



Citation for published version:

Rees, RW, Flood, J, Hasan, Y, Wills, MA & Cooper, RM 2012, 'Ganoderma boninense basidiospores in oil palm plantations: evaluation of their possible role in stem rots of *Elaeis guineensis*', *Plant Pathology*, vol. 61, no. 3, pp. 567-578. <https://doi.org/10.1111/j.1365-3059.2011.02533.x>

DOI:

[10.1111/j.1365-3059.2011.02533.x](https://doi.org/10.1111/j.1365-3059.2011.02533.x)

Publication date:

2012

Document Version

Peer reviewed version

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1 ***Ganoderma boninense* basidiospores in oil palm plantations:**
2 **evaluation of their possible role in stem rots of *Elaeis guineensis***

3

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29 ***Ganoderma boninense* basidiospores in oil palm plantations:**
30 **evaluation of their possible role in stem rots of *Elaeis guineensis***

31

32 **R. W. Rees^a, J. Flood^b, Y. Hasan^c, and R. M. Cooper^{a*}**

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34

35 Basidiospores are implicated in the distribution and genetic diversity of *Ganoderma*
36 *boninense*, cause of basal stem rot (BSR) and upper stem rot (USR) of oil palm
37 (*Elaeis guineensis*). Measurement of aerial basidiospores within plantations in North
38 Sumatra showed continuous and high production over 24 h (range c. 2-11,000
39 spores/m³) with maximum release during early evening. Basidiospores applied to cut
40 surfaces of fronds, peduncles and stems germinated *in situ*. Equivalent, extensive
41 wounds are created during plantation harvesting and management and represent
42 potential sites for formation of infective heterokaryons following mating of haploid
43 basidiospore germlings. Notably, use of spore-sized micro-beads showed that
44 basidiospores could be pulled up to 10 cm into severed xylem vessels, where they are
45 relatively protected from dehydration, UV irradiation and competing microflora.
46 Diversity of isolates from five locations on two plantations was assessed by RAMS
47 fingerprinting. Isolates from within individual palms with USR were identical and
48 represent single infections, but different USR infections had unique band patterns and
49 reveal separate infections. Some BSR affected trees contained >1 isolate, thus had
50 multiple infections. There was one example of adjacent BSR palms with the same
51 isolate, indicative of vegetative spread, but there were no identical genets from BSR
52 infections and adjacent fallen palms. Isolate diversity was as great within a plantation
53 as between plantations. It is evident that basidiospores play a major role in spread and
54 genetic variability of *G. boninense*. Evidence for direct basidiospore infection *via* cut
55 fronds, indirectly through roots *via* colonized debris and less frequently, infection by
56 vegetative, clonal spread is considered.

57

58 *Keywords:* white rot, host-pathogen interaction, sexual recombination, tetrapolar
59 mating, DNA markers, pathogen dispersal

60

61

62 **Introduction**

63

64 Considerable yield losses and often death of palms continue to be inflicted on the oil
65 palm (*Elaies guinensis*) in South East Asia and Papua New Guinea by the white-rot
66 fungus *Ganoderma boninense* (Corley & Tinker, 2003; Pilotti *et al.*, 2002; Rees *et al.*,
67 2007). Basal stem rot (BSR) involves decay of the lower stem and sometimes the root
68 system, leading to severe symptoms such as flattening of the crown and unopened
69 spear leaves. Basidiocarps characteristically emerge from the lower stem. Upper Stem
70 Rot (USR) is generally considered to be a less frequent manifestation although in
71 some estates in North Sumatra, the incidence is increasing (unpublished data,
72 LONSUM). USR as described here is defined as a decay of the upper stem and
73 basidiocarp formation ≥ 2 m above ground level. If affected palms are felled and
74 dissected, USR infection is revealed to be unconnected to BSR; in severe infections,
75 stem fracture can result (Hasan *et al.*, 2005; Pilotti, 2005; Rees *et al.*, 2007).

76 Elucidating the route of infection and extent of pathogen diversity is crucial in order
77 to enable development of successful management practices for disease control. BSR
78 infection can result from root infection, presumably following root contact with soil
79 inoculum or other infected roots. Oil palm roots from mature palms can extend up to
80 four planting rows, so root contact will be frequent (Miller *et al.*, 1999). Rees *et al.*
81 (2007; 2009) clearly showed that controlled root infection leads to typical symptoms
82 and can occur in the field. In contrast, others have questioned the role of root
83 infection, based on the high genetic diversity of *G. boninense* isolates (Miller *et al.*
84 1999; Pilotti *et al.*, 2003).

85 Other root-infecting basidiomycetes such as *Heterobasidion annosum* and
86 *Armillaria mellea* spread from tree to tree through soil by vegetative growth, often as
87 a single genet (Woodward *et al.*, 1998). However, genetic studies of *G. boninense* in
88 Malaysia and Papua New Guinea reveal considerable diversity in oil palm plantations
89 according to mitochondrial DNA markers, mating alleles and somatic (or vegetative)
90 compatibility (Miller *et al.*, 1999; Pilotti, 2005). Infections by separate genotypes
91 must have arisen through sexual recombination and subsequent dispersal *via* spread of

92 basidiospores. Pilotti *et al.* (2000) revealed the great diversity in monokaryons, based
93 on RAPDs, such that no two isolates showed an identical RAPD genotype. Also,
94 outbreaks of BSR in new plantations where *G. boninense* inoculum is not present in
95 debris or soil, implies introduction by spores. Basidiospores have been detected in
96 very high numbers under single basidiocarps (Sanderson, 2005). Outcrossing is
97 favoured thanks to *G. boninense* being heterothallic and tetrapolar with multiple
98 alleles at both mating type loci (Pilotti *et al.*, 2002).

