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1 Metabolite production by species of *Stemphylium*

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- 10

11 Abstract

- 12 Morphology and phylogeny has been used to distinguish members of the plant pathogenic fungal 13 genus Stemphylium. A third method for distinguishing species is by chemotaxonomy. The main goal of 14 the present study was to investigate the chemical potential of *Stemphylium* via HPLC-UV-MS analysis, 15 while also exploring the potential of chemotaxonomy as a robust identification method for 16 Stemphylium. Several species were found to have species-specific metabolites, while other species 17 were distinguishable by a broader metabolic profile rather than specific metabolites. Many previously 18 described metabolites were found to be important for distinguishing species, while some unknown 19 metabolites were also found to have important roles in distinguishing species of Stemphylium. This 20 study is the first of its kind to investigate the chemical potential of Stemphylium across the whole 21 genus.
- 22

23 Keywords:

Antibacterial metabolites, chemotaxonomy, host specific toxins, morphology, orobol, phytotoxins
 25

26 **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

32	a broad range, such as <i>S. vesicarium</i> , which causes purple spot of asparagus and brown spot of pear
33	but is also able to live as a saprobe on plant debris (Graf et al. 2016; Köhl et al. 2009; Puig et al. 2015).
34	Some species, like S. botryosum, S. eturmiunum and S. vesicarium, can also occur on food products
35	such as beans, pulses, tomato, apple, pear and cereal grain (Pitt and Hocking 2009; Samson et al.
36	2010; Snowdon 1990). Though Stemphylium metabolites have been detected in mouldy tomatoes
37	(Andersen and Frisvad 2004), no mycotoxins sensu stricto have been associated with Stemphylium
38	food spoilage.
39	
40	Morphologically, Stemphylium is easy to distinguish from its relatives, Alternaria Nees and Ulocladium
41	Preuss, by its percurrent or annellidic proliferation often with a distinct terminal swelling (Simmons
42	1967). Phylogenetically, the genus is also easy to delimit from Alternaria and Ulocladium (Ariyawansa
43	et al. 2015). Within Stemphylium some species such as S. botryosum and S. globuliferum or S.
44	eturmiunum and S. vesicarium appear similar and may be mixed up and misidentified using
45	morphology alone whereas some taxa previously recognized as distinct species such as S. alfalfa, S.
46	herbarum, S. vesicarium and others, fall in the same phylogenetic clade (Câmara et al. 2002;
47	Inderbitzin et al. 2009) and are now based on molecular data synonymized as S. vesicarium
48	(Woudenberg et al. 2017).

49

50 Chemically, individual *Stemphylium* strains have been shown to produce a broad variety of secondary 51 metabolites, of which many probably play a role during host plant infection as phytotoxins or host-52 specific toxins (Trigos et al. 2011). Culture extracts of different strains of *S. vesicarium* have, for

53	instance, been shown to be pathogenic to either European pear cultivars or Japanese pear cultivars,
54	but never both (Singh et al. 1999). The extracts contained host-specific toxins (SV-toxins I and II),
55	compounds that have not been structurally elucidated (Tanahashi et al. 2017). Other research has
56	shown that two endophytic strains of <i>S. globuliferum</i> produced alterporriols H and K, altersolanol L,
57	stemphypyrone (Debbab et al. 2009) and alterporriols D and E, altersolanol A (= stemphylin),
58	altersolanols B and C, and macrosporin (Liu et al. 2015), while an endophytic strain of S. botryosum
59	produced altersolanol A (= stemphylin), curvularin, dehydrocurvularin, macrosporin and
60	stemphyperylenol (Aly et al. 2010). Another study has shown a strain of S. herbarum (later identified
61	as Stemphylium sp. by Kurose et al. 2015) that produced alterporriols D-G and altersolanol A
62	(Kanamaru et al. 2012). Recently, it has also been shown that Stemphylium metabolites have
63	biological activities, such as cytotoxic and antibacterial effects (Debbab et al. 2009; Liu et al. 2015)
64	that may be of interest to the pharmaceutical industry.
65	
66	Chemotaxonomy as reviewed by Frisvad et al. (2008) has only been attempted in a few cases on
67	Stemphylium (Andersen et al. 1995) and with little success. However, the study showed that S.
68	majusculum and some strains of S. botryosum produced stemphol (Andersen et al. 1995).
69	Chemotaxonomy has previously been useful in saprobic genera such as Aspergillus and Penicillium
70	(Kim et al. 2012; Kozlovskii et al. 2017) and host-specific plant pathogenic Alternaria (Andersen et al.
71	2008; Brun et al. 2013), but less successful in saprobic or non-pathogenic species of Alternaria
72	(Andersen et al. 2009) and Fusarium (de Kuppler et al. 2011). One purpose of this study was to

