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# Performance of leaf extract media in culturing mycorrhizal mushroom mycelium

In-vitro culture of mycorrhizal mushroom (MM) species in southern Africa remains largely unexplored, particularly using tree-derived media. In this study, a *Julbernardia globiflora* [(Benth.) Troupin] leaf infusion was tested for its ability to promote MM mycelial growth. *Amanita loosii*, *Cantharellus miomboensis* and *Cantharellus heinemannianus* isolates were incubated at a pH of 2, 3, 4, 5, 6 or 7 and at 25 °C in six leaf extract agar (LEA) infusion concentrations of 150, 175, 200, 225 or 250 grams of leaves/L distilled water, with potato dextrose agar (PDA) as a standard. We determined mycelium growth rates for all treatment combinations. Mycelium growth rate was found to be optimal at a pH between 4 and 6 in all leaf infusion concentrations tested. Significant ( $p < 0.001$ ) linear regressions of *A. loosii* and *C. miomboensis* were found for pH only ( $R^2 = 0.837$  and  $0.8582$ , respectively) and a significant ( $p < 0.001$ ) regression was found for *C. heinemannianus* ( $R^2 = 0.293$ ). *Amanita loosii* and *C. heinemannianus* had faster ( $p < 0.001$ ) growth in PDA than in LEA, while *C. miomboensis* had similar growth rates in the two media. Growth characteristics observed were attributed to acid phosphatase mediated physiological processes in mycelium for the different MM species with an optimum pH of 4–6. MM mycelia were white, mycelia for *A. loosii* and *C. miomboensis* were loose and for *C. heinemannianus* were thin filaments. LEA proved to be a potential alternative medium for culturing MM species.

**Significance:**

- A novel miombo tree extract medium was tested with three miombo mycorrhizal mushrooms.
- Our findings show the new medium to be a possible alternative to, but not as viable as, potato dextrose agar.
- The findings of his study widen the scope of use for the forest tree derived media and demonstrate the cultivability of miombo mycorrhizal mushroom species.
- Our findings improve the possibility of enhancing food security through culturing and possibly cultivating the less explored African mycorrhizal mushrooms.

## Introduction

Unlike for temperate habitats, the biology of edible mycorrhizal mushrooms (MMs) of the miombo biome is little understood and documented. Although mushroom consumption dates to 900 BCE and cultivation of saprotrophic species started around 1650 CE for *Agaricus bisporus* in China, in 600 CE for *Auricularia auricula-judae* and in 1100 CE for *Lentinula edodes*<sup>1</sup>, MMs have largely eluded ex-situ cultivation. Over the millennia, saprotrophic mushroom cultivation has been modernised through specialised microbiology, hence their present significant contribution towards global food security.<sup>2</sup> The same effort for MM cultivation has been hampered by their requisite close association with woody plant hosts.<sup>3</sup> Although many temperate MMs have been successfully cultured in synthetic media containing a single carbon source<sup>4</sup>, few tropical MM species have to date been tested.

Mushrooms reproduce and multiply sexually by releasing basidiospores which fuse and germinate into hyphae, which eventually form complex networks of vegetative mycelia and, subsequently, their fruiting bodies. Mushroom cultivation has avoided the use of basidiospores owing to non-reliability of the resultant product quality and trueness to type. Hence, modern mushroom cultivation involves two asexual phases of spawning materials, namely, primary spawn production and grain spawn running, giving a more farmer-usable material.<sup>5</sup> Mushroom primary spawning material has traditionally been grown in agar-based media to obtain their pure cultures. Although pure MM cultures of *Tuber* sp. (truffles) have been successfully used for infecting the roots of a variety of tree species<sup>6</sup>, similar technology has not been attempted with edible miombo MM, thereby keeping them outside formal agriculture.

Several agar-based general media are used for fungal culture, including potato dextrose agar (PDA), malt extract agar, Czapek-Dox agar and yeast extract agar<sup>7–9</sup>, but no new suitable media have been developed for tropical MM mushroom culture. PDA is, therefore, still the most widely used medium for mushroom spawn culture<sup>10</sup> for such mushrooms as *Pleurotus*, *Agaricus*, *Auricularia* and *Volvariella* species.<sup>11,12</sup> Gamborg, modified Melin-Norkrans, and Murashige and Skoog media have successfully been used on mycorrhizal *Phlebopus portentosus*, suggesting that suitable MM culture media development is possible.<sup>13,14</sup> However, these media have not completely succeeded for those MMs requiring more specific growing requirements<sup>15</sup> and thus the difficulty in obtaining viable in-vitro cultures.<sup>16,17</sup> In natural growing habitats, MM mycelia successfully grow under narrow pH ranges, for example, a pH of 4.0 for *P. portentosus* and pH 6.0 for *Coprinus phlyctidosporus*.<sup>18</sup> Media pH is critical in activation of most metabolic enzymes, including endoglucanase, cellobiohydrolase,<sup>17,19,20</sup> invertase, endoglucanase, and acid phosphatase.<sup>21–23</sup> Mycorrhizal mycelia have the capacity to release acid phosphatase, irrespective of available organic phosphorus (P) in the growing substrate, with general specificity to soil habitats pH.<sup>23,24</sup> Li et al.<sup>25</sup>, however, found low substrate P to induce higher acid phosphatase activity, whereas Costa et al.<sup>24</sup> found organic phosphate to suppress synthesis of acid phosphatase in *Pisolithus microcarpus*, making it necessary to examine suitable media