99 Monokaryotic mycelium from basidiospores can colonize palm wood but is non-
100 infective (Rees *et al.*, 2007); anastomosis with a compatible mating type is required to
101 form the potentially invasive and faster growing heterokaryon. Basidiospore
102 germlings readily anastomose (Pilotti, 2005) and mating could occur either on the
103 palm surface, or during colonization of organic debris in soil (Flood, *et al.*, 2002).
104 Nevertheless, many and various attempts to infect mature palms and seedlings with
105 basidiospores have failed (see Hasan *et al.* 2005). Also we have previously
106 demonstrated the very weak, competitive saprotrophic ability of *G. boninense* in soil
107 and in the substantial organic debris that accumulates at the frond-stem junction of oil
108 palms (Rees *et al.*, 2007). Therefore the question arises as to where a heterokaryon
109 might form and flourish to create a sufficient inoculum. It is important to note that
110 infection of palm roots requires a substantial inoculum (Rees *et al.*, 2007).

111 In an attempt to address some of these anomalies, here we quantify for the first time
112 airborne basidiospore concentrations and their diurnal fluctuations in plantation air
113 samples. We assess the capacity of basidiospores to germinate on and in potential
114 wound sites on oil palms,

115 We extend the study of *G. boninense* genetic diversity by molecular
116 characterization, using nuclear DNA markers, of isolates from BSR and USR
117 infections and from isolates taken from fallen palms (those killed and left within
118 established plantations) at five locations in North Sumatra. Few studies have
119 addressed relationships between USR and BSR affected palms and fallen palms (FPs).
120 FPs become heavily colonized with *Ganoderma* and may be an important source of
121 inoculum. Also Sumatra potentially offers a different pathogen population from
122 Malaysia and PNG.

123 Numerous molecular tools were considered, based on sensitivity, isolate
124 discrimination, facilities and available skill levels and cost. Randomly amplified
125 microsatellites (RAMS) was eventually chosen. RAMS has now been used in a

126 number of studies on fungal diversity including isolates of *Phlebiopsis gigantea* from
127 Europe and North America (Vainio & Hantula, 2000; Vainio *et al.*, 1998) and pine
128 rusts (*Cronartium flaccidum* and *Peridermium pini*) (Hantula *et al.*, 2002) This work
129 shows the importance of basidiospores in dissemination and infection but suggests
130 several mode(s) of infection are in operation.

131

132

133 **Materials and Methods**

134

135 **Quantification of airborne spores within plantations**

136 A Biotester® RCS centrifugal air sampler (Biotest UK) was used to sample the
137 concentration of airborne spores within oil palm plantations at Bah Lias Research
138 Station (BLRS), Sumatra. Water agar was loaded into the sampler which was run for
139 8 min per sampling, performed in the mid point of interrows and held at 2.0 m height.
140 Agar blocks were then removed and observed microscopically for trapped spores.
141 Four samples were taken for each time point and location to provide mean
142 concentration according to: spores/m³ air = no. basidiospores on agar strip x
143 1000/volume of air sampled (in an 8 min period 320 litres of air was sampled). It was
144 not possible to differentiate spores of different *Ganoderma* species, which often have
145 very subtle differences. Nevertheless, c. 90% of the *Ganoderma* basidiocarps within
146 the plantations studied are from *G. boninense* (unpublished observations of the senior
147 author); the characteristic morphology (basidiospore dimensions were 9.8 to 4.5 µM
148 (mean), and reddish brown, narrow ellipsoid with a visible hilar appendage and often
149 containing a large vacuole (see Fig. 3), characteristics of *G. boninense* (see Pilotti *et*
150 *al.* (2004)) enabled easy discrimination from contaminating fungal spores of other
151 genera and from some other basidiospores. *Ganoderma* basidiospores typically
152 comprised c. 25% of the collected fungal spores.

153

154 **Collection of basidiospores and preparation of spore suspensions**

155 Basidiospores were collected from fresh basidiocarps of *G. boninense* emanating from
156 trunks of mature, diseased oil palm. Spore collection was conducted between 06:00
157 and 10:00 h by placing Whatman filter paper, held in place by aluminium foil, directly

158 below the pores of active fruiting bodies. Spores were then air dried for 10-15 min.
159 The spore-coated filter paper was then cut into small pieces and added to SDW (pH
160 5.5) to make a suspension. Spore concentration was calculated with a haemocytometer
161 and in all cases spores were inoculated onto wounded oil palm tissues and also tested
162 for viability *in vitro*, on the same day as collection.
163 Basidiospore viability was quantified microscopically after 48 and 72 h from 100 μ l
164 of 1×10^6 / ml spores spread on three replicate plates of water agar (pH 5.5).

165

166 **Scanning Electron Microscopy**

167

168 Samples (≤ 5 mm maximum dimension) of palm tissues previously inoculated with
169 basidiospores were collected and placed in 3.5% glutaraldehyde (Agar Scientific) in
170 0.05 M piperazine-*N, N'*-bis (2-ethanesulfonic acid) (PIPES) buffer at pH 8.0. Tissue
171 was then cut into 3x3x1 mm pieces and, still immersed, exposed to vacuum for 16-20
172 h. Samples were then viewed with low temperature scanning electron microscopy
173 (cryo-SEM) on a JEOL SEM6310 model scanning electron microscope fitted with an
174 Oxford Instruments Cryotrans 1500 system attachment.

175

176 **Spore germination on exposed tissue surfaces of oil palm**

177

178 Germination of basidiospores, combined after collection from two basidiocarps on
179 two spatially separated palms, was assessed on cut surfaces of oil palm fronds and
180 peduncles. This exposed tissue represents the most extensive and frequent wound sites
181 created in plantations as at harvest, fruit bunches are removed and fronds are also
182 excised to allow access to the bunches. Also germination was determined within
183 xylem vessels of fronds, severed 10–15 cm from the main stem, and on wounded
184 trunk epidermis, which was cut to a depth of *ca.* 5cm.