73 examine if profiles of secondary metabolites are species-specific according to the latest phylogeny

74 (Woudenberg et al. 2017) and thereby would distinguish phylogenetically and/or morphologically

75 similar species. Another purpose was to examine if individual metabolites are associated with specific

- 76 host plants across species.
- 77
- 78 **2. Materials and methods**
- 79 2.1 Fungal strains

80 Eighty-seven Stemphylium strains were used in this study. Table 1 gives the identification numbers,

- 81 original and new identity, host and origin of these strains. The strains were selected to include as
- 82 many different species and habitats as possible and as many strains as possible that had been

83 investigated in previous studies (Câmara et al. 2002; Inderbitzin et al. 2009; Woudenberg et al. 2017).

84 An extended version of table 1 is available in supporting material table S1 giving strain numbers in

85 other collections and other papers.

- 86
- 87 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

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

95	Amsterdam, Holland) were placed 40 cm from the plates. The DRYES and PDA plates (9 cm diameter,
96	plastic) were placed in perforated plastic bags and incubated for 14 days in the dark at 25 °C. The
97	micro-morphological characteristics of the strains were observed from PCA and V8 plates after 7 days
98	of growth. Recording of primary conidiophore length, conidial size and shape (L/W ratio), colour and
99	ornamentation were done at X200 magnification using slide preparations made in Shear's mounting
100	liquid with clear Scotch tape as described in Samson et al. (2010). The PCA plates were then stored in
101	the dark at 7 °C and checked for ascomata after 6 months. Colony characteristics (e.g. colour, texture
102	and diameter) were recorded from DRYES plates after 7 days of growth. The morphological
103	characteristics of each strain were registered and compared to reference strains.
104	
105	2.3 Chemical extraction
106	The metabolite profiling was done on the 14-day-old DRYES and PDA cultures using a micro-scale
107	extraction method modified for Alternaria metabolites (Andersen et al. 2005). Five agar plugs (6 mm
108	ID) were cut from the two media and placed in a 2 ml screw top vial. Then 1.0 ml ethyl
109	acetate/dichloromethane/methanol (3:2:1, vol/vol/vol) containing formic acid (1:100, vol/vol) was
110	added to each vial and the plugs were extracted by ultra-sonication for 60 min. The extract was
111	transferred to a clean 2 ml vial, evaporated to dryness in a gentle stream of N_2 and re-dissolved in 400
112	μ l methanol. The methanol extract was filtered through a 0.45 μ m filter into a clean 2 ml vial and kept
113	at -18 °C prior to HPLC analysis.

114

115 2.4 Chemical analyses

116	Analyses were performed using ultra-high-performance liquid chromatography (UPHLC) with a diode
117	array detector (DAD) and high-resolution maXis 3G QTOF mass spectrometer (MS) (Bruker Daltonics,
118	Bremen, Germany), equipped with an ESI source and connected to an Ultimate 3000 UHPLC system
119	(Dionex, Sunnyvale, CA, USA) equipped with a Kinetex 2.6- μ m C18, 100 mm × 2.1mm column
120	(Phenomenex, Torrance, CA, USA) (Klitgaard et al. 2014). A linear water-acetonitrile gradient was used
121	(buffered with 20 mM formic acid) starting from 15% (vol/vol) acetonitrile and increased to 100% in
122	10 min, maintained for 3 min before returning to the starting conditions. MS was performed in ESI+ in
123	the scan range m/z 100–1250, with a mass accuracy < 1.5 ppm (Klitgaard et al. 2014). The mass
124	spectrum of sodium formate was used for calibration at the beginning (0.3-0.4 min) of each
125	chromatogram by injection with a divert valve. UV/VIS spectra were collected at wavelengths from
126	200 to 700 nm. Data processing was performed using DataAnalysis 4.0 and Target Analysis 1.2 (Bruker
127	Daltonics, Bremen, Germany) by the aggressive dereplication approach (Klitgaard et al. 2014), using a
128	database of 297 known and putative Alternaria and Stemphylium compounds, tentatively identifying
129	them based on accurate mass (deviation < 1.5 ppm) (Klitgaard et al. 2014) and if applicable an UV/VIS
130	spectrum. All major peaks observed in the base peak chromatograms, not tentatively identified by
131	this approach, were added to the search list of unknown compounds for mapping. All major peaks
132	(known and unknown) for the 87 extracts were subsequently ordered in a data matrix.

