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ORIGINAL ARTICLE

Ganoderma basidiospore germination responses as affected by spore density, temperature and nutrient media

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Abstract The present study investigated the axenic basidiospore germination of *Ganoderma australe*, *G. mastoporum* and *G. philippii* at three spore densities in five nutrient media encompassing a range of carbohydrate complexity, in combination with sawdust and/or ethanol as medium additives. Five incubation temperatures ranging from 10 to 35 °C were used, totalling 300 treatment combinations. A Weibull model, with the asymptote occurring at 50 to 75 h, provided good fit to percentage spore germination data at each treatment combination for all three *Ganoderma* species. *Ganoderma australe* and *G. mastoporum* basidiospores germinated on all media, whereas *G. philippii* basidiospores required media that contained 2% ethanol. The best medium for *G. australe* and *G. mastoporum* basidiospore germination was rice dextrose agar with a mixture of *Eucalyptus* and *Acacia* sawdust, whereas for *G. philippii* it was 1% malt extract agar plus ethanol, with or without sawdust. Spore density was also critical to achieving the best germination rate, with ~400 spores/cm^2 optimal for all three species. As *Ganoderma* root rot disease affects commercial *Acacia mangium* and *Eucalyptus pellita* plantations, the greater understanding of basidiospore germination gained from the current study should assist in developing strategies to contain the dispersal and spread of root rot in Indonesia and other southeast Asian countries where these species have been planted.

Keywords *Ganoderma australe* • *Ganoderma mastoporum* • *Ganoderma philippii* • nonlinear regression

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Introduction

Ganoderma spp. are frequently associated with root rots of tropical *Acacia* and *Eucalyptus* plantations and *Ganoderma philippii* (Bres. & Henn. ex Sacc.) Bres. These have been identified as the dominant pathogen causing red root rot disease in both *Acacia mangium* Willd. and *Eucalyptus pellita* F. Muell. plantations in Indonesia and Malaysia (Coetzee et al. 2011; Mohammed et al. 2012, 2014; Agustini et al. 2014; Yuskianti et al. 2014). *Ganoderma australe* (Fr.) Pat. and *Ganoderma mastoporum* (Lév.) Pat. are also associated with *A. mangium* plantations in Sumatra (Glen et al. 2009). *Ganoderma mastoporum* produces abundant sporocarps and is occasionally isolated from *A. mangium* roots (Yuskianti et al. 2014), but in the absence of pathogenicity tests it is unclear whether this species can act as a primary pathogen or is merely a secondary coloniser of damaged roots. *Ganoderma australe* represents a diverse species complex that is globally widespread (Moncalvo and Buchanan 2008). It is primarily known as a decay agent of dead wood.

Knowledge of the biology and ecology of these three *Ganoderma* species is limited compared with *Ganoderma boninense* Pat., an important pathogen of oil palm that causes Basal Stem Rot (Flood et al. 2000; Cooper et al. 2011). The genetic diversity in *G. philippii* populations is also poorly

understood. Disease incidence surveys indicate that vegetative transmission may occur (Francis et al. 2014); however, the role of basidiospores in transmission is yet to be established. Previous studies generally failed to induce germination of *G. philippii* basidiospores *in vitro* (Lim 1977). For *Ganoderma* spp. in general, mature basidiospores are not easy to obtain from detached polypore sporocarps, but can be collected *in situ* during the fruiting season (Hilton 1961; Kadowaki et al. 2010). Maximum spore release occurs overnight for *G. boninense* (Ho and Nawawi 1986a; Rees et al. 2012) and *G. applanatum* (Sreeramulu 1959, 1963). Compared with the oil palm pathogen *G. boninense* (Ho and Nawawi 1986b) and the commercially valuable *G. lucidum* (Liu and Chung 2001, Srivastava et al. 2010, Karadeniz et al. 2013, Magday et al. 2014), relatively few studies investigated factors that influence basidiospore germination in the three *Ganoderma* species in this study. For example, Lim (1977) attempted germination of *G. philippii* basidiospores but no studies into germination of *G. mastoporum* or *G. australe* basidiospores were found. Experimental proof of the conditions required to break dormancy in *Ganoderma* spores have been hampered by the difficulty in demonstrating the ability of spores to germinate *in vitro* (Brown and Merrill 1973, Bazzalo and Wright 1982, Adaskaveg and Gilbertson 1986). However, manipulation of nutritional and environmental conditions, sometimes combined with treatments such as grinding, enzymolysis or passage through insect guts have successfully induced axenic germinations of *G. boninense* (Ho and Nawawi 1986b), *G. lucidum* (Curtis) P. Karst. (Liu and Chung 2001), and *G. philippii* (Lim 1977) basidiospores.

Exogenous nutrients absorbed from the environment also affect spore germination, thus helping to define their ecological niches. Germinating fungal spores utilise organic sources of carbon with varying degrees of success among species (Gao et al. 2007). Niederpruem and Dennen (1966) reported variable rates of axenic germination in *Schizophyllum commune* Fr. when provided with soluble simple carbohydrates. The carbohydrate sources that stimulated earliest germination (between 15 and 20 h) included glucose, xylose, fructose, mannose, turanose, cellobiose, sucrose and galactose. Ho and Nawawi (1986a) determined that the incidence of germination of *G. boninense* basidiospores was greater on complex media such as lima bean agar and led to rapid and more profuse mycelial development compared with germination on defined media.