185 Fronds and peduncles were cut near the stem junction by machete (ethanol washed) to
186 leave a smooth, near-horizontal surface onto which 5 ml of a freshly prepared (<1 h)
187 spore suspension (1×10^8 spores/ml) was applied immediately, or in some cases two
188 weeks after wounding. The trunk outer layer was breached and a wedge excised to
189 create a horizontal surface. Cut tissues were then covered with a plastic bag to prevent

190 removal of spores by rainfall and to maintain high RH. After 48 and 72h, treated
191 tissues were excised with a scalpel and sectioned (transverse sections [TS] for surface
192 examination and also longitudinal sections [LS] made to examine spores in xylem
193 vessels) and fixed for subsequent analysis by cryo-SEM. For assessment of spores
194 drawn into xylem, eosin dye (2 mg/ml) was added to spore suspensions. This method
195 revealed the location of functional vessels and guided sectioning along the resulting
196 red vascular tissue.

197 Five trees of the same age (5 years) and from the same plantation were used and one
198 frond and peduncle from each palm was assessed for each day of the trial. Trunk
199 wounds were made on five 15 year old palms.

200

201 **Xylem vessel length**

202

203 A suspension containing distilled water, eosin dye and spore-sized (2-20 μ m)
204 fluorescent vinyl particles (Elgersma *et al.*, 1972) was prepared and applied to the
205 freshly cut surface of oil palm fronds. The suspension was added to the cut surface of
206 three different length fronds at 15 min intervals maintaining an excess of suspension
207 on the cut surface at least for 1 h. Thin hand-cut sections were prepared after 24 h
208 every 1 cm using a razor blade and examined microscopically (Leica DMIRB
209 microscope with fw4000 imaging software). Particles fluoresced bright red under
210 incident light

211

212 **Sampling of isolates.**

213

214 *Ganoderma* isolates used for molecular characterisation were isolated from
215 basidiocarps and necrotic tissue from BSR and USR affected palms from five
216 plantings (plots) at two LONSUM owned estates located at 23 kms distance: Bah Lias
217 and Sungei Bejanker. USR samples were taken from felled palms, which were
218 selected for felling if they had evidence of USR 2m or greater from the base of the
219 tree, with no evidence of BSR.

220 Isolates used for sequencing and fingerprinting (57 isolates) are listed in Table 1.

221 Plots were given a numerical code; the first two numbers are the year of planting and
222 the three subsequent numbers represent the number of trees in the planting. Plots 86-
223 200, 85-200 and 88-300 were from Bah Lias Estate and 84-300 and 86-400 were from
224 Sungei Bejanker Estate. Mature plots were chosen so that incidence of BSR would not
225 be limiting with numerous fallen palms located adjacent to standing BSR infections.
226 Essentially sampling involved obtaining isolates from adjacent palms which had a
227 fallen palm as the focus. Thus in Table 1, isolates coded T1, T2 or T3 would be from
228 three adjacent trees.

229 One BSR palm was felled in each plot and isolation was attempted from rotting tissue
230 and basidiocarps using a *Ganoderma*-selective medium (GSM) as described by Rees
231 *et al.* (2007). Other than antibacterial components, antimicrobial components
232 comprised (g/l) pentachloronitrobenzene (285), Ridomil (130), Benlate (150) and
233 tannic acid (1.25). Sampling was also attempted from host tissue and basidiocarp
234 ground tissue from at least one adjacent fallen palm in each plot and from
235 basidiocarps on any nearby BSR infected palms. These palms were not felled because
236 of financial considerations and therefore sampling from rotting tissue was not
237 possible. One USR tree was also felled from each plot with sampling from tissue and
238 basidiocarps.

239 Success of *Ganoderma* isolation from palm tissue was not high and was particularly
240 low from fallen palms, but was most easily obtained from basidiocarps. Initial
241 isolation was facilitated by use of GSM. Isolates were then subcultured onto PDA
242 before extraction for DNA, sequencing and fingerprinting as described above.
243 Multiple isolates of the cultures were stored on PDA on slants covered with sterile
244 mineral oil or sterile water at room temperature and refrigerated at $\leq 6^{\circ}\text{C}$.

245

246 **Harvesting *G. boninense* mycelia and DNA extraction**

247

248 *Ganoderma* isolates were grown on PDA for 1 wk. 1 cm² plugs were taken from the
249 leading edge of the mycelium, placed in 60 ml of 3% malt extract (Oxoid) in 250 ml
250 conical flasks then incubated at 28°C on a rotary incubator at 120 rpm, for 4-5 d.
251 Mycelium was then removed, filtered and washed in SDW, then frozen in liquid
252 nitrogen and ground to a fine powder in a mortar and pestle. 100 mg of the powder
253 was then used for DNA extraction and the remainder was stored at -70°C for future
254 extractions.

255 DNA extraction was achieved from 57 isolates using the DNeasy® plant DNA
256 extraction kit (Qiagen) as described in manufacturer's instructions.

257

258 PCR

259

260 Universal fungal rDNA primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4
261 (5'TCCTCCGCTTATTGATATGC) were used to amplify the ITS1, 5.8S rDNA gene
262 and ITS2 region of *G. boninense* yielding a product of approximately 650 bp (Latiffah
263 *et al.*, 2002). PCR amplification conditions are described under Supplementary
264 Information.