pH for tropical MMs. In particular, pH was found to regulate extracellular proteases to release nitrogen (N) in amino acids for *Amanita muscaria*, an MM associated with pine,<sup>20</sup> making it necessary to find the right pH when developing culture media for tropical/subtropical MMs.

Concentration of macro- and micronutrients in culture media is critical in MM mycelium growth, some of which they naturally obtain from their woody hosts – their associated saprotrophic fungal partners.<sup>26</sup> As most MMs are incapable of using external sucrose as a carbon (C) source, preferring glucose and/or fructose,<sup>27</sup> standard media such as PDA are likely to favour MM mycelium growth only, but no balanced nutrients, as found in their native materials.<sup>28–30</sup> *Phlebotus portentosus* was successfully cultured in a medium with a C:N ratio of 10:1<sup>18</sup>, unlike the optimum C:N ratio of 1:4 found for non-MM *Pleurotus tuberregium*, suggesting that conventional media for non-MM species may not be suitable for MM culture. However, no similar studies have been documented for miombo MM, particularly *Amanita loosii*, *Cantharellus miomboensis* and *Cantharellus heinemannianus*, which are popular foods among communities in southern Africa.<sup>31</sup> The objectives of this study were to: (1) assess suitability of *Julbernardia globiflora* leaf extract in agar as an alternative medium for culturing MMs; (2) compare growth rates of the three MM species' mycelia when cultured in the leaf extract agar and PDA; and (3) develop a predictive model which relates MM mycelium growth rates with media pH and leaf extract concentration as independent variables; and hence, describe the growth form and appearance of mycelia of the three MM species as compared to *Pleurotus ostreatus*.

## Materials and methods

### Source of reagents and mushroom specimens

All experiments were conducted at Lupane State University (S19.15616°, E029.79517°) at an elevation of 862 m above sea level. Analytical grade PDA (Biolab, Merck 63725), Agar-agar (Philip Harris Education), 32% HCl (Merck), 99.8% NaOH (SkyLabs, South Africa), 90 mm plastic Petri dishes (Boxmore Plastics, South Africa) and sticky labels were obtained from Krain Laboratories (Bulawayo), and fresh *A. loosii*, *C. miomboensis* and *C. heinemannianus* sporocarps were harvested at Mtao Forest. Pure mycelial cultures were prepared in standard PDA by isolating 2 mm mushroom context disks in an aseptic method. Pure cultures were stored in the dark at 4 °C for 5 months.

### Source and preparation of leaf litter

Mature whole leaves of *J. globiflora* were harvested at Masenyane Village, Lupane, Zimbabwe, from two mature 20-year-old *J. globiflora* trees in winter. Fresh leaf samples were thoroughly mixed and sun dried on a metal sheet for 36 h to simulate field conditions. The samples were then oven dried for 24 h at 105 °C. A quantity of 1000 g of oven-dried leaves was soaked in 10 L distilled water for 72 h to leach out water-soluble compounds to simulate rainfall effects in mushroom habitats.<sup>32</sup> After decanting the water, leaves were oven dried in batches for 24 h each at 105 °C.

### Preparation and chemical analysis of leaf extract

Leaf extract preparation followed the protocol for PDA preparation by HiMedia, India (<https://himedialabs.com/TD/GM043.pdf>), with modifications in drying of the leaf litter, leaching it and oven drying before infusion. Oven-dried leached leaf portions of 150, 175, 200, 225 and 250 g were each immersed in 1000 mL distilled water at 96 °C for 30 min, giving different extract concentrations. Supernatants were transferred to 250 mL Erlenmeyer Academy flasks and pH (measured at 50 ± 2 °C) of each extract was determined using an electronic pH meter (Greisinger GMH 3500 Series) before adding 22 g/L agar-agar (determined by adjusting agar-agar concentration to attain media solidification from a preliminary test). The mixture was thoroughly stirred with a glass rod and autoclaved at 121 °C and 103.4214 kPa for 15 min in a Classic Prestige Medical autoclave. The autoclaved preparations were allowed to cool to 55 °C and pH was adjusted to 2, 3, 4, 5, 6 or 7<sup>18,29</sup> by addition of 1M HCl or 1M NaOH and thorough stirring with a glass rod<sup>11,33,34</sup>. Pre-run preparation for pH adjustment using 1M HCl or 1M NaOH was done with