The ability of basidiospores to germinate depends largely on the rehydration and activity of the intracellular water in spores. As basidiospores have a low water content, poor germination levels are generally observed at low to medium relative humidity (RH), and some species will only germinate at high RH or in the presence of free water (Kuhlman and Pepper 1994; Dong et al. 2006). Water uptake is an active process and requires a change in permeability of the spore wall (Fries 1984). Srivastava et al. (2010) recorded a substantial increase in germination of *G. lucidum* spores provided with a 2% ethanol media additive, and suggested that this was associated with chemical degradation of the sporoderm. Increased axenic germination has been recorded in *G. philippii* (Lim 1977) and *G. lucidum* (Srivastava et al. 2010) basidiospores when wood was present as a media substrate.

This study details a simple and efficient method for the collection and axenic spore germination of basidiospores of *G. australe*, *G. mastoporum* and *G. philippii* under different nutritional and physical conditions. A Weibull function was used to model germination dynamics under the influence of spore density, soluble simple and complex carbohydrates, the presence of ethanol and woody substrates, and incubation temperature on the course of germination.

Materials and methods

Spore collection

Spores were collected opportunistically and non-destructively from basidiocarps at three locations: Langgam (0°07′48″N/101°36′0″E), two *G. philippii* basidiocarps from separate stumps ca. 1 km apart in a *E. pellita* stand; Logas (0°17′24″S/101°16′12″E), one *G. mastoporum* basidiocarp growing in a mature-age stand of *A. mangium*; Baserah (0°12′36″S/101°28′48″E), two *G. australe* basidiocarps from two mature-age *A. mangium* wildling trees, approximately 750 m apart in a mature-age stand. Basidiocarps were tentatively identified according to morphological characters as previously described (Glen et al. 2009).

A sterilised 50-mm diameter Whatman filter paper disk was pinned just below the hymenial surface of each basidiocarp, 5–10 mm below the pores (Rees et al. 2012). To minimise airborne contamination, the entire basidiocarp and collection apparatus were wrapped in aluminium foil. Collection occurred overnight between 18 h00 and 08 h00. Collection disks were unpinned from the basidiocarp and individually stored for transport face up in sterile 50 mm Petri dishes. The basidiocarps were removed from the tree, placed in paper bags, dried for two weeks and then vacuum sealed for storage (Herbarium Accession Nos. BO22945-BO22949 to be added). DNA was extracted from each parent basidiocarp and species confirmed using species-specific PCR tests for *G. philippii* and *G. mastoporum* (Yuskianti et al. 2014) and PCR and rDNA ITS sequencing for *G. australe* (Glen et al. 2014). The sequences for the *G. australe* collections and isolates used in this study had over 99% sequence similarity (2 mismatches over 660 bp) to KJ654369.1 and many other accessions of *G. australe,* with the next best match being 94% similarity to KY708881.1, *G. meredithiae.*

Filter papers were air-dried for 15 min, and then stored in sealed dark containers at room temperature. Spores were suspended by placing a section of the spore collection disc in 10 mL of sterile distilled water (SDW) and mixing thoroughly using a vortex. The spore suspension was incubated in the dark for 4 h at 27 °C in a solution containing, per litre, 50 mg penicillin, 50 mg streptomycin, 25 mg polymixin, 1 mL thiabendazole solution (23 mg mL^{-1} in lactic acid). Spores were then collected onto sterile 5 μm filter paper using a perforated ceramic funnel filter, and re-suspended in 50 mL SDW.

Experimental design

A full factorial experiment to investigate the effect of the four factors resulted in 300 treatment combinations for each species (three spore densities × five nutrient media × four medium additive combinations × five incubation temperatures), as follows: Spore density (1×10^4, 3×10^4, 8×10^4 spores/mL in SDW); Nutrient media of high and low concentrations of simple and complex soluble carbohydrates (five different base media, see Table S1); Media additives (the presence/absence of 2% ethanol and 10 g sawdust in all four combinations, NIL = neither present, SAW = sawdust only, ETH = ethanol only, SAW & ETH = both sawdust and ethanol, the sawdust prepared from a mixture of ground-up *Eucalyptus* and *Acacia dealbata* wood and roots); Incubation temperature (10, 22, 27, 31, 35 °C). Each of the 300 treatment combinations was replicated 4 times (twice in each of two runs). All replicates were prepared using the same spore suspensions. Plates were equilibrated to their respective incubation temperatures for 24 h prior to inoculation.

Collection of germination data

Germination data were collected directly after inoculation (t = 0 h), to ensure no germination had occurred during preparation, and every 24 h for a period of up to and including 4 d (t = 96 h). Germination was measured by counting 300 basidiospores in a random transect over each Petri dish under 10× ocular/50× phase contrast magnification using a Zeiss Axioskop. Spores were counted as germinated when the germ tube reached a length equal to the shorter diameter of the germinating spore. For each observation time, a cumulative germination percentage ($G_{\%}$) was recorded. This was also converted to average germinated spores per cm^2 by multiplying $G_{\%}$ by the expected number of spores applied to each plate (2500, 7500 or 20,000) and dividing by the surface area of the plate (19.6 cm^2).