133

134 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

original identification is given in Table 1 together with information on host and origin. Table 1 also
gives the new identification of individual strains based on our overall findings using morphology,
chemistry and names/synonyms proposed by Woudenberg et al. (2017). A supplementary table gives
all known identification numbers for each strain according to Câmara et al. (2002), Inderbitzin et al.
(2009) and Woudenberg et al. (2017). Sixteen species of *Stemphylium* are represented in this study.

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

158	most conidial shapes became more elongated on V8 compared to PCA, however, S. globuliferum, S.
159	loti and S. sarciniforme, maintained their L/W ratio best. Conidial size and L/W ratio varied within the
160	same culture and therefore the following conidial sizes are the maximum sizes on PCA. Conidial
161	measurements for all strains, except the two S. majusculum, were within the limits of the respective
162	species descriptions given in the literature (Câmara et al. 2002; Pei et al. 2011; Simmons 1969, 1985,
163	1989).
164	
165	Common characteristics for S. callistephi, S. lancipes, S. lycopersici, S. majusculum and S. solani were
166	their pointed conidia, production of ascomata and L/W ratio (> 1.9). Conidial size varied greatly from
167	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.
168	lycopersici). Stemphylium majusculum had a conidial size of 40-42 × 21-22 μ m, an L/W ratio of 1.9 and
169	the presence of ascomata. Stemphylium trifolii also had pointed conidia and an L/W ratio of 2.0, but
170	much smaller (25-28 x 12-14 $\mu\text{m})$ and production of ascomata. Colony diameter on DRYES also varied
171	from 31-33 mm (S. majusculum), over 27 mm (S. callistephi) and 26 mm (S. lycopersici) to 21-16 mm
172	(S. solani), 16-22 mm (S. trifolii) and 10-12 mm (S. lancipes).

173

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).

180	With a few exceptions, the rest of the strains (62 in all) identified as S. astragali, S. beticola, S.
181	botryosum, S. eturmiunum, S. simmonsii, S. vesicarium (including former S. alfalfae and S. herbarum)
182	and strains with no species identification were more or less similar. Common for all of them was the
183	production of ascomata, conidial size of 24-45 × 13-23 μm (average: 31 x 17 μm), L/W ratios between
184	1.3 and 2.5 (average: 1.8), but no clear species segregation was seen. Figure 2 shows the morphology
185	of a selection of strains from this cluster. One of the exceptions was S. vesicarium # 25 (ex-type
186	culture of <i>S. herbarum</i> (CBS 191.86)). It did not produce ascomata, produced only a few conidia and
187	was very restricted in its growth on DRYES.
188	
189	3.2 Chemistry
190	The cluster analysis in Figure 3 is based on 219 secondary metabolites of both known and unknown
191	structure and shows that S. globuliferum, S. gracilariae, S. lancipes, S. loti, S. majusculum, S.
192	sarciniforme, S. solani and S. trifolii form their own distinct clusters based on the production of
193	species-specific metabolites or unique combinations of metabolites. However, several species were
194	not completely separated. Cluster 1 contains strains identified as S. botryosum, S. eturmiunum, S.
195	lycopersici and S. astragali, while Cluster 2 contains strains identified as S. callistephi, S. vesicarium
196	including strains originally identified as S. alfalfae and S. herbarum. Stemphylium strains in Cluster 2
197	and S. trifolii had the broadest metabolite profile producing between 72 and 93 detectable
198	metabolites, while <i>S. lancipes</i> and <i>S. sarciniforme</i> produced between 25 and 30 metabolites.
199	

200	Table 2 gives the production of the known metabolites by Stemphylium species with two or more
201	stains together with selected species-specific metabolites of unknown structure. Table 3 gives the
202	Mass [M+H], putative formula and retention time (RT) for each of the unknown metabolites in Table
203	2.
204	
205	Stemphypyrone was the only known metabolite produced by all 87 strains, whereas only two of the
206	known metabolites, orobol and solanapyrone A, were species specific for S. trifolii and S. lancipes,
207	respectively. Stemphyperylenone A was specific to S. beticola and S. simmonsii. All known metabolites
208	could be detected in one or more strains in Clusters 1 and 2 and only strains in Cluster 2 had one
209	species/cluster specific metabolite of unknown structure (Uke23).
210	Four species, represented by only one strain each, are not shown in Table 2, but had the following
211	metabolite profiles: S. astragali produced alterporriol G/H, altersolanol K/L, macrosporin, stemphylin,
212	stemphyltoxins I to III and stemphyperylenol; S. callistephi produced altersolanol K/L, macrosporin,
213	stemphol, stemphylin, stemphyltoxins I to III and stemphyperylenol; S. lycopersici produced
214	macrosporin and stemphylin; and S. simmonsii produced GsS-1, stemphol, stemphyltoxins I to III and
215	stemphyperylenol. <i>Stemphylium vesicarium</i> #25 (ex-type culture of <i>S. herbarum</i> CBS 191.86) is not
216	included in Cluster 2 in Table 2, because it produced only half of the metabolites that other S.
217	vesicarium and Stemphylium sp2 strains produced, which included alterporriol G/H, altersolanol K/L,
218	dehydrocurvularin, GsS-1, macrosporin, stemphol, stemphone, stemphylin, stemphyloxin I/II,
219	stemphyltoxins I to III and stemphyperylenol.