a dropping pipette to determine the required amounts for each targeted media pH. Standard buffer solutions for pH 1, 4, 6 and 9 were used to verify the adjusted media pH. The media were poured into plastic Petri dishes in 20 mL volumes and allowed to set, and the Petri plates were placed in a refrigerator at 4 °C for 24 h before inoculation. The positive controls were agar-agar plates while negative controls were blanks of leaf extract agar (LEA).

The atomic absorption spectrophotometer (AAS; Varian Spectr AA 200) was used to analyse the mineral content of the LEA and potato infusion in order to explain the difference in mycelium growth rates in the LEA medium. The potato extract was prepared using the standard protocol (HiMedia, 11 May 2017) with variation in filtering infusions through mutton cloth instead of cheese cloth. Table 1 gives the composition of the crude leaf extract and crude potato extracts analysed with AAS. Organic nitrogen analysis was done using the Kjeldahl method<sup>35</sup>, while organic carbon was analysed using the Nelson and Sommers (1996) protocol<sup>36–38</sup>.

Qualitative analyses showed that both the crude leaf and crude potato extracts contained reducing sugars, where crude potato extract contained starch with high levels of amino acids but no monosaccharides (Table 2).

### Inoculation and experimental design

Aseptic inoculation in the centre of the plates<sup>39</sup> was done using the tip of a flamed scalpel blade with pure cultures of the three mushroom species for all pH–concentration treatment combinations. Treatments were three MM species (*A. loosii*, *C. heinemannianus* and *C. miomboensis*), six levels of pH (pH 2, 3, 4, 5, 6 and 7), six levels of leaf infusion strengths (infusion dry mass g/L: 0, 150, 175, 200, 225 and 250) making 108 treatment combinations. Each treatment combination was done in triplicate. Two negative (uninoculated) controls were used for each pH versus concentration combination. After inoculation, all plates were sealed with parafilm (Bemis Flexible Packaging, Neenah, WI54956) and placed in a completely randomised design in an incubator (Genlab INC/150/DIG) at 25 °C.<sup>18</sup>

To compare mean growth rates in LEA at each MM determined optimum pH and leaf extract concentration ( $d$ ) with standard PDA for the three MMs, a completely randomised design was used with 15 replicates each and five negative controls, and they were incubated at 25 °C for 4 days taken by *C. heinemannianus* to complete growth coverage of the plate surface.

### Data collection and analysis

Simple linear regressions and a standard multiple regression were used to determine predictive ability of pH and  $d$  (all controls were excluded in analyses) on MM mycelium growth rate at 3 days after inoculation (DAI) in SPSS Version 20 (IBM Corporation 1989, 2011). Variables of pH and  $d$ <sup>40</sup> were used to test their predictive ability on mycelium growth rates in a general multiple regression model:

$$\gamma = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon \quad \text{Equation 1}$$

where  $\gamma$  is the predicted variable (mycelium growth rate),  $X_1$  is the pH variable,  $X_2$  is  $d$  in agar-agar,  $\varepsilon$  is the error term (assumed to be zero),  $\beta_0$  is the constant and  $\beta_1$  and  $\beta_2$  are the coefficients.

To determine the contribution of each of the two independent variables – pH and media concentration – on the response variable before selecting the regression model variables of mycelium growth rate for each mushroom species, daily growth curves were fitted to the data in SPSS 20.0 and the best-fit model was selected based on its significance ( $p < 0.05$ ),  $R^2$  and F-value.

A *t*-test for independent samples was used to compare mean mycelium growth rates for each MM species when grown at an optimal LEA pH and extract concentration, and PDA at 3 DAI. The appearance of the mycelium and patterns of its growth were described for each species and growing media, and photographs were taken.

**Table 1:** Elemental composition of potato and *Julbernardia globiflora* leaf crude extracts (mg/L)

Element	Substrate	
	Potato extract	Leaf extract
Phosphorus	57.39	20.98
Nitrogen (%)	0.09	0.08
Carbon (%)	0.17	0.18
Sulfur	116.10	87.99
Magnesium	108.176	96.912
Calcium	33.67	337.95
Iron	0.346	8.2
Nickel	0.064	0.079
Manganese	0.179	7.02
Lead	0.27	1.200
Copper	0.190	0.253
Cobalt	0.036	0.004
Zinc	0.159	0.202

## Results

### Curve fitting

When curves were fitted to the data, the three fungal species showed varying responses to the explanatory variables, media pH and concentration in the leaf extract agar media (Figure 1a–d with the best fit lines highlighted in red, and  $R^2$  and F values given on the caption notes).