Statistical modelling

All statistical modelling and tests were undertaken using the SAS System for Windows Vers. 9.2 (SAS Institute). Preliminary trials with nonlinear regression models including the logistic, Gompertz and Weibull models, resulted in the Weibull model ranking first. This takes the form:

$$G_{\%}(t) = M\left(1-e^{\left\{-\left(\frac{t}{b}\right)^c\right\}}\right) \quad (1)$$

where $G_{\%}(t)$ is the cumulative percentage of germinated spores at time t, M is the maximum cumulative percentage germination, b is the time taken in hours for $G_{\%}$ to reach 63.2% of the maximum M, and c is a dimensionless shape parameter. The higher the value of c (holding the other parameters constant), the greater the slope of the response curve in the region where $t = b$. Other workers, for example Koide and Yasokawa (2008), used exactly the same model to describe the growth of the mycelial mat and fruiting zone diameters of *Aspergillus niger* subjected to temperature changes. Eq. (1) can be made more general using an additional time parameter t_0 which estimates the lag phase, i.e. the time to germination onset (Brown 1987),

$$G_{\%}(t) = M\left(1-e^{\left\{-\left(\frac{t-t_0}{b}\right)^c\right\}}\right) \quad (2)$$

This extended form of the model was used in other studies, e.g. by Wu et al. (1999) to model the effects of temperature and wetness duration on the infection of peanut cultivars by *Cercospora arachidicola* Hori, and by Furuya et al. (2009) to model spring onion infection by *Puccinia allii* (DC.) F. Rudolphi urediniospores, but as the additional time parameter did not substantially improve the fit for the data in the present study, the simpler Eq. (1) was used to model the cumulative percentage of germinated spores for the 300 treatment combinations. Model fitting was carried out using the procedure PROC NLIN of SAS Vers. 9.2, with parameter estimates calculated using ordinary least squares estimated by the Gauss iterative algorithm as described by Seber and Wild (2005). The goodness-of-fit of Eq. (1) to the data for cumulative percentage of germinated spores at each treatment combination was assessed using root mean squared error (RMSE), a measure of the average deviation between the observed data and the fitted model.

Modelling the cumulative percentage of germinated spores at each of the 300 combinations of the four design factors and the three species produced estimates of the parameters M, b and c for each treatment combination. These answer such questions as which treatment combination achieves the highest percentage germination (via the asymptote M), what is the time required (via the

parameter *b*) to reach 63.2% of that asymptote, and what is the overall shape (via the parameter *c*) of the sigmoidal response curve. Analysis of variance (ANOVA) of the individual estimates of *M* was carried out using the procedure PROC GLM.

Results

Modelling percentage germination

Not all of the 300 treatment combinations resulted in successful germination within 96 h, with most of the failures involving spore densities of 1×10^4 spores/mL and 8×10^4 spores/mL for each of the *Ganoderma* species. At these spore densities, significantly lower percentage germination was recorded (Table 1). At the lower concentration, an increased time to the onset of germination was also observed, whereas, at the higher spore concentration, *G. australe* and *G. mastoporum* commenced germination within 24 h, in contrast to *G. philippii,* which did not show any signs of germination until after 48 h. Graphs from the fitting of Eq. (1) to the data at the most successful spore density of 3×10^4 spores/mL are shown in Figs. 1–3. We examine each species separately.

Ganoderma australe Maximum percentage germination of *G. australe* basidiospores occurred on Rice Dextrose Agar (RDA) combined with sawdust additive at a temperature of 22 °C, with 27 °C a close second (Fig. 1). In all combinations, either 22 or 27 °C was generally the best temperature, with 31 °C occasionally the second best. Cumulative germination was generally poor at 10 °C and also at 35 °C. At all temperatures, the shape of the cumulative germination curve was generally sigmoidal, the curve having a positive slope for times up to ca. 40–50 h, where an inflection point usually occurred followed by a levelling off (Fig. 1).

Ganoderma mastoporum The results for *G. mastoporum* (Fig. 2) had some similarities to those for *G. australe*, but also some noticeable differences. Successful germination was not achieved at either 10 °C or 35 °C, the optimum temperature almost always being 27 °C. A clearly perceptible difference between the shapes of the curves in Fig. 2 compared with those in Fig. 1 are the very steep slopes at 24 h or less, the percentage germination almost always reaching close to the maximum by 48 h. As with *G. australe*, the single best treatment combination was RDA with sawdust additive. The addition of alcohol had either a small positive (e.g. on DWA) or no effect. This contrasts with *G. australe* (Fig. 1), where the addition of ethanol, with or without sawdust, tended to have a small, but sometimes significant, inhibitory effect on spore germination.

Ganoderma philippii The results for *G. philippii* were very different from those for the other species (Fig. 3). Malt Extract Agar (MA1) with sawdust plus ethanol as additives resulted in a cumulative germination incidence having a maximum of 51% at 27 °C, with no other treatment combination reaching 40% germination. As with *G. mastoporum*, germination was not observed at either 10 °C or 35 °C, but the shape of the germinating curve was more often like that of *G. australe* (i.e. gradually sigmoidal) rather than that of *G. mastoporum* (i.e. steeply sigmoidal). This difference can largely be attributed to the delay in germination of *G. australe* and *G. philippii,* both of which had zero or close to zero germination in the first 24 h, whereas *G. mastoporum*, in most cases, was already close to maximum germination percentage.