221 3.3 Host specificity

- 222 Comparison between *Stemphylium* species and host (Table 1) did not give any strong connection
- 223 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
- 225 particular metabolites (known as well as unknown) and host plant.

226

4. Discussion

228 4.1 Taxonomy/Nomenclature and Morphology

In recent years, several papers (Câmara et al. 2002; Inderbitzin et al. 2009; Köhl et al. 2009) have

230 suggested that *S. alfalfae*, *S. herbarum* and *S. vesicarium* together with other taxa represent the same

231 species based on molecular data. Our morphological and chemical results are in agreement.

232 Woudenberg et al. (2017) synonymised these species under the oldest name S. vesicarium (see

233 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*.

235

236 Conidial measurements alone have always been problematic to use for identification of *Stemphylium* 237 species. Size and shape of the conidia can vary within the same culture depending on the age. Most 238 young *Stemphylium* conidia are small, spherical/ovoid, with one or few transverse septa. These 239 juvenile conidia become mature within a day or so, developing darker, multiseptate dictyoconidia and 240 assume the shape and size characteristic of its species. The medium also has an influence on conidial 241 size and shape. Our results show that growth on PCA, SNA and V8 yield quite different appearances

242	(Fig. 1), which might contribute to the uncertainty of morphological identifications. For comparison, it
243	is important to use the same medium. In this study, we have used both PCA and V8 since both media
244	have been used in past descriptions (Simmons 1969, 1989, 2001). However, since SNA is a well-
245	defined medium compared to PCA and V8, experiments should be conducted to see if useful
246	characteristics are preserved on SNA, thus replacing PCA and V8.
247	
248	Morphologically, species with oblong pointy conidia can be somewhat difficult to distinguish based on
249	measurements of conidia alone, but other characteristics make it possible to distinguish these
250	species. Strains of S. lancipes can be distinguished by their lanceolate, irregular conidia with several
251	transverse constrictions and often having secondary conidiophores that emerge from the apex of the
252	conidia. Stemphylium callistephi, S. lycopersici and S. solani are similar in conidial shape and size, but
253	other characteristics make them distinct. In this study, S. callistephi never produced secondary
254	conidia, while S. lycopersici grew secondary conidiophores, but only from the apex of the conidia and
255	S. solani produced secondary conidiophores from all cells of the conidial body. Also, S. lycopersici tend
256	to have a rectangular base compared to the other two species.

257

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.
- 265
- 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.*
- 269 globuliferum (41-69 mm). The conidia of S. beticola and S. simmonsii are similar to those of S.
- 270 globuliferum and S. loti and therefore other methods like phylogeny used by Woudenberg et al.
- 271 (2017) or chemotaxonomy should be used for distinguishing these species. Juvenile conidia of S.
- 272 gracilariae are often ellipsoidal compared with the subglobose juvenile conidia of S. globuliferum and
- 273 can be used to distinguish between the two species.
- 274
- 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).
- 280
- 281 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

represented by one strain such as *S. astragali, S. callistephi* and *S. lycopersici* must be studied further
with at least one other strain in order to find species-specific metabolites.