#### *Amanita loosii*

For *A. loosii*, the fitted cubic model shows a pH of 6 to be optimum (Figure 1a) and no lag phase is found within the lower end of the pH range examined.

#### *Cantharellus heinemannianus*

Mycelium growth rate for *C. heinemannianus* was optimum at a pH of 6, with an anomalous decrease from pH 2 to pH 3 (Figure 1b).

For media concentration, the fitted quadratic curve for *C. heinemannianus* coincides with the cubic model, showing optimum media concentration to be about 215 g/L (Figure 1d). Beyond this concentration, the growth rate falls rather than levelling off.

#### *Cantharellus miomboensis*

Growth rate for *C. miomboensis* also showed a cubic model for pH with an optimum at pH 6 but with a plateau between pH 5 and pH 7, slowly tapering down beyond the examined pH range. This model clearly shows there was no growth below pH 2 (Figure 1c).

### Regression results

Using the models for best-fit curves in Figure 1a–d, a standard multiple regression was done to assess the ability of pH and medium concentration ( $\alpha$ ) to predict mycelium growth rate in millimetres per day determined at 3 DAI in the LEA medium for pH 2 to 7 and  $\alpha$  of 150, 175, 200, 225 and 250 g/L for the three MM species *A. loosii*, *C. heinemannianus* and *C. miomboensis*. Preliminary checks showed the data complied with linearity, non-multicollinearity and homoscedasticity but failed normality tests for all available transformations.

A significant regression model (Equation 2) was found when *A. loosii* mycelium growth rate (mm/day) was regressed on pH:

**Table 2:** Qualitative analysis results of tests for starch, sugars and amino acids in leaf and potato extracts

Test substance	Iodine ST test	Benedict's RS test	Barfoed's MS test	Ninhydrin PR test
Potato extract	++	+	-	+++++
Leaf extract	-	++	+	+
Distilled water	-	-	-	-
Glucose	-	+++++	++	-

- negative reaction; + positive reaction: the number of + signs shows the score relative to the magnitude of the positive result

ST, starch; RS, reducing sugar; MS, monosaccharides; PR, protein

$F(1, 89) = 457.567; p < 0.001$ . The adjusted coefficient of determination ( $R^2$ ) was 0.837. However,  $\alpha$  was not a significant ( $p > 0.05$ ) predictor for *A. loosii* mycelium growth rate.

A significant regression model (Equation 3) was found when *C. heinemannianus* mycelium growth rate (mm/day) was regressed on pH and leaf extract concentration:  $F(2, 88) = 18.025; p < 0.001$  for pH and  $\alpha$  and  $R^2$  was 0.293.

A significant regression model (Equation 4) was also found when *C. miomboensis* mycelium growth rate (mm/day) was regressed on pH:  $F(1, 89) = 533.781; p < 0.001$  for pH with  $R^2$  of 0.858.  $\alpha$  was not a significant ( $p > 0.05$ ) predictor of *C. miomboensis* mycelium growth rate.

Equation 2 implies that for every unit increase in pH, mycelium growth rate increased by 2.897 mm/day within the specified pH limits.

$$\hat{Y} = 2.897X - 0.685 \quad \text{Equation 2}$$

where  $\hat{Y}$  is mycelium growth rate in mm/day and  $X$  is pH with  $2 \leq X \leq 7$  for *A. loosii*, implying that for each unit increase in pH, mycelium growth rate increased by 2.897 mm/day, irrespective of  $\alpha$  between 150 and 250 g/L.

Equation 3 implies that for every unit increase in pH, mycelium growth rate increased by 1.726 mm/day when  $\alpha$  was kept constant, and mycelium growth rate increased by 0.042 mm/day for each unit increase in  $\alpha$  when pH was kept constant. The model was true for the stated pH and  $\alpha$  limits.

$$\hat{Y} = 6.05 + 1.726X_1 + 0.042X_2 \quad \text{Equation 3}$$

where  $\hat{Y}$  is mycelium growth rate in mm/day and  $X_1$  is pH with  $2 \leq X_1 \leq 7$  and  $X_2$  is  $\alpha$  with  $150 \text{ g/L} \leq X_2 \leq 250 \text{ g/L}$  for *C. heinemannianus*.