Table 1 Average cumulative germination rates and average spores per cm^2 of *Ganoderma australe*, *G. mastoporum* and *G. philippii* basidiospores, plated at concentrations of 1×10^4, 3×10^4 and 8×10^4 spores mL^{-1} over 96 h

Species	Spore conc. ($\times 10^4$ mL^{-1})[a]	Cumulative % germination (SE) at time t hours[b]					Average spores per cm^2
		0	24 h	48 h	72 h	96 h	
Ganoderma australe	1	0	0	0	10.6 (0.48)	10.5 (0.62)	127
	3	0	0.7 (0.21)	21.7 (1.82)	26.6 (1.74)	26.1 (1.55)	382
	8	0	2.9 (0.85)	7.7 (0.64)	10.6 (0.69)	11.8 (0.70)	1019
Ganoderma mastoporum	1	0	0	11.2 (0.76)	11.5 (0.71)	14.3 (0.86)	127
	3	0	17.6 (1.76)	24.7 (1.68)	27.7 (1.71)	29.7 (1.79)	382
	8	0	10.7 (1.07)	11.3 (0.77)	12.6 (0.78)	13.5 (0.82)	1019
Ganoderma philippii	1	0	0	0	0.1 (0.03)	7.8 (1.51)	127
	3	0	0	7.0 (1.30)	8.5 (1.48)	8.6 (1.49)	382
	8	0	0	0	0.3 (0.05)	2.0 (0.41)	1019

[a] Entries for each spore concentration for a given observation time for each species are calculated as the mean of the average of the four replicates of the 60 treatment combinations across two experimental runs of nutrient medium (DWA, LBA, MA1, PDA, RDA), media additive (ETH, NIL, SAW, SAW & ETH) and three temperatures (22, 27, 31 °C). [b] SE (standard error of the mean) in parentheses

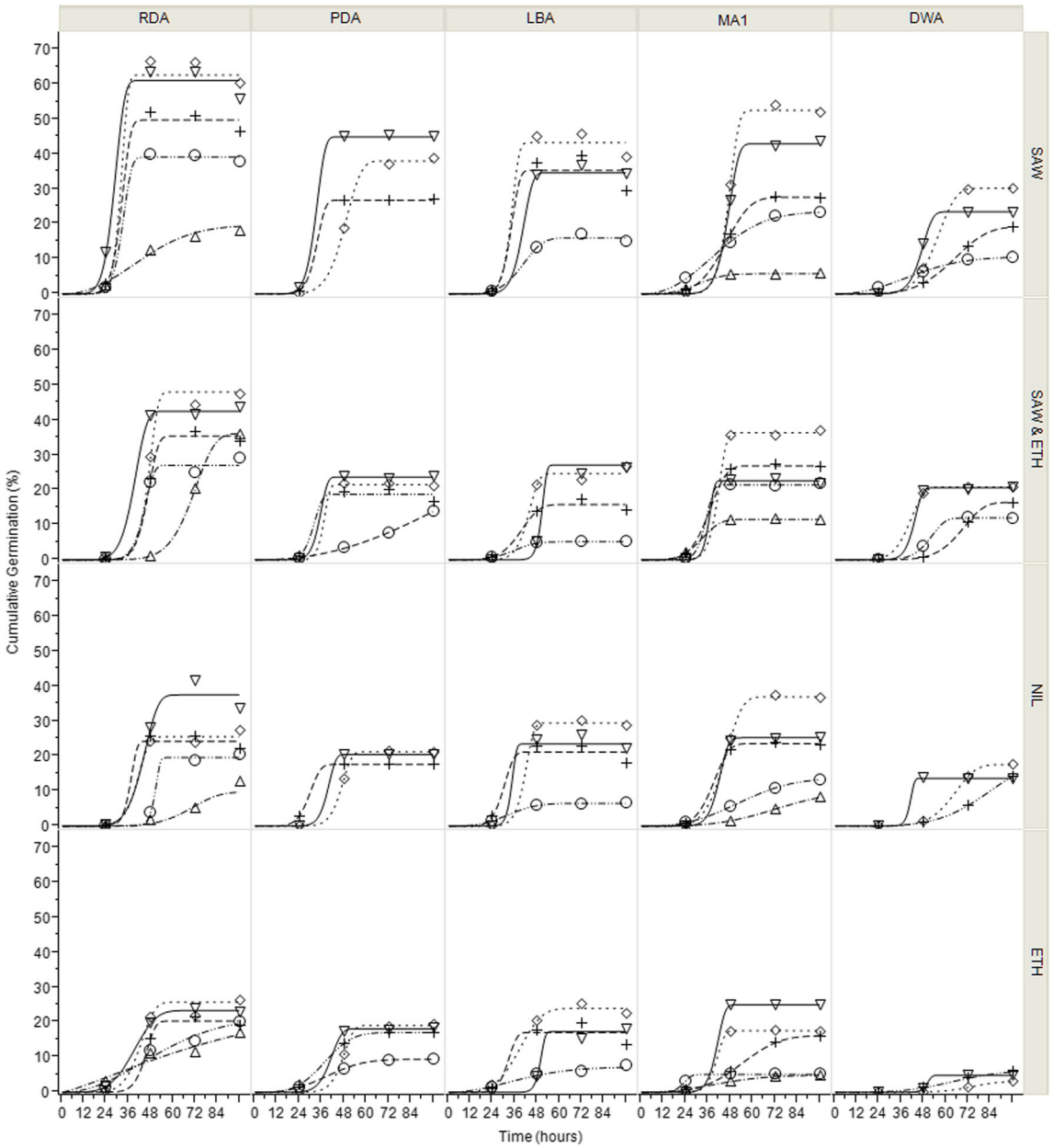

Fig. 1 Cumulative percentage germination of *Ganoderma australe* basidiospores, plated at 3×10^4 spores mL^{-1}, over a 96 h period, fitted by the Weibull-type model, Eq. (1). Effects of five different nutrient media: Rice Dextrose Agar (RDA); Potato Dextrose Agar (PDA); Lima Bean Agar (LBA); 1% Malt Extract Agar (MA1); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: sawdust (SAW), 2% ethanol (ETH), both sawdust and ethanol (SAW & ETH) or none (NIL), is presented, from top to bottom, in combination with the medium in approximate order of decreasing germination success. Points represent observed data at the five different incubation temperatures Δ 10 ◦ 22 ∇ 27 + 31 o 35, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at five constant incubation temperatures signified by -· − ·10 – 27 - -·31 ··· − 35; see Table S2 for parameter estimates and goodness of fit. Missing data points represent treatment combinations where no germination was recorded and hence, the data could not be modelled

