (part of the IBT collection); CBS: Centraalbureau voor Schimmelcultures, The Netherlands; IBT and FIP: Department of Bioengineering, Technical University of Denmark. *Strains also treated in Woudenberg et al. (2017). All known identification numbers for each strain can be found in supplementary material **Table S1**.

[b] New name corresponding to the morphological and chemical findings in this study and the phylogeny by Woudenberg et al. (2017). *Stemphylium* sp. 1 and 2 refer to the location in cluster 1 and 2, respectively, of the strain in Figure 1.

[c] The original name/identity the culture arrived with from the culture collection.

517 **Table 2.** Production of known metabolites and unknown species-specific metabolites by different
518 *Stemphylium* species (n= number of strains). Clu 1 contains *S. botryosum, S. eturmiunum* and
519 *Stemphylium* sp1 strains and Clu 2 contains *S. vesicarium* (including *S. alfalfae* and *S. herbarum*) and
520 *Stemphylium* sp2 strains.

Metabolite[a]	beti (n=2)	glob (n=5)	grac (n=5)	lanc (n=2)	loti (n=4)	maju (n=2)	sarc (n=4)	sola (n=4)	trif (n=5)	Clu 1 (n=20)	Clu 2 (n=29)
Alterporriol G/H	-	5	-	1	-	-	-	4	4	8	12
Alterporriol I/J	-	-	-	-	-	-	-	3	-	-	3
Altersolanol A (=Stemphylin)	-	5	5	1	-	1	-	4	5	17	25
Altersolanol K/L	-	5	3	1	4	-	-	4	4	14	15
Altersolanol M	-	3	-	-	-	-	-	2	-	2	1
Altertoxin II (= stemphyltoxin II)	1	4	5	1	-	2	-	-	1	17	28
Curvularin	-	-	-	-	-	-	-	-	-	1	12
Dehydrocurvularin	-	-	-	-	-	-	-	-	-	1	12
Macrosporin	-	5	4	2	-	-	-	4	5	15	19
Orobol	-	-	-	-	-	-	-	-	5	-	-
Pyrenophorin	-	-	-	-	4	1	-	-	-	7	1
Pyrenophorol	-	-	-	-	4	-	-	-	-	2	-
Solanapyrone A	-	-	-	2	-	-	-	-	-	-	-
Stemphol	2	2	2	-	4	2	2	4	-	18	17
Stemphone	1	-	-	-	1	-	4	-	5	4	7
Stemphyloxin I/II	-	-	-	-	-	-	-	-	1	3	2
Stemphyltoxin I	1	4	5	1	-	-	-	-	-	11	20
Stemphyltoxin III	1	5	5	1	-	2	-	-	1	17	25
Stemphyperylenol	2	5	5	1	-	2	-	1	5	20	28
Stemphypyrone	2	5	5	2	4	2	4	4	5	20	29
Ukn095	2	-	-	-	-	-	-	-	-	-	-
Ukn185 [b]	2	-	-	-	-	-	-	-	-	-	-
Ukn212 [b]	2	-	-	-	-	-	-	-	-	-	-
Ukn074	-	5	5	-	-	-	-	-	-	-	-
Ukn094	-	-	5	-	-	-	-	-	-	-	-
Ukn287	-	-	5	-	4	-	-	-	-	-	-
Ukn063	-	-	-	-	4	-	-	-	-	-	-
Ukn191	-	-	-	2	4	2	-	-	-	-	-
Ukn210	-	-	-	2	-	-	-	-	-	-	-
Ukn054	-	-	-	-	-	-	-	-	5	-	-
Ukn184	-	-	-	-	-	-	4	-	5	-	-
Ukn116	-	-	-	-	-	-	4	-	-	-	-
Ukn196	-	-	-	-	-	-	-	4	-	-	-
Ukn224	-	-	-	-	-	-	-	-	-	-	23

521 [a] Metabolite identification are based on comparison of UV-spectrum and exact mass.

522 [b] Ukn185 and Ukn212 are identical to metabolites 1010 and 1120 in Andersen et al. 2009.
523

524 **Table 3.** Retention time (RT), *m/z* of the $[M+H]^+$ adduct and a proposed molecular formula for the
525 unknown species specific *Stemphylium* metabolites given in Table 2.