265

266 Sequencing

267

268 Sequencing reactions were performed in 5 µl volumes using 96 well PCR plates (AB
269 Gene) according to manufacturer's instructions for Bigdye® (Applied Biosystems).
270 Twelve millilitres of milliQ water was added to each well, sealed, vortexed and
271 centrifuged at 400 x g for 20 sec. Fifty-two microlitres of absolute ethanol and 3 mM
272 sodium acetate (50:2 v/v) was then added to each well and mixed. The plate was then
273 chilled in a -20°C freezer for 30 min before centrifugation at 1350 x g for 30 min. The
274 plate was then blotted onto paper tissue and centrifuged inverted on paper tissue for
275 20 sec at 200 x g. 150 µl of 70% ethanol was added to each well, sealed and
276 centrifuged at 1350 x g for 30 min. After centrifugation the plate was blotted on
277 paper tissue and centrifuged inverted on paper tissue at 200 x g for 20 sec. The plate
278 was then air dried and sealed before sequencing.

279

280 DNA Fingerprinting

281

282 Fingerprinting of *G. boninense* was carried out using randomly amplified
283 microsatellites (RAMS) as described (Dai *et al.*, 2003). Degenerate primers
284 5'DHB(CGA)₅ and 5'HBH(GAG)₅, where D=A/G/T, H=A/C/T, B=C/G/T, were used
285 to amplify microsatellite DNA. PCR amplifications were as above with an initial
286 denaturation of 10 min at 95°C followed by 37 x [30 sec denaturation at 95°C, 45 s

287 annealing at 61°C] and 2 min extension at 72°C, followed by a final 10 min extension
288 at 72°C. 20 µl of the reaction mixture was analysed on a 2% w/v agarose gel.

289

290 **Statistical Analysis**

291

292 At least three RAMS amplifications were performed on separate occasions using the
293 same DNA sample for each *Ganoderma* isolate and only amplicons that reproduced
294 consistently were scored for presence (1) or absence (0). Identical banding patterns
295 were regarded as genetically identical and were only introduced to the matrix once for
296 statistical analysis. The analysis is shown in Supplementary Information.

297

298

299 **Results**

300

301 **Quantification of airborne spores within plantations**

302

303 Accurate quantification of basidiospores in plantation air combined with circadian
304 influence on spore release by *G. boninense* has never previously been determined.
305 Four successive 8 min air samples (which were considered replicates) were taken with
306 a Biotester® positioned at 2m height in the early morning, midday, early evening and
307 midnight over a 4 day period and mean basidiospore no./m³ was determined as
308 described above.

309 Basidiospores were detected in high numbers throughout 24 h periods, but greatest
310 spore release occurred in the early evening (Figure 1). Basidiospore density was lowest
311 at 07.00 h with mean ca. 2,000/m³, doubling by 12.00 h, then peaking at 19.00 h with
312 ca. 11,000 spores/ m³ and declining by 24.00 h to c. 4,500 spores/ m³.

313 Samples collected with the Biotester® at a distance of 10 cm beneath young, active
314 brackets with a pore surface of approximately 10 cm² from 07:00 h to 23:00 h
315 revealed a basidiospore release rate of c. 140,000 spores/min.

316 Airborne spore concentrations are likely to depend upon abundance of basidiocarps,
317 which will reflect presence of infected and dead palms.. Therefore air samples were
318 also taken at 12.00 h (on successive days under rain-free conditions) from 8 and 17

319 year-old plantings and from a replanted area containing windrowed trunks felled 3
320 years previously.. Windrowing involves uprooting of previous bole and trunk tissues
321 and stacking these along the inter-rows (Viridiana *et al.*, 2010). Mean basidiospore
322 concentrations were greatest in the oldest planting (c. 4,500 spores/m³ (SE 507)),
323 lower in the 8 year stand (c. 3,000 spores/m³ (SE 1014)) and lowest in the windrow
324 samples (c. 2,000 spores/m³ (SE 725)), but differences were not significant (one-way
325 ANOVA ($P < 0.239$, $df = 2$)).

326

327

328 ***In vivo* spore germination**

329

330 In order to mimic possible infection conditions, i.e. encourage anastomosis and
331 formation of potentially infective dikaryons, basidiospores from two different
332 basidiocarps on spatially separated, infected trees within a mature oil palm block,
333 were mixed and applied concurrently to the cut host surfaces. Basidiospores were
334 added immediately to cut surfaces or to surfaces two weeks after wounding (3
335 replicates per treatment).

336 Spores germinated readily on water agar (pH 5.5) with germination ranging from
337 57-85% (data not shown). Germination *in vitro* was not significantly different after 72
338 h than 48 h, or when suspended in eosin dye (used later to reveal functioning xylem
339 vessels). Germination sometimes varied with spores from brackets on spatially
340 separated palms and in one experiment appeared greater when the spore suspensions
341 were mixed (data not shown), but differences were not significant (Tukey-Kramer
342 HSD $p = 0.05$). Thus spores were viable at the time of inoculation of palm tissues.

343 Basidiospores germinated readily on wounded surfaces in the field (Figure 2). All
344 wounded surfaces: peduncles, trunk tissue and fronds, supported very high
345 germination rates. Some basidiospores had been pulled into xylem vessels and
346 germination was similarly high to that on the exposed frond surface. Wounded trunk
347 tissue and peduncles showed markedly more microbial contamination than frond
348 tissue and were more difficult to analyze microscopically. All the surfaces left for two
349 weeks after wounding became colonized with diverse microorganisms and were more
350 difficult to assess basidiospore germination.

351

352 **Vessel lengths in oil palm fronds**

353

354 Severed xylem vessels are exposed at pruning cuts to fronds and petioles and
355 represent a considerable surface area for possible infection. As a result of negative
356 tension in xylem, solutions and suspensions will be withdrawn into vessels from the
357 cut surface. This offers a potential route for basidiospores to penetrate deep within the
358 palm. The extent of this access will be dictated by the length of vessels, which
359 terminate at end walls and function to limit the progression of embolisms and particles
360 such as fungal spores (Cooper, 1981).