Weibull-type model assessment

The goodness-of-fit of the Weibull-type model (Eq. 1) to the cumulative germination frequencies for the spore density 3×10^4 spores/mL is shown graphically (Figs. 1–3) and quantitatively (Tables S2–S4). Depending upon whether convergence was achieved or not, and whether that convergence was conditional, the results for parameters *M, b* and *c* fell into three broad groups. For the first group, the convergence criterion was met unconditionally. For these data sets, all parameter estimates are meaningful. In the second the predicted germination was the same for the last two, three or four times (i.e. 24, 48, 72 and 96 h) at which measurements were made. Here, only conditional convergence was obtained and only the least-squares estimate of the

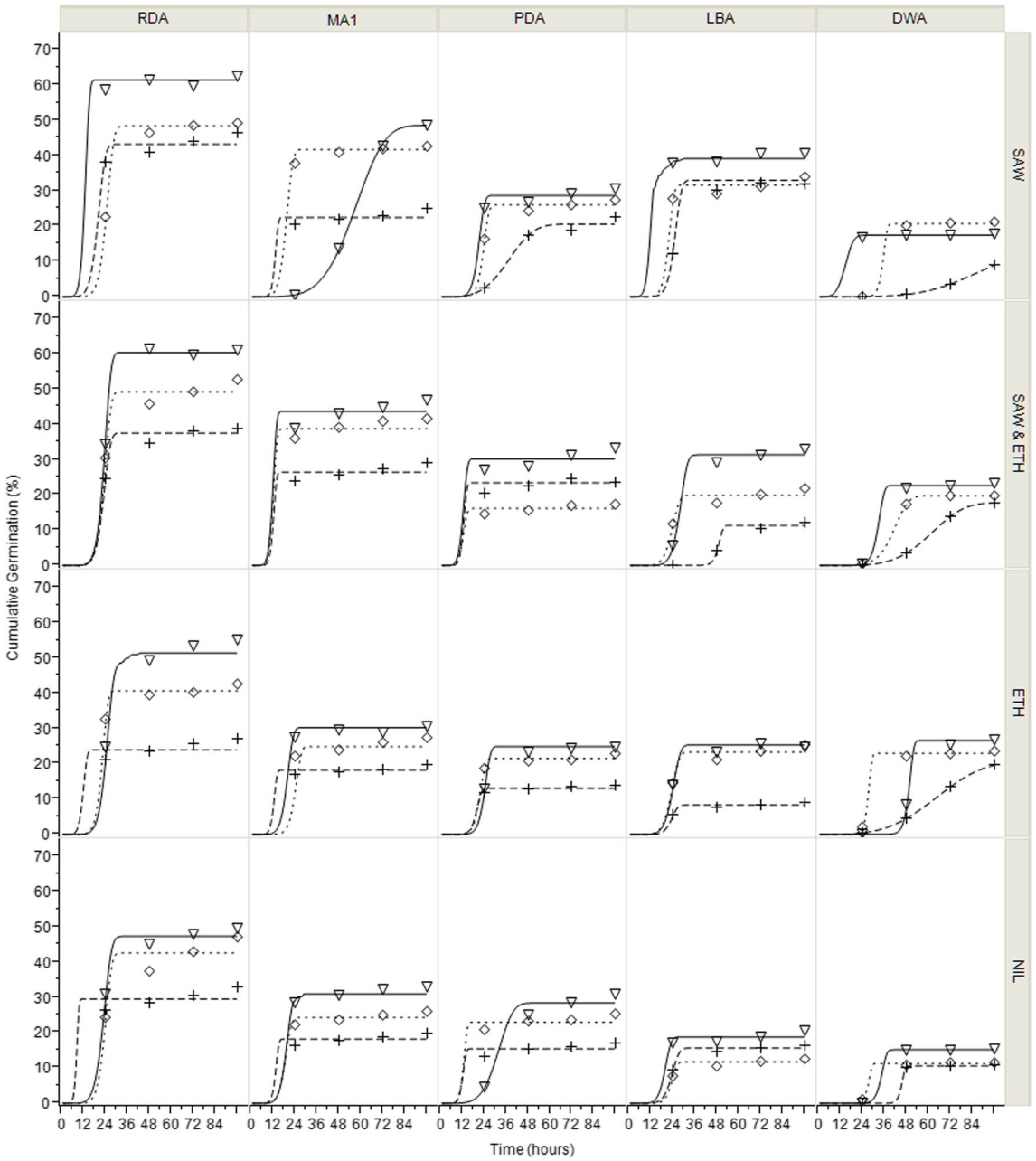

Fig. 2 Cumulative percentage germination of *Ganoderma mastoporum* basidiospores, plated at 3×10^4 spores mL^{-1}, over a 96 h period, fitted by the Weibull-type model, Eq. (1). Effects of five different nutrient media: Rice Dextrose Agar (RDA); 1% Malt Extract Agar (MA1); Potato Dextrose Agar (PDA); Lima Bean Agar (LBA); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: sawdust (SAW), 2% ethanol (ETH), both sawdust and ethanol (SAW & ETH) or none (NIL), is presented, from top to bottom, in combination with the medium in approximate order of decreasing germination success. Points represent observed data at three different incubation temperatures ◦ 22 ∇ 27 + 31, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at three constant incubation temperatures signified by ····22 –27 -· − ·31.; see Table S3 for parameter estimates and goodness of fit