Metabolite	RT (min)	Mass $[M+H]^+$	Putative formula
Ukn095	4.7	205.086	$C_{12}H_{12}O_3$
Ukn185	6.7	409.165	$C_{24}H_{24}O_6$
Ukn212	7.4	409.165	$C_{24}H_{24}O_6$
Ukn074	4.2	235.060	$C_{12}H_{10}O_5$
Ukn094	4.7	319.227	$C_{20}H_{30}O_3$
Ukn287	10.6	273.258	$C_{20}H_{32}$
Ukn063	3.9	184.097	$C_9H_{13}NO_3$
Ukn191	6.8	375.180	$C_{21}H_{26}O_6$
Ukn210	7.4	345.170	$C_{20}H_{24}O_5$
Ukn054	3.8	286.155	$C_{16}H_{19}N_3O_2$
Ukn184	6.7	471.274	$C_{28}H_{38}O_6$
Ukn116	5.3	836.362	$C_{29}H_{45}N_{19}O_{11}$
Ukn196	6.8	430.224	$C_{25}H_{27}N_5O_2$
Ukn224	8	365.316	$C_{22}H_{40}N_2O_2$

526
527

528 **Figure captions**

529

530 **Fig. 1.** Morphology of selected *Stemphylium* strains after 7 days of growth on SNA (A, B and C), PCA
531 (D, E and F) and V8 (G, H and I). A, D and G are *Stemphylium* sp. (#76), B, E and H are *S. sarciniforme*
532 (#40) and C, F and I are *S. gracilariae* (#24). Scale bar is 50 µm.