361 Eosin dye applied to a wounded frond surface allowed visualisation of functional
362 xylem vessels and progressed >20 cm into the tissue, but as a solution it is not
363 restricted by the presence of end walls (Figure 3a). In order to determine xylem vessel
364 length, and therefore how far a basidiospore may be pulled into wounded fronds, red
365 vinyl, spore-sized fluorescent particles were mixed with eosin and applied to cut
366 surfaces. UV microscopy of sections revealed red particles were withdrawn up to 10
367 cm (Figure 3b). Frequency of particles was greatest nearest the cut surface, showing
368 that frond vessel lengths comprise a variable population but do not extend beyond 10
369 cm. Figure 3c shows vessels terminating (end walls) in a longitudinal section made
370 near a cut frond surface.

371 .

372 .

373 **Sequencing of *Ganoderma* ITS1, 5.8S rDNA and ITS2**

374

375 Sequencing confirmed the identity of most of the isolates as *G. boninense* after
376 BLAST analysis using the NCBI database. Previous sampling of *G. boninense* in
377 Indonesia by Utomo *et al.* (2005) and numerous species of the *G. lucidum* complex by
378 Moncalvo *et al.* (1995 a; b) provided many sequences for comparison.

379 All isolates obtained from BSR and USR infected standing palms were found to be
380 *G. boninense*; however sequences from several isolates from FPs revealed different
381 *Ganoderma* species. For example, isolate FPA B1 S2 from plot 85-200 had most
382 homology to *G. fornicatum* isolates from Taiwan and FPC R4 S2 was most closely
383 related to *G. gibbosum* from mainland China. Thus, in this study, only *G. boninense*

384 was found to cause infection of oil palm whilst other *Ganoderma* species were
385 saprotrophs of fallen *palm tissue*. It should be noted that other spp. of *Ganoderma*
386 especially *G. zonatum* and *G. tormatum*, have been linked with palm diseases, but
387 they were not revealed in this analysis. Complete identity of the 5.8S rDNA was
388 observed in all isolates from Sumatra and also with *G. boninense* isolates from oil
389 palm in Indonesia (Utomo *et al.*, 2005). ITS1 and ITS2 are more variable than 5.8S
390 rDNA and have been used for numerous interspecific phylogeny studies (Moncalvo *et*
391 *al.*, 1995b, Smith & Sivasithamparam, 2000). Three residues in ITS1 and one residue
392 in ITS2 showed variability, but this was not sufficient for determination between
393 individuals (data not shown). Inability to differentiate between closely related isolates
394 was also observed by Latiffah *et al.* (2002) using RFLP of ITS1 and ITS2 on
395 populations within oil palm and coconut. For greater discrimination, randomly
396 amplified microsatellites (RAMS) was used to fingerprint isolates.

397

398 **Randomly amplified microsatellites (RAMS)**

399

400 Fingerprinting based on patterns of similarity in RAMS profiles of isolates was
401 conducted to address key questions relating to infection, spread and diversity of *G.*
402 *boninense*: i) Are neighbouring palms infected by the same isolate of *G. boninense*?
403 ii) Is the same isolate of *G. boninense* found in palms and adjacent fallen palms? iii)
404 Are BSR and USR infections in a palm the result of a single infection event and thus
405 contain only one *G. boninense* genet? iv) What is the extent of diversity within
406 plantations and are distinct populations found on different plantations?

407 The RAMS amplification adapted from Hantula *et al.* (1996; 2002) provided 6-12
408 clear bands per amplification, ranging from 400-1500 base pairs for each *G.*
409 *boninense* isolate. Gel images were scored manually and identical band patterns were
410 regarded as the same genet; a binary matrix was compiled from the different profiles
411 (Table 2). This technique was reproducible and revealed differences between isolates
412 that were inseparable by ITS sequencing.

413 Examination of the RAMS profiles did not reveal any identical genets between BSR
414 infected trees and FPs whether adjacent or from any of the plots (Figure 4a).
415 Therefore, there was no evidence to indicate secondary vegetative spread of the
416 disease from FPs to neighbouring palms.

417 Only one plot contained two adjacent palms infected with BSR that appeared to
418 have identical fingerprints. BSR T4 B1 had an identical band pattern to BSR palm
419 BSR T3 B2 in plot 86:400 from Sungei Bejanker Estate (Figure 4b); in all other cases
420 RAMS profiles from isolates obtained from separate palms were unique, indicating
421 infection by separate genets.

422 Isolates obtained from individual USR infections i.e. from different palms, each had
423 unique band patterns, but within each palm infected by USR, the RAMS banding
424 profile was identical (Figure 4c); this pattern suggests that a single infection event
425 causes USR infections.

426 In contrast, in three of the seven BSR infected palms more than one RAMS profile
427 was evident: Isolates BSR T1R1 and BSR T1 R2 had distinct banding patterns within
428 an infected palm (plot 88-300), BSR T3 B2 was distinct from other isolates within the
429 same palm in (plot 86-400) and BSR T2 B2 was unique from other isolates within the
430 same palm (plot 86-200). This indicates that multiple infections involving more than
431 one isolate can occur within a single palm affected by BSR. However, Fig. 4d reveals
432 that seven isolates from a single BSR infected palm show the same RAMS profile,
433 indicating that in this instance isolates were clonal.