286

287	Our results show a distinct <i>S. globuliferum</i> 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

305 *Exserohilum* sp. (Li et al. 2014), and thus stemphypyrone can be used as a chemical marker for the 306 genus Stemphylium. Most of the known metabolites detected in this study (Table 2) have previously 307 been found in strains of Stemphylium. Our results show that the production of known metabolites is 308 not consistent in all stains of the same species (e.g. S. gracilariae) and often occurs in more than one 309 species (e.g. macrosporin). On the other hand, all species in Table 2 were able to produce species-310 specific metabolites of unknown structure that could distinguish them from other species. Several 311 novel connections have been made. All four strains of *S. loti* produced pyrenophorin and 312 pyrenophorol, which are also produced by *Phoma* sp. and have antimicrobial activities (Zhang et al. 313 2008). All five strains of S. trifolii produced orobol, an isoflavone produced in red clover (Trifolium 314 pratense (Klejdus et al. 2001)), which is interesting, since all five strains were isolated from clover. 315 Stemphylium trifolii seems to be particularly adapted to Trifolium spp. in that both fungus and plant 316 produce orobol. Other species, like S. globuliferum and S. simmonsii, also isolated from Trifolium spp., 317 did not produce orobol. Two metabolites (Ukn185 and Ukn212) of unknown structure, but with 318 recognizable UV-spectra, mass and RT (Table 3), were produced in large quantities by S. beticola and 319 S. simmonsii. These two metabolites have previously been detected in species of Chalastospora as 320 metabolites 1010 and 1120, respectively (Andersen et al. 2009). Unknown metabolites with 321 phytotoxic activity have been reported from Stemphylium, such as SV- and SS-toxins (Zheng et al. 322 2010; Tanahashi et al. 2017), but no molecular information has been given, so direct comparison is 323 not possible.

325	Metabolite profiling can be a powerful tool in fungal identification, but it has its limitations when it
326	comes to strains that have been maintained and re-cultured for many years in culture collections. Our
327	strain of the ex-type culture of <i>S. herbarum</i> , #25 (EGS 36-138 = CBS191.86), now <i>S. vesicarium</i> , has
328	stopped sporulating and is also losing its ability to produce metabolites. The same phenomenon has
329	been observed in Alternaria (Andersen et al. 2008). Only strains that can be unequivocally identified
330	morphologically should be used in the selection process of species-specific metabolites or
331	chemotaxonomic markers.
332	
333	4.3 Host specificity
334	No connections were made between individual species and host plants. Some Stemphylium species,
335	such as S. globuliferum, S. sarciniforme and S. trifolii, were isolated from species of alfalfa, clover,
336	lentils, and pea (Table 1). Other species/taxa, like S. eturmiunum, S. vesicarium and Stemphylium sp.
337	2, have a broader host range comprising Amaryllidaceae, Apiaceae, Brassicaceae, Poaceae, Rosaceae
338	and Solanaceae (Table 1). A search in U.S. National Fungus Collections shows that the species S.
339	vesicarium (including S. alfalfae and S. herbarum) will have an extremely broad host range (Farr and
340	Rossman 2017). One reason that a species can have such a broad host range could be that all strains
341	produce that same non-host-specific metabolites. Trigos et al. (2011) proposed that macrosporin is a
342	non-host specific toxin that plays a role in leaf necrosis due to its photosensitizing ability. Since
343	macrosporin is a non-species-specific metabolite produced by 58 (67 %) of the tested strains, this
344	metabolite might be a contributing factor to the broad host range of Stemphylium, especially among
345	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

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372 References

- 373 Aly AH, Debbab A, Edrada-Ebel RA, Müller WEG, Kubbutat MHG, Wray V, Ebel R, Proksch P, 2010.
- 374 Protein kinase inhibitors and other cytotoxic metabolites from the fungal endophyte
- 375 Stemphylium botryosum isolated from Chenopodium album. Mycosphere 1: 153-162.
- 376 Andersen B, Frisvad JC, 2004. Natural occurrence of fungi and fungal metabolites in moldy tomatoes.
- 377 Journal of agricultural and food chemistry **52**: 7507-7513.
- 378 Andersen B, Dongo A, Pryor BM, 2008. Secondary metabolite profiling of Alternaria dauci, A. porri, A.
- 379 solani, and A. tomatophila. Mycological Research **112**: 241-250.
- 380 Andersen B, Hollensted M, 2008. Metabolite production by different *Ulocladium* species.
- 381 International journal of food microbiology **126**: 172-179.
- 382 Andersen B, Hansen ME, Smedsgaard J, 2005. Automated and unbiased image analyses as tools in
- 383 phenotypic classification of small-spored *Alternaria* spp. *Phytopathology* **95**: 1021-1029.
- 384 Andersen B, Solfrizzo M, Visconti A, 1995. Metabolite profiles of common *Stemphylium* species.

385 Mycological Research 99: 672-676.

- 386 Andersen B, Sørensen JL, Nielsen KF, Gerrits van den Ende B, de Hoog S, 2009. A polyphasic approach
- 387 to the taxonomy of the Alternaria infectoria species-group. Fungal Genetics and Biology **46**:
- 388 642**–**656.
- 389 Ariyawansa HA, Thambugala KM, Manamgoda DS, Jayawardena R, Camporesi E, Boonmee S,
- 390 Wanasinghe DN, Phookamsak R, Hongsanan S, Singtripop C, Chukeatirote E, Kang JC, Gareth
- 391 Jones EB, Hyde KD, 2015. Towards a natural classification and backbone tree for *Pleosporaceae*.
- 392 *Fungal Diversity* **71**: 85-139.