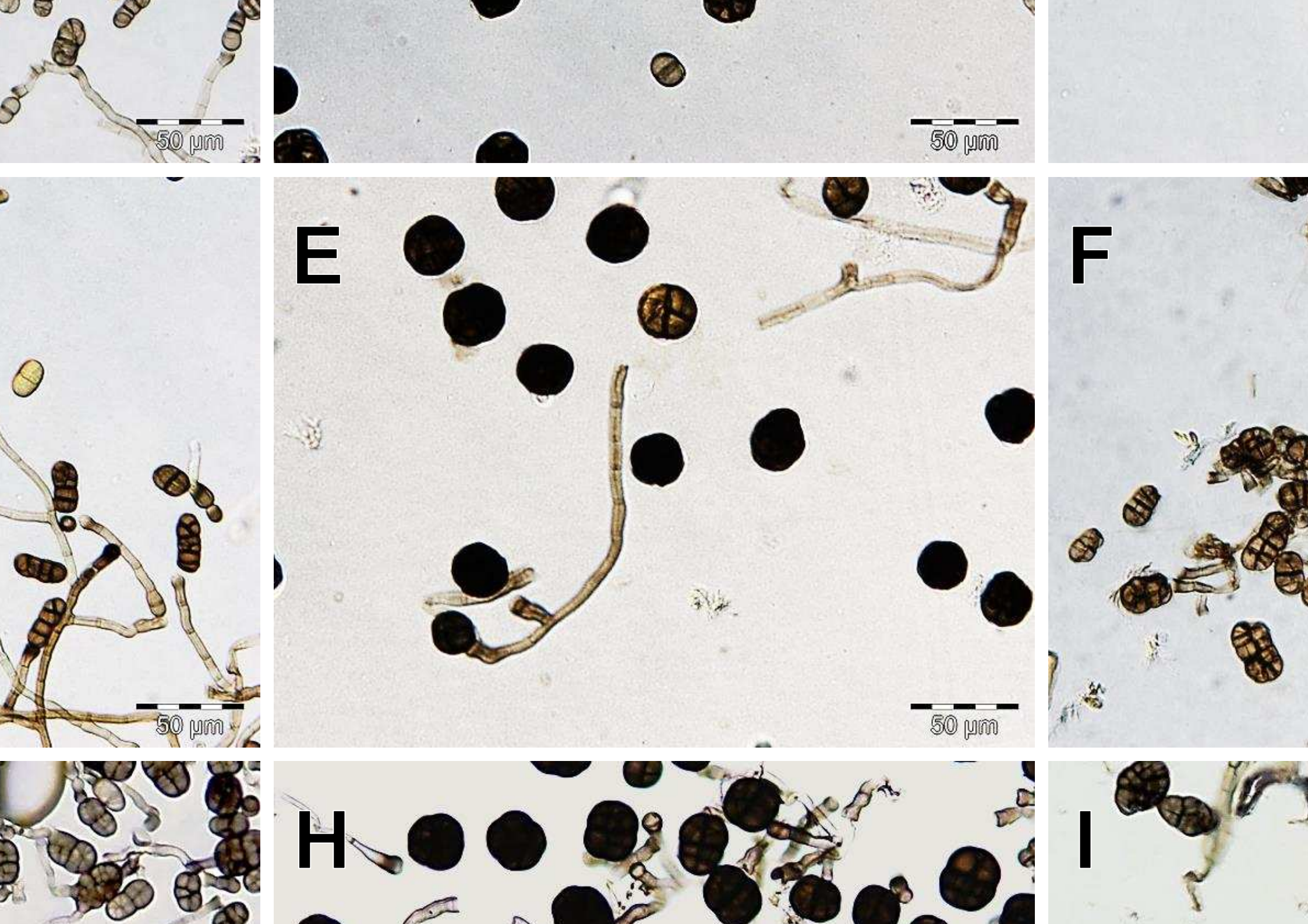
533

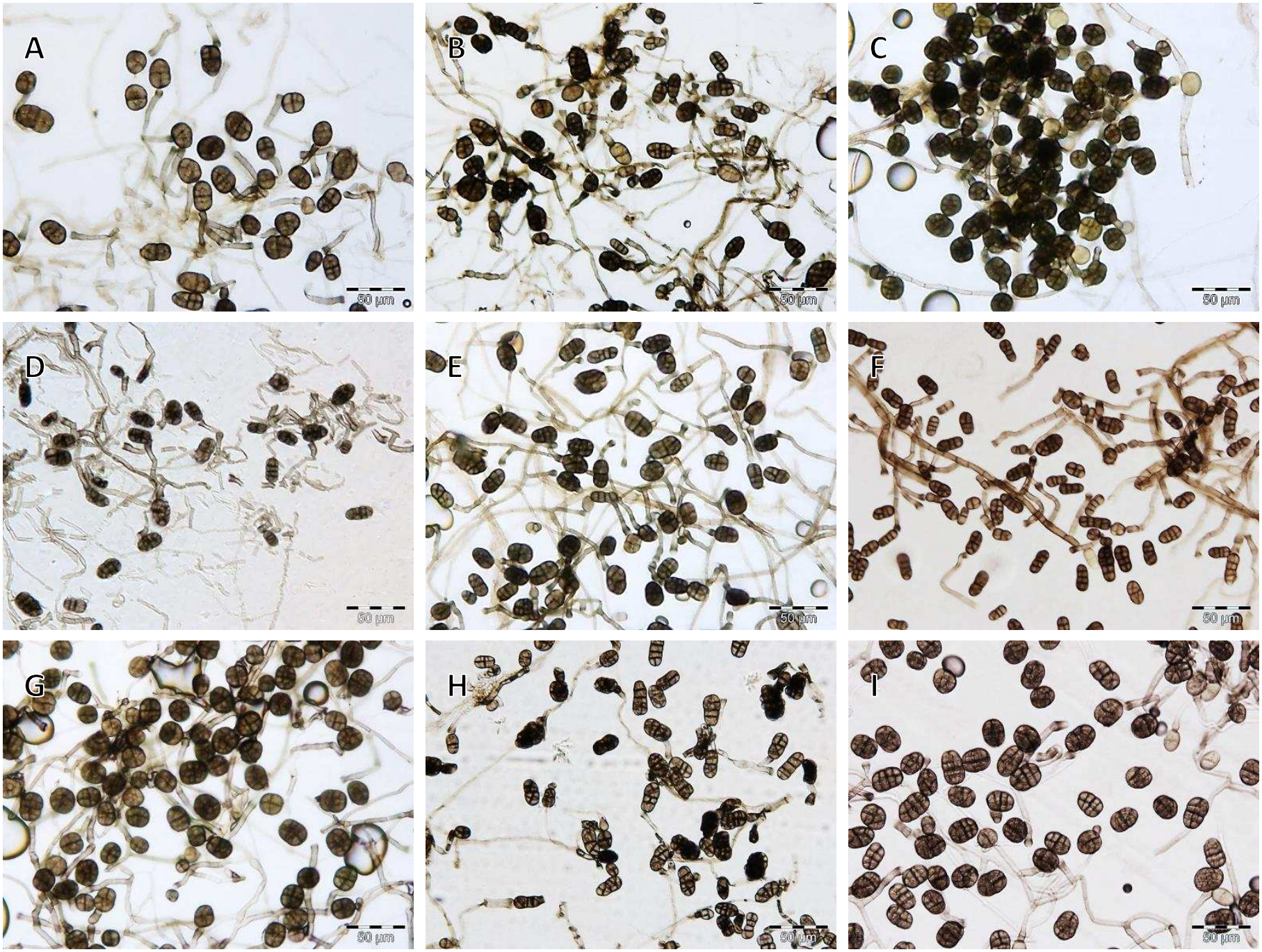
534 **Fig. 2.** Morphology of selected *Stemphylium* strains after 7 days of growth on PCA. A: *S. botryosum*
535 (#60), B: *Stemphylium* sp. 2 (#62), C: *S. botryosum* (#73), D: *S. vesicarium* (#84), E: *S. vesicarium* (#03),
536 F: *S. vesicarium* (#85), G: *S. simmonsii* (#70), H: *S. eturmiunum* (#13) and I: *S. globuliferum* (#19). Scale
537 bar is 50 µm.