434 Examination of the matrix using cluster analysis showed that individuals within a
435 single planting did not cluster together more than those from different plantings
436 (Figure 5). In addition, isolates from Sungei Bejanker were equally likely to cluster
437 with isolates from Bah Lias estate as they were to cluster with those within the same
438 estate. For example, *G. boninense* isolates obtained from palm BSR T2 in plot 86-200
439 in Bah Lias, have more similarity (based on number of shared bands) to isolates from
440 fallen palms FP T1 & T2 in Sungei Bejanker plot 84-300 than to other isolates from
441 Bah Lias. . The cluster analysis also confirmed that the only identical isolates from
442 neighbouring palms are BSR T4B1 and BSR T3B2 in plot 86-400.

443

444

445 **Discussion**

446

447 Evidence based on the physical and genetic discontinuity of USR from BSR
448 infections and especially the high genetic diversity of *G. boninense* isolates within
449 plantations, even between most neighbouring trees with BSR, suggests that
450 basidiospores play a key role in the development of both manifestations of stem rot.

451 This is the first record of temporal quantification of basidiospore release in air
452 samples from a plantation. Basidiospores are produced in prolific numbers throughout
453 the sampling period, with maximal release in the evening, earlier than the midnight
454 maximum release reported by Ho and Nawawi (1986). Clearly there will be constant
455 potential inoculum to colonize wounds and palm debris throughout the plantation.
456 Previous assessments of basidiospore release were restricted to individual
457 basidiocarps, when production was estimated by Sanderson (2005) at *ca.* 2 million
458 spores per minute from a 5 cm² bracket. Similarly, we detected mean *ca.* 1.4 x10⁵
459 spores per minute from 10 cm² pore surface area during daylight hours.

460 In spite of the evidence for their apparent involvement, there have been no
461 successful attempts at infecting oil palm with basidiospores (Hasan *et al.*, 2005; Idris
462 pers. comm; Thompson, 1931; Yeong, 1992 cited in Miller *et al.*, 2000). This
463 presumably reflects the relatively low aggressiveness of *G. boninense* and the need for
464 large inoculum, as discussed by Rees *et al.* (2007). Some other fungal tree pathogens
465 can infect, directly or indirectly by spores, such as *H. annosum* via conifer stumps
466 (Woodward *et al.*, 1998) and *Cryphonectria parasitica* via wounds in chestnut bark
467 (Nuss, 1992).

468 The potential for infection sites in plantation palms is considerable, with extensive
469 wounds created by routine harvesting (severing the fruit bunch peduncle) and pruning
470 (of frond base to free the fruit bunch). Also trunk wounds are more likely in older
471 palms as harvesting becomes more difficult at greater height. Here we show for the
472 first time that basidiospores can germinate abundantly on cut surfaces under
473 plantation conditions. Spores contaminating cut frond surfaces are withdrawn into
474 xylem as a result of negative tension within functional vessels (Cooper, 1981). The
475 potential distance of ingress is *ca.* 10 cm, reflecting vessel length, as dictated by
476 vessel end walls (Cooper 1981). Here, basidiospores would be relatively protected
477 from dehydration, microbial competition and solar radiation. Spores readily mate
478 according to Pilotti (2005) and anastomosis was apparent *in situ* from our cryo-SEM
479 images. Resulting heterokaryon formation, a prerequisite for formation of infective
480 mycelium, could result in a lesion which extends into the palm trunk.

481 Some workers have implicated or provided indirect evidence for trunk infection *via*
482 wounded surfaces of fronds. Initially, Thompson (1931) surmised that spores entered
483 stem through old leaf bases or through pruning wounds. Sanderson & Pilotti (1997)
484 cut back the rachis of decayed frond bases and followed lesions into the stem base.

485 Following stem expansion this initial infection would appear to have originated near
486 the centre of palm base. Panchal & Bridge (2005) using PCR primer GanET detected
487 *Ganoderma* in frond base material (sampling at 0.25-1 cm depth) with 73% of
488 detections from the most recently pruned fronds, as might be expected in view of the
489 considerable number of aerial basidiospores. 71% of detections were from frond bases
490 near ground level where palms were beginning to show BSR symptoms, with the
491 remainder on upper frond bases. Infection of wounded frond surfaces can occur
492 according to Lim *et al.* (1992), but *G. boninense*-colonised oats was used as the
493 inoculum source. In contrast, Hasan *et al.* (2005) failed to reproduce USR, even using
494 *Ganoderma*-infested rubber wood.

495 USR is not linked to BSR (Hasan *et al.*, 2005; Pilotti, 2005). RAMS analysis of *G.*
496 *boninense*, showed individual USR infections only contained a single isolate of *G.*
497 *boninense* and each isolate was genetically distinct. This pattern would be expected if
498 infections in upper stems are exceptional events and derive from basidiospores. Pilotti
499 (2005) recorded USR occurrence in only 0.01% of trees in PNG

500 Some BSR infections derive from root infection, as clearly evidenced by Rees *et al.*
501 (2007). The inoculum might derive from contact with infected roots from
502 neighbouring palms or from colonized debris. Infection of seedlings can occur from
503 nearby colonized oil palm trunks with those seedlings nearer to the colonized trunks
504 became diseased more quickly (Flood *et al.*, 2005). Seedlings also become infected
505 when planted near infected stumps from the previous planting (Hasan & Turner,
506 1998). Infection at replanting from colonized debris or from windrows remaining in
507 the field is suggested by the reduced infection following fallowing and various
508 windrow treatments (Virdiana *et al.*, 2010), or when increased incidence of infection
509 occurs following poor land preparation with infected boles left in the ground (H.
510 Foster, pers. comm.).