Equation 4 shows that for every unit increase in pH, mycelium growth rate increased by 4.115 mm/day within the specified pH limits.

$$\hat{Y} = 4.115X - 5.595 \quad \text{Equation 4}$$

where  $\hat{Y}$  is mycelium growth rate in mm/day and  $X$  is pH with  $2 \leq X \leq 7$  for *C. miomboensis*, that is, mycelium growth rate increased by 4.115 mm/day for a unit increase in pH irrespective of change in  $\alpha$  between 150 and 250 g/L.

### Comparative performance of *A. loosii*, *C. miomboensis* and *C. heinemannianus* when cultured in PDA versus LEA

The *t*-test results of mycelium growth rate taken 3 DAI showed a higher growth rate ( $p < 0.001$ ) in PDA for *A. loosii* and *C. heinemannianus*, but similar growth rates in both media for *C. miomboensis* (Table 3). Optimum mycelium growth rates recorded were between pH 4 and pH 7.

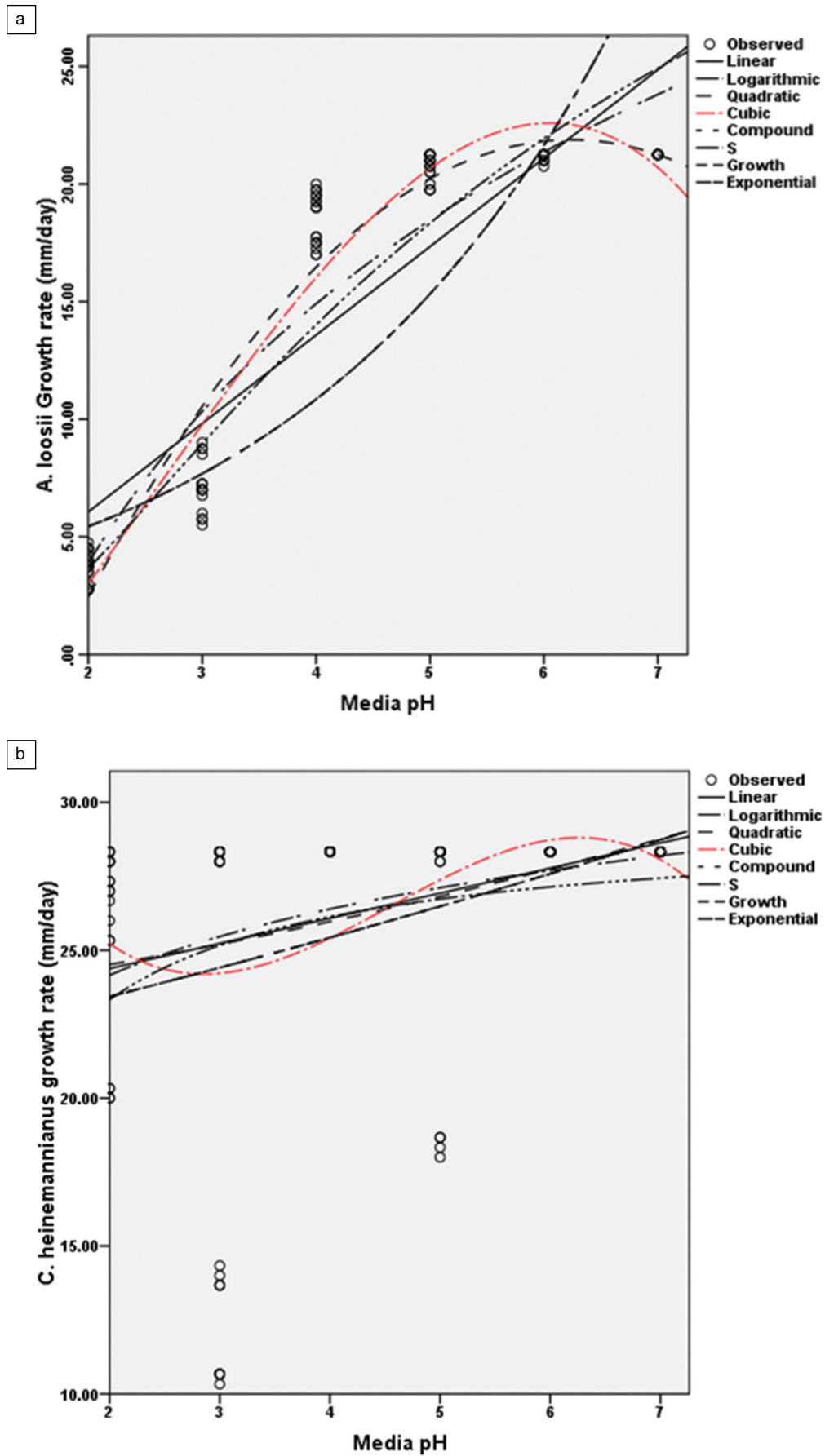
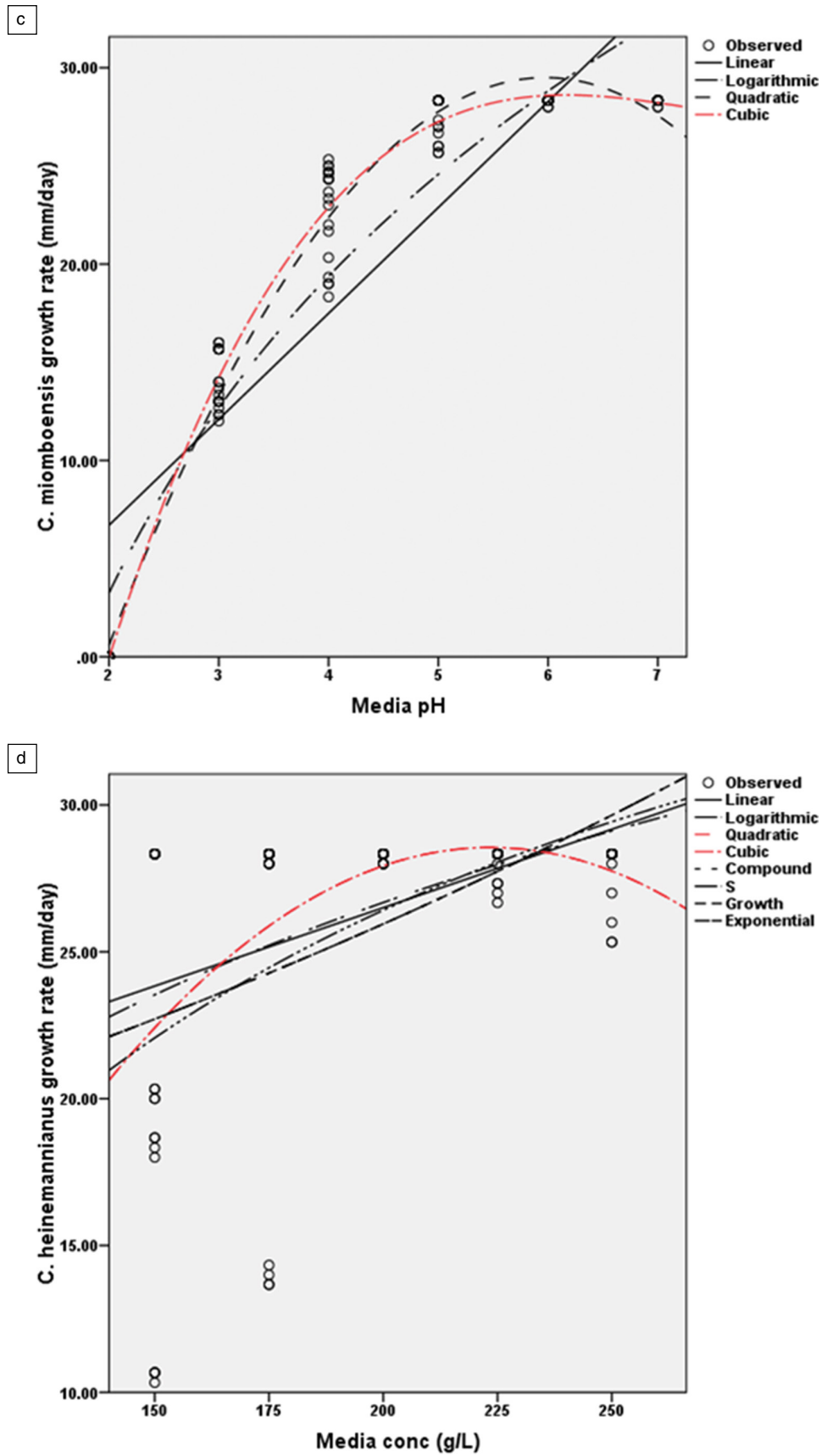


Figure 1 continues...



**Figure 1:** (a–c) Fitted curves for growth rate with media pH as the explanatory variable with the cubic curve as the best-fit significant ( $p < 0.05$ ): (a) *Amanita loosie*  $R^2=0.938$  and  $F=585.934$  (media concentration was not significant and was therefore omitted); (b) *Cantharellus heinemannianus*  $R^2=0.136$  and  $F=6.084$ ; (c) *Cantharellus miomboensis*  $R^2=0.987$  and  $F=2854.139$  (media concentration was not significant and was therefore omitted). (d) Fitted curves for growth rate for *Cantharellus heinemannianus* with media concentration as the explanatory variable with the quadratic curve as the best-fit significant ( $p < 0.05$ )  $R^2=0.251$  and  $F=19.563$ .