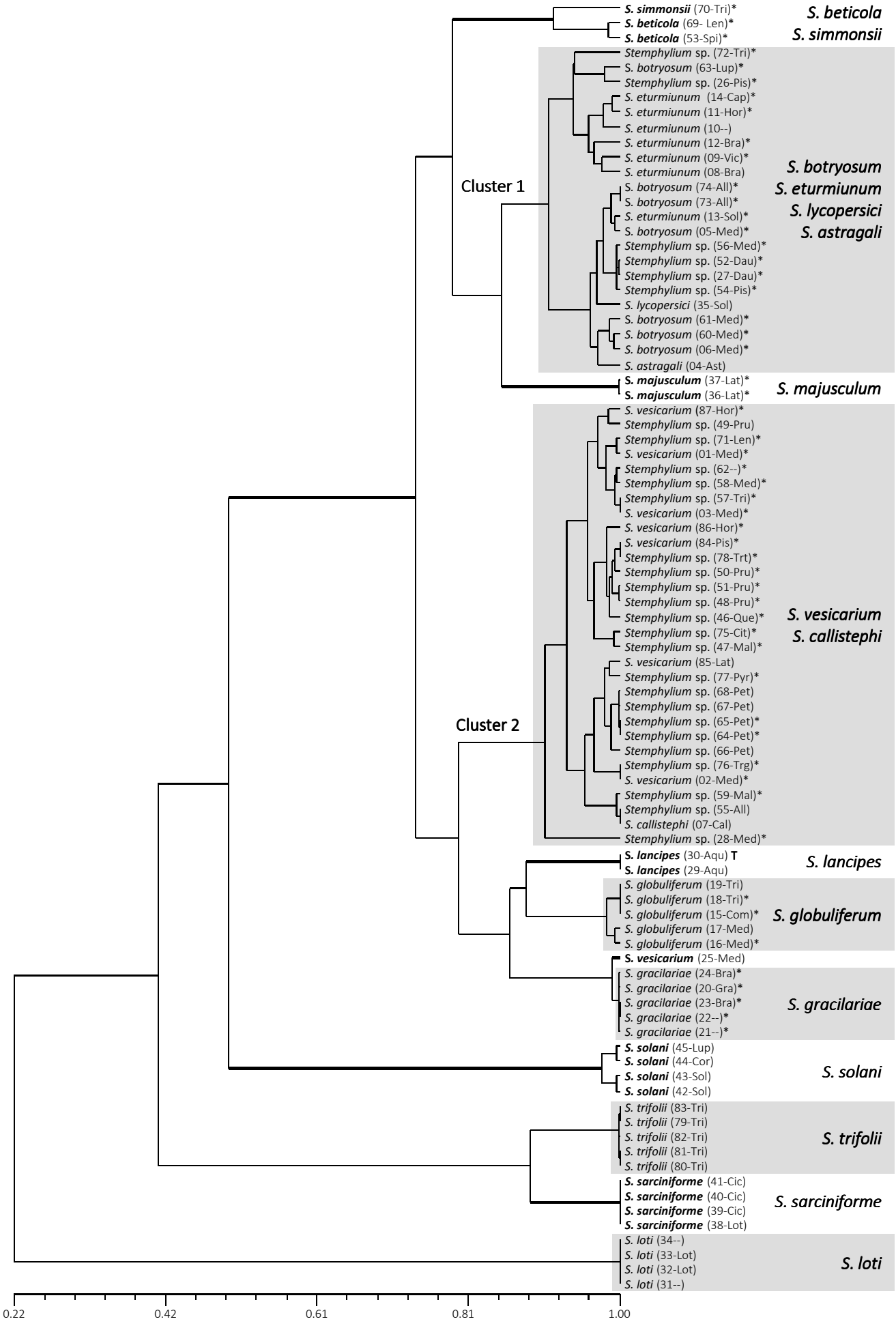
538

539 **Fig. 3.** Dendrogram based on a cluster analysis of 87 Stemphylium strains and 219 known and
540 unknown metabolites. Strain labels: strain ID (analysis number-host) as given in Table 1. T: type
541 culture. *: ascomata produced on PCA. The dendrogram is calculated using the Yule correlation
542 coefficient and UPGMA as the clustering method and the axis shows the correlation coefficient.