511 A pattern of expanding clusters of affected palms might be predicted from root to
512 root spread. There is only one such published report (Singh, 1991), although field
513 observations in Malaysia still suggest its occurrence, with pattern dependent on first
514 generation or replanting and if clean clearing has been practised (G S Thind, pers.
515 com.). *Ganoderma* stem infections of amenity palms including oil palm, in Singapore,
516 showed no obvious clustering and it was concluded that basidiospores are the means
517 for dissemination and infection (Lim & Fong, 2005). Also recent GPS positioning of
518 BSR-infected palms shows mostly random distribution of BSR in several estates in

519 Sabah (N. Hisham, pers. com.). Based on genetic diversity of isolates, some consider
520 that root infection and secondary vegetative infection between trees is of minor
521 importance (Miller *et al.* 1999; Ariffin *et al.*, 1996; Pilotti *et al.*, 2003).

522 Our data partly concur with isolate diversity in BSR, as three of seven affected
523 palms contained more than one isolate of *G. boninense*, based on RAMS profiles.
524 Likewise, a Malaysian study by Miller *et al.* (1999) showed six of eight BSR palm
525 isolates had different somatic compatibilities and mtDNA RFLP profiles, and Pilotti
526 (2005) found multiple isolates in single palms. This pattern clearly indicates multiple
527 infections rather than clonal spread. Nevertheless, clonal colonization can occur, as
528 revealed by all seven isolates from a single BSR infected palm with an identical
529 RAMS profile.

530 Molecular evidence for mycelial spread of the disease from FPs or BSR infected
531 palms to neighbouring palms was not strong in this study. RAMS profiles did not link
532 BSR infected trees to neighbouring FPs. Similarly, none of the band patterns from
533 FPs was shared with isolates from adjacent FPs. The data reveal a genetically variable
534 population in North Sumatra and the importance of basidiospores, which concurs with
535 previous studies in Malaysia (Miller *et al.*, 2000) and PNG (Pilotti, 2005; Pilotti *et al.*,
536 2003). However, isolates from within two adjacent BSR infected palms (plot 86:400,
537 Sungei Bejanker) did share identical profiles concurring with findings of Miller *et al.*
538 (2000) where adjacent BSR palm isolates displayed the same mtDNA RFLP band
539 pattern. Pilotti (2005) also obtained 2/15 isolates from adjacent palms with the same
540 somatic compatibility. Therefore vegetative spread of the pathogen does occur.

541 However, these apparently conflicting mechanisms are not mutually exclusive.
542 Rees *et al.* (2007) showed multiple, natural infections of different roots in a single
543 palm and this could explain some of the diversity of isolates within BSR lesions. It is
544 tenable that, based on the continual spore deposition in plantations and the extent of
545 potential substrates, that a single trunk may become colonised with genetically
546 distinct isolates. Colonisation of woody substrates by diverse genotypes resulting
547 from basidiospores was demonstrated for pine root rot (*H. annosum*) and the
548 biocontrol fungus *Phlebiopsis gigantea*. Colonization of a single pine stump by
549 several genetically distinct individuals of *P. gigantea*, was based on morphological
550 characters, pairing experiments and RAMS fingerprinting (Vainio *et al.*, 2001).
551 Similarly, Swedjemark & Stenlid (2001) isolated 27 genets of *H. annosum* from
552 within a single pine stump over two years based on somatic incompatibility studies.

553 The extent of variation within and between plantations is considerable and must
554 reflect the tetrapolar mating system of *G. boninense*, which provides inbreeding
555 restriction of 25% thus ensuring the pathogen acquires maximum diversification.
556 Once dikaryons are formed, they maintain their integrity such that isolates found in
557 plantations are individuals and will remain so. Cytoplasmic and nuclear exchange is
558 prevented by somatic incompatibility mechanisms (Pilotti *et al.*, 2002). Cluster
559 analysis conducted on the binary matrix produced from banding patterns showed that
560 isolates from a single plot often did not cluster together more than those from
561 different plantings or even from a different estates at 23km distance. These data agree
562 with the high genetic variability observed in Malaysia and PNG. For example Miller
563 *et al.* (1999) identified 34 of 39 from one plot as distinct somatic incompatibility
564 groups. Sexual compatibility studies revealed great variation within an area studied in
565 PNG by Pilotti *et al.* (2003), with 81A and 83B mating alleles identified, and more
566 genetic relatedness between isolates 15-17 km distant than between adjacent
567 individuals. Somatic incompatibility studies yielded the same conclusions. Pilotti
568 (2005) suggests that migration of spores from outside planting areas explains how
569 new alleles are being detected every year.

570 Commercial oil palm is propagated as tenera seed produced from crosses between
571 *dura* x *pisifera*; thus, other than the small proportion of clonal palms planted, these
572 segregating populations present a heterogeneous host. Such heterogeneity could create
573 additional selection pressure for *G. boninense*, which is ideally designed through out-
574 crossing and prolific propagule generation to segregate and adapt for aggressiveness
575 traits (Miller *et al.*, 2000; Sanderson & Pilotti, 1997).

576 In summary, a model is emerging of multiple modes of infection by *G. boninense*.
577 However, infection based on initial substrate colonization conflicts with the very
578 weak competitive saprotrophic ability of *G. boninense* in soil and organic debris,
579 shown by Rees *et al.* (2007). Not only does the ability of spores to infect wounds need
580 to be fully investigated, but so too does their capacity to colonize palm wood as felled
581 trunks or as remaining debris in soil.

582 Whilst disease control by the development of resistant material and methods to
583 reduce inoculum at replanting must continue to be pursued, where practicable,
584 management strategies should ideally include routine removal of basidiocarps (Hunt
585 & Pilotti, 2004).

586 Whilst disease control by the development of resistant material and methods to
587 reduce inoculum at replanting must continue to be pursued, management strategies
588 involving routine removal of basidiocarps could be investigated in some
589 circumstances, as recommended by Hunt & Pilotti(2004). In first plantings with low
590 levels of infection and few basidiocarps this should be beneficial, but this option
591 might become impracticable in some second and third plantings with high basidiocarp
592 frequency, as often found in Sumatra.