**Table 3:** *Amanita loosii*, *Cantharellus miomboensis* and *C. heinemannianus* mean mycelium growth rates in potato dextrose agar (PDA) versus leaf extract agar (LEA) in mm/day at 3 days after inoculation

Media	<i>A. loosii</i>	<i>C. miomboensis</i>	<i>C. heinemannianus</i>
PDA	18.934	14.267	28.330
LEA	15.578	14.360	16.983
<i>n</i>	15	15	15
<i>t</i> -value	6.051	-0.136	36.600
<i>p</i> -value	< 0.001	0.893	< 0.001

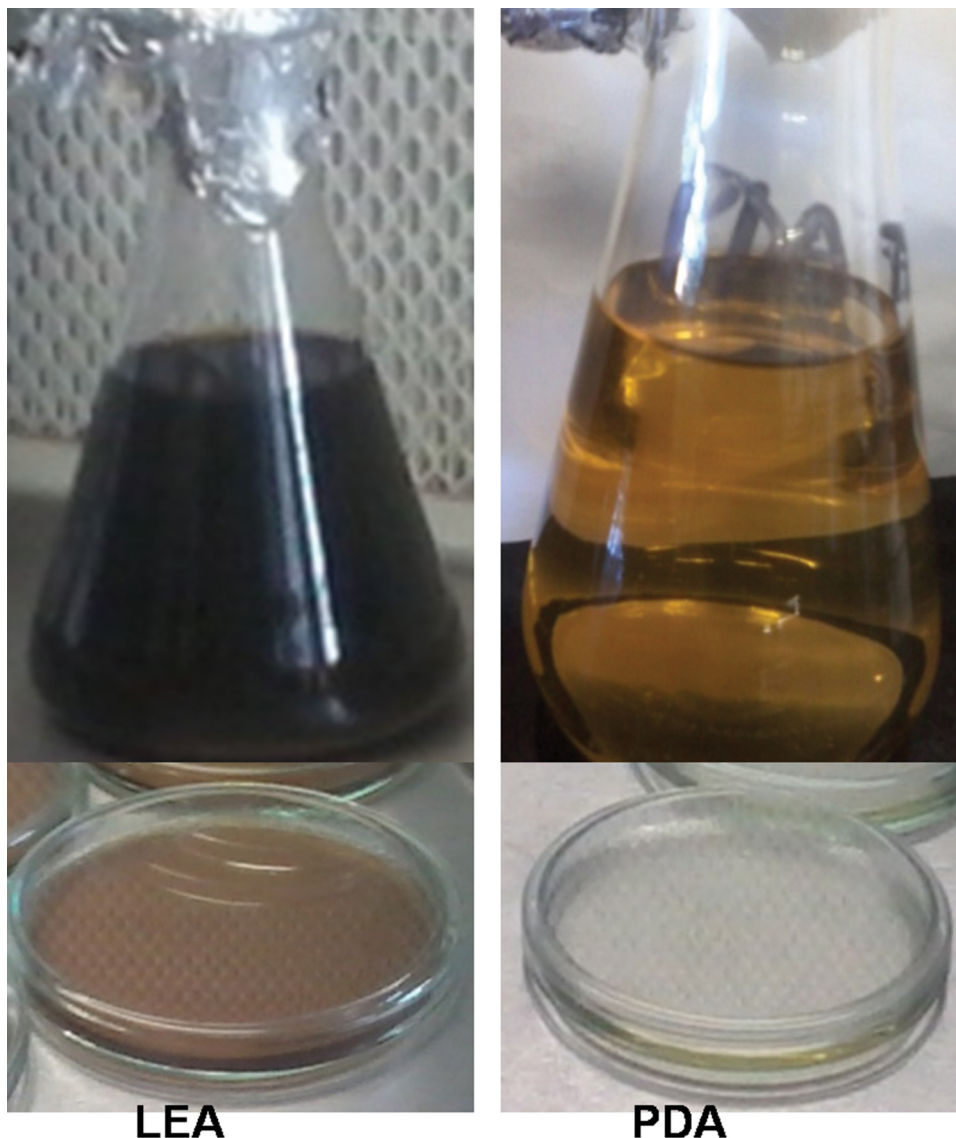
The novel medium, LEA, was light brown compared to the light yellow colour of PDA after autoclaving (Figure 2). Mycelia patterns differed for the same MM mycelia when cultured in the two different media (Figure 3).