parameter *M* is meaningful. The third is where the convergence process did not get beyond step 0. In this case, an estimate of *M* was obtained as the mean of the 16 observations at the times 24, 48, 72 and 96 h, and the standard error of the mean as the standard error of those 16 observations. For *G. australe*, 57 of 84 data sets converged unconditionally, compared with only seven of the 60 data sets for *G. mastoporum* and 19 of 30 data sets for *G. philippii*. Although mean values for the estimates of the parameters *b* and *c* for each species can be calculated from the values listed in Tables S2–S4 that converged unconditionally, caution is needed in drawing conclusions about the population values of these parameters because of the imbalance among the species that resulted from the large number of data sets that converged only conditionally or failed to converge.

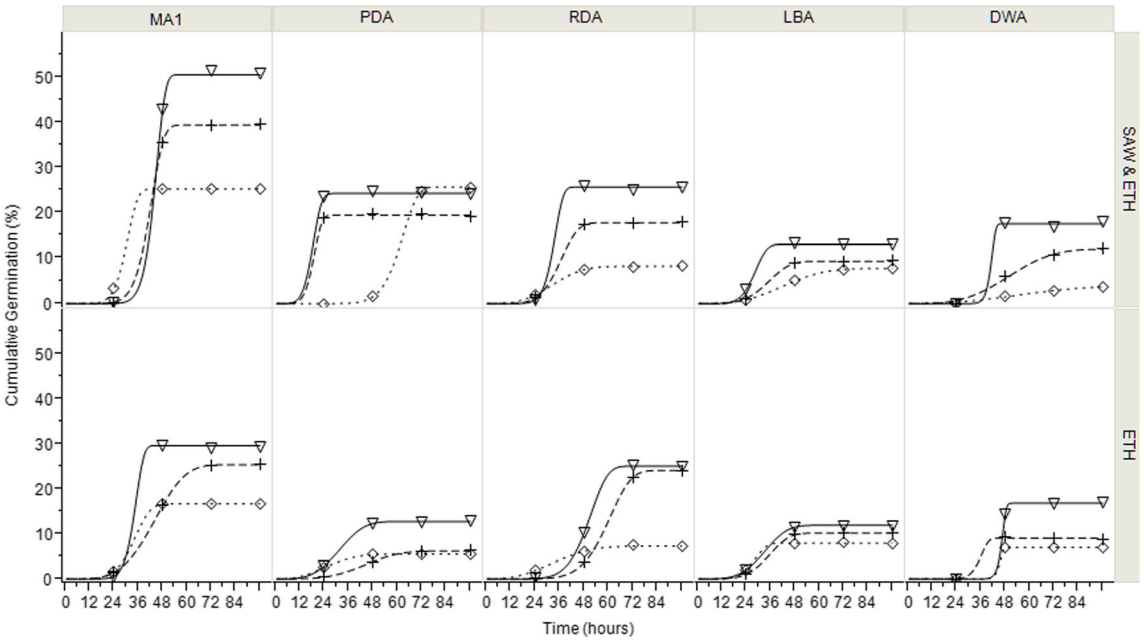

Fig. 3 Cumulative percentage germination of *Ganoderma philippii* basidiospores, plated at 3×10^4 spores mL^{-1}, over a 96 h period, fitted by the Weibull-type model, Eq. (1). Effects of five different nutrient media: 1% Malt Extract Agar (MA1); Potato Dextrose Agar (PDA); Rice Dextrose Agar (RDA); Lima Bean Agar (LBA); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: 2% ethanol (ETH) and both sawdust and ethanol (SAW & ETH) is presented, from top to bottom, in approximate order of decreasing germination success. Points represent observed data◦ 22 ∇ 27 + 31, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at three constant incubation temperatures signified by ····22 –27 -· − ·31; see Table S4 for parameter estimates and goodness of fit

Germination dynamics

For each *Ganoderma* species separately, only a small percentage of treatment combinations for basidiospore concentrations of 1×10^4 mL^{-1} and 8×10^4 mL^{-1} successfully germinated. Therefore, attention was focussed on the germination dynamics for basidiospore concentrations of 3×10^4 spores/mL only. Of the 100 treatment combinations for each *Ganoderma* species, successful germination at the temperature extremes of 10 °C and 35 °C was obtained only for a handful of runs for *G. australe* and never for *G. mastoporum* or *G. philippii*. We therefore now look only at the 60 treatment combinations for each species where the five nutrient media are combined with four media additives at the three most successful temperatures, viz. 22, 27 and 31 °C. Table S5 reports the results of the ANOVA on parameter *M*, the parameter for which meaningful estimates were obtained for each treatment combination. For each species, there was a significant interaction ($P < 0.01$) between medium and media additive, but the table reports the main effect means with a view towards seeing which treatment levels, on average, perform better or worse than others. We examine each species separately.