593 .

594 **Acknowledgements**

595

596 R.W.R. was supported by a BBSRC Industrial CASE Studentship with CABI, linked
597 with P.T.P.P. London Sumatra Indonesia Tbk (LONSUM). We thank Hugh Foster
598 and Stephen Nelson for their considerable support and advice and for providing
599 facilities and support staff at Bah Lias Research Station (BLRS), Sumatra. Thanks to
600 Ursula Potter, Centre for Electron Optical Studies, University of Bath, for excellent
601 technical advice with cryo-SEM. We appreciate the guidance of Dr Matt Wills
602 (Department of Biology & Biochemistry, University of Bath) on genetic analysis of
603 isolates of *G. boninense*. We wish to thank LONSUM for permission to publish this
604 paper. This work was performed under DEFRA Licence PHL 188A/6287.

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FIGURE LEGENDS

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724 **Figure 1. Diurnal fluctuation of aerial basidiospore numbers in a 17-year old**725 **plot.** Error bars represent standard deviation of the mean of four samples at each time

726 point over 4 d, constituting 16 readings.

727

728

729 **Figure 2 a-f. Germination of *G. boninense* basidiospores on wounded fronds,**730 **peduncles and trunk tissue.** a, b Germination of *Ganoderma* basidiospores on and in

731 cut frond (petioles) parenchyma cells. c. Basidiospore germination in xylem of cut

732 frond. d. Spore germination on cut fruit bunch stalks (peduncles). e. Mass of

733 germinating spores with germ tubes and hyphae in very close association (apparent

734 anastomosis), on wounded trunk surface. f. Initial stages of basidiospore germination

735 on wounded trunk surface. Note the characteristic basidiospore morphology with the

736 truncated apex. All are cryo-SEM images and show spores 48 h post inoculation to

737 wounded surface. Scale bars represent 10 μ M. Germination of these spores *in vitro*738 was $\geq 57\%$.

739

740 **Figure 3 a-c. Vascular anatomy and length in oil palm fronds revealed by**741 **fluorescent particles and eosin dye.** a. Longitudinal section of cut frond showing

742 functional xylem vessels stained with eosin applied immediately to the cut surface. b.

743 Red fluorescent particles (arrow) within a xylem vessel ca. 10 cm below a cut surface

744 reveal the maximum vessel length, based on passage distance of particles unable to

745 traverse vessel end walls. c. LS of frond showing termination of a wide xylem

746 vessel, and a narrower adjacent vessel. Pit fields are evident as the vessels tapers to

747 end wall (arrows). Spores (and fluorescent particles, see Fig 4 b) would be trapped

748 here. d. Vascular bundle in control frond, transverse section showing arrangement of

749 xylem vessels. Scale bars represent 100 μ M.

750

751 **Figure 4 a-d. RAMS profiles of *Ganoderma boninense* isolates.**

752 Band sizes were estimated with a 100bp ladder (Invitrogen); the 3 brightest markets
753 show 600, 1500 and 2000 bp. For a-c, two lanes represent two PCR reactions for a
754 single *G. boninense* isolate.

755 Gel images are representative of a cross section of the 57 isolates; images from
756 isolates not included here were satisfactory for reading and translating to the binary
757 matrix.

758 (a) Microsatellite profiles from a single BSR infected palm and two adjacent fallen
759 palms from plot 85-200. Lanes 1&2 = BSR T2B1, 3&4 = BSR T2B2, 5&6 = FPA
760 T1R1, 7&8 = FPA T1R2.

761 (b) Microsatellite fingerprints from adjacent BSR infected palms in plot 86-400 and
762 four FPs from plot 84-300. Lanes 1&2 = BSR T4B1, 3&4 = BSR T3B3, 5&6 = FP
763 T1B1, 7&8 = FP T1B3, 9&10 = FP T2R2, 11&12 = FP T2B1.

764 (c) Fingerprints of *G. boninense* isolates from a USR infected palm and fallen palms
765 in plot 84-300. Each lane is the result of a PCR amplification. Lane 1 = FP T2R1, 2 =
766 FP T2R2, 3 = FP T2R3, 4 = FP T2B1, 5 = USR T1R1, 6 = USR T1R2, 7 = USR
767 T1R3, 8 = USR T1R4, 9 = USR T1R5

768 (d) Seven *G. boninense* isolates from a single BSR infected palm from plot 85-200 are
769 identical. Lanes 1&2 = BSR T1R1, 3&4 = BSR T1R2, 5&6 = BSR T1R3, 7&8 = BSR
770 T1B1, 9&10 = BSR T1B2, 11&12 = BSR T1B3.

771 Isolates were from: FP-fallen palm; BSR basal stem rot; USR upper stem rot; R rot; B
772 basidiocarp

773

774 **Figure 5. Hierarchical clustering of *Ganoderma* isolates.** Clustering produced
775 using *pvclust* in R. Distances were binary (based only on shared presences, not
776 absences), and clusters were formed using average linkage. Numbers above internal
777 nodes indicate approximate *p*-values (%) based on 10,000 bootstrap re-samplings of
778 characters. The first (left) number is the Approximately Unbiased (AU) value, and the
779 second (right) is the conventional Bootstrap Probability (BP) value. AU values are
780 computed using multiscale bootstrap re-sampling, and constitute a better
781 approximation to unbiased *p*-values than those obtained using conventional
782 bootstrapping (Schimodaira, 2004). Values are consistent with expectations for the
783 modest ratio of variables to objects (isolates) (6:5). Further details and references are
784 in Supplementary information

785