393	Bradley DJ, Gilbert GS, Parker IM, 2003. Susceptibility of clover species to fungal infection: the
394	interaction of leaf surface traits and environment. American Journal of Botany 90: 857-864.
395	Brun S, Madrid H, Van Den Ende BG, Andersen B, Marinach-Patrice C, Mazier D, De Hoog GS, 2013.
396	Multilocus phylogeny and MALDI-TOF analysis of the plant pathogenic species Alternaria dauci
397	and relatives. Fungal Biology 117: 32-40.
398	Câmara MP, O'Neill NR, Van Berkum P, 2002. Phylogeny of Stemphylium spp. based on ITS and
399	glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia 94: 660-672.
400	Christensen KB, Van Klink JW, Weavers RT, Larsen TO, Andersen B, Phipps RK, 2005. Novel
401	chemotaxonomic markers of the Alternaria infectoria species-group. Journal of agricultural and
402	food chemistry 53 : 9431-9435.
403	de Kuppler ALM, Steiner U, Sulyok M, Krska R, Oerke EC, 2011. Genotyping and phenotyping of
404	Fusarium graminearum isolates from Germany related to their mycotoxin biosynthesis.
405	International Journal of Food Microbiology 151 : 78-86.
406	Debbab A, Aly AH, Edrada-Ebel R, Wray V, Müller WE, Totzke F, Zirrgiebel U, Schächtele C, Kubbutat
407	MHG, Lin WH, Mosadak M, Hakiki A, Proksch P, Ebel R, 2009. Bioactive metabolites from the
408	endophytic fungus Stemphylium globuliferum isolated from Mentha pulegium. Journal of
409	Natural Products 72 : 626-631.
410	Farr DF, Rossman AY, 2017. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved
411	October 6, 2017, from https://nt.ars-grin.gov/fungaldatabases/
412	Frisvad JC, Andersen B, Thrane U, 2008. The use of secondary metabolite profiling in chemotaxonomy
413	of filamentous fungi. Mycological Research 112: 231-240.

414	Gálvez L, Gil-Serna J, García M, Iglesias C, Palmero D, 2016. Stemphylium leaf blight of garlic (Allium
415	sativum) in Spain: taxonomy and in vitro fungicide response. The Plant Pathology Journal 32 :
416	388-395.
417	Graf S, Bohlen-Janssen H, Miessner S, Wichura A, Stammler G, 2016. Differentiation of Stemphylium
418	vesicarium from Stemphylium botryosum as causal agent of the purple spot disease on
419	asparagus in Germany. European Journal of Plant Pathology 144 : 411-418.
420	Graham JH, 1953. A disease on Birdsfoot Trefoil caused by a new species of Stemphylium.
421	Phytopathology 43 : 577-579.
422	Hanse B, Raaijmakers EEM, Schoone AHL, Van Oorschot PMS, 2015. Stemphylium sp., the cause of
423	yellow leaf spot disease in sugar beet (Beta vulgaris L.) in the Netherlands. European Journal of
424	Plant Pathology 142 : 319-330.
425	Inderbitzin P, Mehta YR, Berbee ML, 2009. Pleospora species with Stemphylium anamorphs: a four-
426	locus phylogeny resolves new lineages yet does not distinguish among species in the Pleospora
427	herbarum clade. Mycologia 101: 329-339.
428	Kanamaru S, Honma M, Murakami T, Tsushima T, Kudo S, Tanaka K, Nihei KI, Nehira T, Hashimoto M,
429	2012. Absolute stereochemistry of altersolanol A and alterporriols. <i>Chirality</i> 24: 137-146.
430	Kim HY, Park HM, Lee CH, 2012. Mass spectrometry-based chemotaxonomic classification of
431	Penicillium species (P. echinulatum, P. expansum, P. solitum, and P. oxalicum) and its correlation
432	with antioxidant activity. Journal of Microbiological Methods 90: 327-335.