Ganoderma australe The best overall nutrient medium is RDA, the best additive is sawdust, and the best temperature is 22 °C, although the percentage germinated is not significantly different from that at 27 °C (Table 2). The best individual treatment combination is RDA plus sawdust at 22 °C, with the same medium plus the same additive at 27 °C coming second (Fig. 1). The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) resulted in one significant interaction, that being Medium*Additive ($P = 0.0063$) with mean values for the treatment combinations given in Table S5. From that table, the highest overall mean percentage germinated is 57.9%, for the combination RDA with sawdust, averaged over temperature. Therefore, all indicators point to the best germination occurring on RDA with added sawdust, with temperatures of 22 °C or 27 °C being almost equally efficacious.

Gandoderma mastoporum Results obtained were similar to those for *G. australe*, with RDA as the best individual nutrient medium, and sawdust the best additive when these factors are averaged over the levels of the other factors (Table 2). However, the optimum temperature is 27 °C, with a clear lead over the second best temperature, 22 °C. Ethanol provides neither benefit nor detriment for *G. mastoporum*, the overall germination mean being unchanged compared to the control (NIL), whereas for *G. australe* it appears to have a depressive effect (Table 2). This is particularly noticeable in Fig. 1, where the combination of RDA and ethanol resulted in a much reduced germination success compared with RDA coupled with the other additives, including the control. Another striking difference between germination success for *G. australe* and *G. mastoporum* is that the response curves of

Table 2 Main effect means for nutrient medium, medium additive, and incubation temperature on the maximum germination (%), as estimated by parameter *M* of the Weibull-type model, of *Ganoderma australe*, *G. mastoporum* and *G. philippii* basidiospores, plated at 3×10^4 spores/mL. The sample size *n* indicates the number of values in the mean for that treatment group. DNG denotes a result where no germination was recorded and hence the data could not be modelled; the asymptote *M* was recorded as zero in this case

Treatment		*M* (percent)		
		G. australe	*G. mastoporum*	*G. philippii*
Medium[a]	RDA	[d]38.1[a]	44.9[a]	9.0[b]
(*n* = 12)	MA1	29.5[b]	30.8[b]	15.6[a]
	LBA	26.2[bc]	22.4[c]	5.0[d]
	PDA	24.0[c]	22.6[c]	7.9[bc]
	DWA	16.3[d]	18.2[d]	5.6[cd]
Additive[b]	SAW	39.6[a]	33.1[a]	DNG[c]
(*n* = 15)	SAW & ETH	26.9[b]	30.0[b]	20.1[a]
	NIL	23.9[b]	22.9[c]	DNG[c]
	ETH	17.0[c]	25.1[c]	14.4[b]
Temperature[c]	22 °C	30.0[a]	27.9[b]	5.8[c]
(*n* = 20)	27 °C	27.7[a]	34.3[a]	11.4[a]
	31 °C	22.8[b]	21.2[c]	8.7[b]

[a] Medium: MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b] Additive: NIL = none; SAW = 10 g dried sterile sawdust; ETH = 2% ethanol; SAW & ETH = 10 g dried sterile sawdust +2% ethanol. [c] Temperatures = 22, 27 and 31 °C. [d] Means followed by the same lower case letter in a column depicting a treatment for each *Ganoderma* species are not significantly different (Fisher's LSD, $\alpha = 0.05$)

the latter approach the asymptote more quickly than the former, as expressed through the scale parameter *b*. The difference is best observed by comparing the curves in Fig. 2, with their steeply rising slopes, against the curves in Fig. 1, which rise more gradually towards the asymptote. The graphs for *G. mastoporum* (Fig. 2) confirm that germination has almost reached its asymptote by 48 h, with a substantial proportion of the asymptote reached at 24 h, whereas for *G. australe*, germination has scarcely begun at 24 h (Fig. 1). The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) gave significant effects of Medium ($P < 0.0001$), Additive ($P < 0.0001$), Temperature ($P < 0.0001$), Medium*Additive ($P = 0.0012$), and Medium*Temp ($P = 0.0142$). The mean values for the treatment combinations are given in Table S5, which support the conclusion from Table 2 that the combination of the best individual treatments (RDA, SAW and 27 °C) results in the highest germination rate.

Ganoderma philippii Results in Table 2 for the separate factors reveal major differences between the cumulative germination successes from those of the other two *Ganoderma* species. Whereas RDA was best for both of the other species, MA1 was better than any other nutrient medium for *G. philippii*. Another major difference is that no germination was observed unless ethanol was present, either with or without sawdust. The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) gave significant effects for Medium ($P < 0.0001$), Additive ($P < 0.0011$), Temperature ($P < 0.0002$), and the Medium*Additive interaction ($P = 0.0027$). Mean values for the treatment combinations are given in Table S5, which confirm that the best overall treatment combination (MA1, SAW&ETH, 27 °C) is what one would obtain by combining the best individual treatments from Table 2.

Effects of simple vs. complex carbohydrates

Each of the three *Ganoderma* species was able to germinate on the entire range of nutrient media; however, germination was always higher on media amended with an additional carbohydrate source compared with the control (Figs. 1–3).

Discussion

The Weibull-type model provided a good fit to the germination data for all three *Ganoderma* species for all nutrient media, media additives and temperatures. Combined with their relatively low values of the root mean square error, these results indicate that the Weibull-type model is suitable for modelling cumulative spore germination data in *Ganoderma* species, and support the Weibull-type model as a biologically relevant means of describing the course of spore germination under each set of conditions, also permitting accurate reconstructions of germination over time for comparison among species.