543


50 µm
50 µm
E
50 µm
F
H
I


A
50 µm
B
50 µm
C
50 µm
D
50 µm
E
50 µm
F
50 µm
G
50 µm
H
50 µm
I
50 µm


S. simmonsii (70-Tri)*
S. beticola (69- Len)*
S. beticola (53-Spi)*
S. beticola
S. simmonsii
Stemphylium sp. (72-Tri)*
S. botryosum (63-Lup)*
Stemphylium sp. (26-Pis)*
S. eturmiunum (14-Cap)*
S. eturmiunum (11-Hor)*
S. eturmiunum (10--)
S. eturmiunum (12-Bra)*
S. eturmiunum (09-Vic)*
S. eturmiunum (08-Bra)
S. botryosum (74-All)*
S. botryosum (73-All)*
S. eturmiunum (13-Sol)*
S. botryosum (05-Med)*
Stemphylium sp. (56-Med)*
Stemphylium sp. (52-Dau)*
Stemphylium sp. (27-Dau)*
Stemphylium sp. (54-Pis)*
S. lycopersici (35-Sol)
S. botryosum (61-Med)*
S. botryosum (60-Med)*
S. botryosum (06-Med)*
S. astragali (04-Ast)
S. botryosum
S. eturmiunum
S. lycopersici
S. astragali
Cluster 1
S. majusculum (37-Lat)*
S. majusculum (36-Lat)*
S. majusculum
S. vesicarium (87-Hor)*
Stemphylium sp. (49-Pru)
Stemphylium sp. (71-Len)*
S. vesicarium (01-Med)*
Stemphylium sp. (62--)*
Stemphylium sp. (58-Med)*
Stemphylium sp. (57-Tri)*
S. vesicarium (03-Med)*
S. vesicarium (86-Hor)*
S. vesicarium (84-Pis)*
Stemphylium sp. (78-Trt)*
Stemphylium sp. (50-Pru)*
Stemphylium sp. (51-Pru)*
Stemphylium sp. (48-Pru)*
Stemphylium sp. (46-Que)*
Stemphylium sp. (75-Cit)*
Stemphylium sp. (47-Mal)*
S. vesicarium (85-Lat)
Stemphylium sp. (77-Pyr)*
Stemphylium sp. (68-Pet)
Stemphylium sp. (67-Pet)
Stemphylium sp. (65-Pet)*
Stemphylium sp. (64-Pet)*
Stemphylium sp. (66-Pet)
Stemphylium sp. (76-Trg)*
S. vesicarium (02-Med)*
Stemphylium sp. (59-Mal)*
Stemphylium sp. (55-All)
S. callistephi (07-Cal)
Stemphylium sp. (28-Med)*
S. vesicarium
S. callistephi
Cluster 2
S. lancipes (30-Aqu) T
S. lancipes (29-Aqu)
S. lancipes
S. globuliferum (19-Tri)
S. globuliferum (18-Tri)*
S. globuliferum (15-Com)*
S. globuliferum (17-Med)
S. globuliferum (16-Med)*
S. globuliferum
S. vesicarium (25-Med)
S. gracilariae (24-Bra)*
S. gracilariae (20-Gra)*
S. gracilariae (23-Bra)*
S. gracilariae (22--)*
S. gracilariae (21--)*
S. gracilariae
S. solani (45-Lup)
S. solani (44-Cor)
S. solani (43-Sol)
S. solani (42-Sol)
S. solani
S. trifolii (83-Tri)
S. trifolii (79-Tri)
S. trifolii (82-Tri)
S. trifolii (81-Tri)
S. trifolii (80-Tri)
S. trifolii
S. sarciniforme (41-Cic)
S. sarciniforme (40-Cic)
S. sarciniforme (39-Cic)
S. sarciniforme (38-Lot)
S. sarciniforme
S. loti (34--)
S. loti (33-Lot)
S. loti (32-Lot)
S. loti (31--)
S. loti
0.22
0.42
0.61
0.81
1.00