433	Klejdus B, Vitamvásová-Štěrbová D, Kubáň V, 2001. Identification of isoflavone conjugates in red
434	clover (Trifolium pratense) by liquid chromatography-mass spectrometry after two-dimensional
435	solid-phase extraction. Analytica Chimica Acta 450 : 81-97.
436	Klitgaard A, Iversen A, Andersen MR, Larsen TO, Frisvad JC, Nielsen KF, 2014. Aggressive dereplication
437	using UHPLC–DAD–QTOF: screening extracts for up to 3000 fungal secondary metabolites.
438	Analytical and Bioanalytical Chemistry 406 : 1933-1943.
439	Köhl J, Groenenboom-de Haas B, Goossen-van de Geijn H, Speksnijder A, Kastelein P, de Hoog S, van
440	den Ende BG, 2009. Pathogenicity of Stemphylium vesicarium from different hosts causing
441	brown spot in pear. European Journal of Plant Pathology 124 : 151-162.
442	Koike ST, O'Neill N, Wolf J, Van Berkum P, Daugovish O, 2013. Stemphylium leaf spot of parsley in
443	California caused by Stemphylium vesicarium. Plant Disease 97: 315-322.
444	Kozlovskii AG, Antipova TV, Zhelifonova VP, Baskunov BP, Ivanushkina NE, Kochkina GA, Ozerskaya
445	SM, 2017. Secondary metabolites of fungi of the Usti section, genus Aspergillus and their
446	application in chemosystematics. <i>Microbiology</i> 86: 176-182.
447	Kurose D, Misawa T, Suzui T, Ichikawa K, Kisaki G, Hoang LH, Furuya N, Tsuchiya K, Tsushima S, Sato T,
448	2015. Taxonomic re-examination of several Japanese Stemphylium strains based on
449	morphological and molecular phylogenetic analyses. Journal of General Plant Pathology 81: 358-
450	367.
451	Laatsch H. AntiBase 2010. Wiley-VCH: Weinheim, Germany, 2010.
452	Li R, Niu S, Guo L, Zhang Y, 2014. Two new pyrone derivatives from the plant endophytic fungus
453	Exserohilum sp. Natural Products Communications 9 : 1497-1498.

454	Liu Y, Marmann A, Abdel-Aziz MS, Wang CY, Müller WE, Lin WH, Mándi A, Kurtán T, Daletos G,
455	Proksch, P, 2015. Tetrahydroanthraquinone derivatives from the endophytic fungus
456	Stemphylium globuliferum. European Journal of Organic Chemistry 2015 : 2646-2653.
457	Nasehi A, Kadir JB, Nasr-Esfahani M, Abed-Ashtiani F, Wong MY, Rambe SK, Golkhandan E, 2014.
458	Analysis of genetic and virulence variability of Stemphylium lycopersici associated with leaf spot
459	of vegetable crops. <i>European Journal of Plant Pathology</i> 140 : 261-273.
460	Neergaard P, 1945. Danish species of Alternaria and Stemphylium. Oxford university press, London,
461	UK.
462	Pei Y, Wang Y, Geng Y, O'Neill N, Zhang X, 2011. Three novel species of Stemphylium form Sinkiang,
463	China: Their morphological and molecular characterization. <i>Mycological Progress</i> 10 : 163-173.
464	Pitt JI, Hocking AD, 2009. Fungi and food spoilage. Springer, New York, USA.
465	Polizzotto R, Andersen B, Martini M, Grisan S, Assante G, Musetti R, 2012. A polyphasic approach for
466	the characterization of endophytic Alternaria strains isolated from grapevines. Journal of
467	Microbiological Methods 88: 162-171.
468	Puig M, Ruz L, Montesinos E, Moragrega C, Llorente I, 2015. Combined morphological and molecular
469	approach for identification of Stemphylium vesicarium inoculum in pear orchards. Fungal
470	<i>Biol</i> ogy 119 : 136-144.
471	Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B, 2010. CBS laboratory manual series 2 -
472	Food and indoor fungi. CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.
473	Simmons EG, 1967. Typification of Alternaria, Stemphylium, and Ulocladium. Mycologia 59: 67-92.