In each *Ganoderma* species, combinations involving spore densities of 1×10^4 spores/mL and 8×10^4 spores/mL had a lower cumulative germination percentage after 96 h than at the optimal spore concentration of 3×10^4 spores/mL. Spore concentration is a critical factor in the germination of many kinds of fungal spore (Feofilova et al. 2004), and low spore densities may be disadvantageous for the conditioning of the spores themselves. On the other hand, high spore densities can also hinder germination through production of self-inhibitors that affect germination of neighbouring spores (Fries 1984). This is in keeping with the theory that germinating spores produce stable metabolites which can retard or inhibit germination of spores of the same species, and yet they may also produce metabolites which stimulate germination (Garrill 1995). An equilibrium between stimulatory and inhibitory effects is suggested here, as an optimal concentration of metabolites for rapid spore germination is frequently found in mycological studies in which the germination time is plotted for a range of spore concentrations (Feofilova et al. 2012). *Ganoderma philippii* responded differently to spore concentration

than did *G. australe* and *G. mastoporum.* In the latter two species, there was no delay of germination onset when basidiospores are plated at 1000 spores/cm^2 compared with 380 spores/cm^2, as is the case with *G. philippii.* This indicates that subsequent basidiospore germination is inhibited by the germinating basidiospores rather than the ungerminated basidiospores as appears to be the case for *G. philippii.* At the lower spore concentration, germination onset is delayed in all three species, indicating that all three produce some unknown substance that promotes basidiospore germination.

Each of the three species achieved highest percentage germination on different media and it is possible that this reflects individual species preferences for different carbon sources; however, the media are all complex and it is uncertain which component of each medium was responsible for stimulated germination. Autoclaving agar produces an organic acid that can inhibit spore germination of some ectomycorrhizal basidiomycetes (Bjurman 1984), so it is possible that the addition of other compounds to the agar provides a protective rather than stimulatory effect *per se*. The apparent difference in carbon preference shown by these species of *Ganoderma* is perhaps understandable when considering the substrates that form their best suited habitats. Both *G. australe* and *G. mastoporum* are generally observed to have low pathogenicity, yet compete well via strong saprotrophic ability in soil and organic debris, forming sufficient heterokaryotic inoculum to persist (Mohammed et al. 2014). In contrast, *G. philippii* appears to be aggressively pathogenic but a poor competitor in the saprophytic stage (Mohammed et al. 2014).

No germination was observed in *G. philippii* unless ethanol was present, either with or without sawdust. Although ethanol stimulates basidiospore germination of *G. lucidum* (Srivastava et al. 2010), it has not been reported as an essential compound. Cochrane et al. (1963) have suggested that it might serve in pure culture as a precursor of some more commonly required compound, e.g. an amino acid; amino acids are known to occur in the soil adjacent to plant roots (Schroth and Hildebrand 1964). Alternatively the ethanol may act as a signal to break dormancy in a similar manner to insect ingestion (Lim 1977). It is unclear how significant such insect ingestion is in nature, or whether some compound in wood can perform this function, despite the fact that the addition of sawdust to the media never resulted in successful germination in the absence of ethanol in this study. The addition of sawdust to DWA enabled the germination of *G. lucidum* basidiospores (Srivastava et al. 2010) but not of the *G. philippii* basidiospores in the current study. It is possible that insect ingestion may modify the sporoderm sufficiently to allow germination (Lim 1977) and that the ethanol may perform a similar function (Srivastava et al. 2010). This behaviour of *G. philippii* is strikingly different from *G. australe* and *G. mastoporum*, which germinated without the aid of ethanol, and for which the addition of sawdust was clearly significantly better than ethanol alone.

For each *Ganoderma* species, the three most successful temperatures were 22, 27 and 31 °C. For *G. mastoporum* and *G. philippii*, the maximum percentage germination was obtained at 27 °C, with significantly lower germination incidences at both higher and lower temperatures. For *G. australe*, the optimum temperature is less clear, as the percentage germination at 22 °C and 27 °C did not differ significantly (Table 2). As germination at the temperature extremes of 10 °C and 35 °C was obtained occasionally for *G. australe*, compared with never for *G. mastoporum* or *G. philippii*, this may reflect the broader geographical and climatic range of *G. australe,* which occurs in temperate through to tropical regions.

This study has successfully demonstrated a simple method for the collection and germination of *G. australe, G. mastoporum* and *G. philippii* basidiospores. The availability of a simple method for axenic spore germination is necessary to obtain monokaryons and study mating systems in fungi (Billiard et al. 2012). The ability to produce monokaryon isolates will also facilitate population genetic studies based on mating type genes (Kile 1983, Pilotti et al. 2003) and development of molecular markers to enable further investigations of the ecology and dispersal mechanisms of the three species in this study (e.g. Nakabonge et al. 2005, Anderson et al. 2006, Franzen et al. 2007). In particular, understanding the population genetics of *G. philippii* and knowledge of spore germination dynamics will contribute to a greater understanding of the modes of infection of this pathogen and its spread in *A. mangium* and *E. pellita* plantations.

Ganoderma phillipi is an intractable pathogen to control and to date the most promising means of management appears to lie in biological control by competitive wood degrading fungi as for *Heterobasidion* spp. (Mohammed et al. 2014). Therefore this germination study also paves the way for studies into competition between *G. philippii* basidiospores and oidia of potential biological control agents on stumps of felled trees, such as have been conducted in other pathosystems (Sun et al. 2009, Oliva et al. 2015).

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