474 Simmons EG, 1969. Perfect states of *Stemphylium. Mycologia* **61**: 1-26.

- 475 Simmons EG, 1985. Perfect states of *Stemphylium* II. *Sydowia* **38**: 284-293.
- 476 Simmons EG, 1989. Perfect states of *Stemphylium* III. *Memoirs of the New York Botanical Garden* 49:
- 477 305-307.
- 478 Simmons EG, 2001. Perfect states of *Stemphylium*—IV. *Harvard Papers in Botany* **6**: 199-208.
- 479 Simmons EG, 2007. *Alternaria an identification manual*. CBS Fungal Biodiversity Centre, Utrecht, The
 480 Netherlands.
- 481 Singh P, Bugiani R, Cavanni P, Nakajima H, Kodama M, Otani H, Kohmoto K, 1999. Purification and
- 482 biological characterization of host-specific SV-toxins from *Stemphylium vesicarium* causing
- 483 brown spot of European pear. *Phytopathology* **89**: 947-953.
- Smedsgaard J, 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite
 production in cultures. *Journal of Chromatography A* 760: 264-270.
- 486 Snowdon AL, 1990. A colour atlas of post-harvest diseases and disorders of fruits and vegetables.
- 487 *Volume 1: General Introduction and Fruits*. Wolfe Scientific Ltd., London, UK.
- 488 Tanahashi M, Okuda S, Miyazaki E, Parada RY, Ishihara A, Otani H, Osaki-Oka K, 2017. Production of
- 489 host-selective SV-toxins by *Stemphylium* sp. Causing Brown Spot of European Pear in Japan.
- 490 *Journal of Phytopathology* **165**: 189-194.
- Trigos Á, Mendoza G, Espinoza C, Salinas A, Fernández JJ, Norte M, 2011. The role of macrosporin in
 necrotic spots. *Phytochemistry Letters* 4: 122-125.
- 493 Woudenberg JHC, Hanse B, van Leeuwen GCM, Groenewald JZ, Crous PW, 2017. Stemphylium
- 494 revisited. *Studies in Mycology* **87**: 77-103.

495	Zhang W, Krohn K, Egold H, Draeger S, Schulz B, 2008. Diversity of antimicrobial pyrenophorol
496	derivatives from an endophytic fungus, Phoma sp. European Journal of Organic Chemistry 2008:
497	4320–4328.
498	Zheng L, Lv R, Huang J, Jiang D, Hsiang T, 2010. Isolation, purification, and biological activity of a
499	phytotoxin produced by <i>Stempyhlium solani</i> . <i>Plant Disease</i> . 94 : 1231-1237.
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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.	<i>Pyrus</i> 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

- ^a Culture collections from where the strain originated. BA: Collection of Birgitte Andersen (part of the
- 508 IBT collection); CBS: Centraalbureau voor Schimmelcultures, The Netherlands; IBT and FIP:
- 509 Department of Bioengineering, Technical University of Denmark. *Strains also treated in
- 510 Woudenberg et al. (2017). All known identification numbers for each strain can be found in
- 511 supplementary material **Table S1**.
- ^b New name corresponding to the morphological and chemical findings in this study and the
- 513 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.

	beti	glob	grac	lanc	loti	maju	sarc	sola	trif	Clu 1	Clu 2
Metabolite ^ª	(n=2)	(n=5)	(n=5)	(n=2)	(n=4)	(n=2)	(n=4)	(n=4)	(n=5)	(n=20)	(n=29)
Alterporriol G/H	-	5	-	1	-	-	-	4	4	8	12
Alterporriol I/J	-	-	-	-	-	-	-	3	-		3
Altersolanol A	-	5	5	1	-	1	-	4	5	17	25
(=Stemphylin)			_								
Altersolanol K/L	-	5	3	1	4	-	-	4	4	14	15
Altersolanol M	-	3	-	-	-	-	-	2	- 7	2	1
Altertoxin II	1	4	5	1	-	2	-		1	17	28
(= stemphyltoxin II)										4	10
	-	-	-	-	-	-	- 🖌		-	1	12
Dehydrocurvularin	-	-	-	-	-	-	Ā		-	1	12
Macrosporin	-	5	4	2	-	-	<u> </u>	4	5	15	19
Orobol	-	-	-	-	-	-	-	-	5	-	-
Pyrenophorin	-	-	-	-	4	1	-	-	-	7	1
Pyrenophorol	-	-	-	-	4		-	-	-	2	-
Solanapyrone A	-	-	-	2	-	<u> </u>	-	-	-	-	-
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	- ,	C	-	-	-	-	-	-	-	-
Ukn212 ^b	2	- /-		-	-	-	-	-	-	-	-
Ukn074	-	5	5	-	-	-	-	-	-	-	-
Ukn094	-		5	-	-	-	-	-	-	-	-
Ukn287	- (-)	5	-	4	-	-	-	-	-	-
Ukn063	-		-	-	4	-	-	-	-	-	-
Ukn191	-) -	-	2	4	2	-	-	-	-	-
Ukn210	-	-	-	2	-	-	-	-	-	-	-
Ukn054	-	-	-	-	-	-	-	-	5	-	-
Ukn184	Y_	-	-	-	-	-	4	-	5	-	-
Ukn116	-	-	-	-	-	-	4	-	-	-	-
Ukn196	-	-	-	-	-	-	-	4	-	-	-
Ukn224	-	-	-	-	-	-	-	-	-	-	23

^a Metabolite identification are based on comparison of UV-spectrum and exact mass.

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

Metabolite RT (min)		lite RT (min) Mass $[M+H]^+$	
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	$C_{22}H_{40}N_2O_2$

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.

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 $\mu m.$

538

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

