In the LEA medium, mycelium of *A. loosii* was found to be septate, branched and growing prolifically flat on the surface of the medium and submerged. Mycelia formed spherical spores on mycelia branches. *C. miomboensis* mycelium was septate and unbranched. Hyphae grew aerially away from the medium's surface and laterally beneath the surface of the medium. Scattered asexual spores were observed. *C. heinemannianus* mycelium was septate, bifurcate branched with

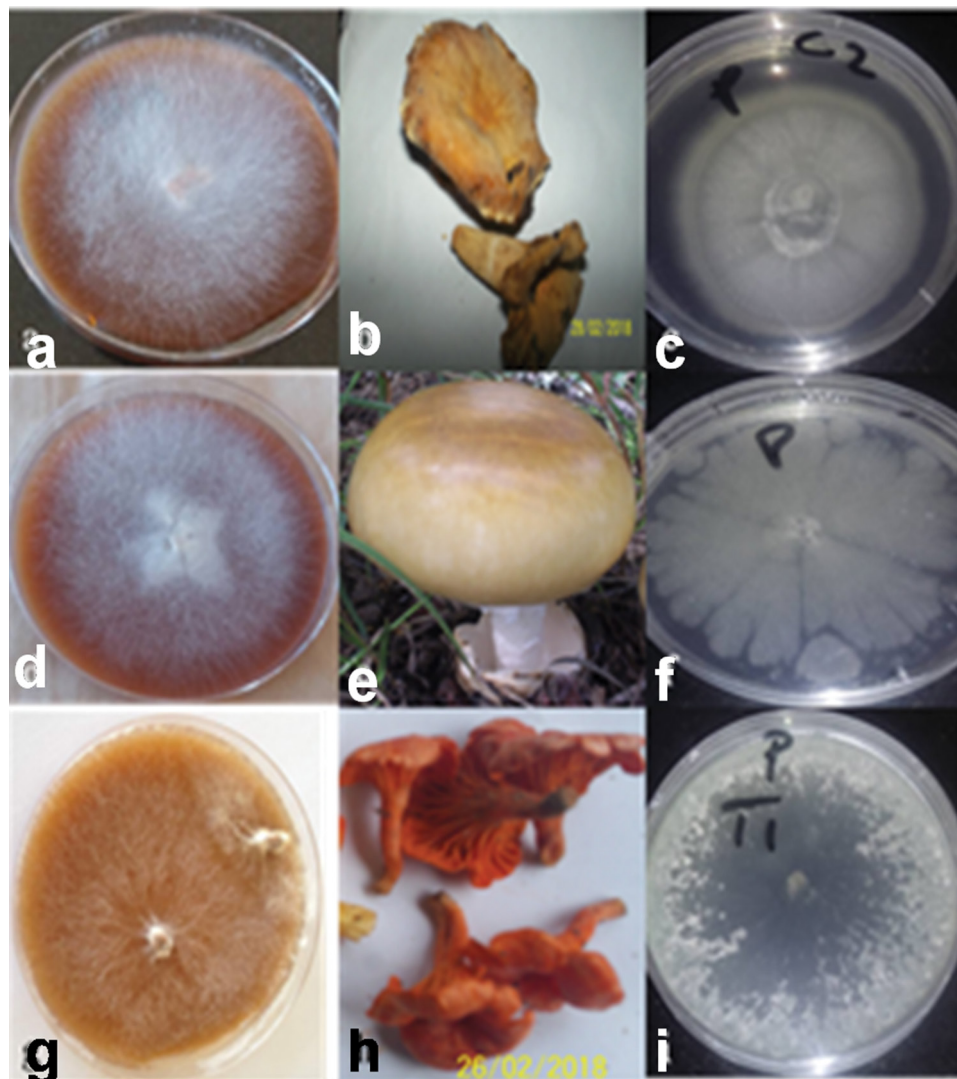
hyphae growing both superficially and submerged, and surface hyphae grew vertically upwards in isolated clumps at pH 5 to pH 7 and grew prostrate at pH 2 and pH 3 (Figure 3). Its mycelium consisted of thick tufted filaments (Figure 3g). At pH extremes, *C. heinemannianus* mycelium seemed to be discontinuous between its edge and its central origin (Figure 3i) in both LEA and PDA media. For all MMs, mycelial strands were more discernible in LEA than in PDA (Figure 2).

## Discussion

We successfully cultured the investigated miombo MM species *A. loosii*, *C. miomboensis* and *C. heinemannianus* in the novel media of *J. globiflora* leaf extract with the inclusion of only the agar-agar fraction. Daily mycelium growth rates for the three mycorrhizal species were differentially influenced by pH to a minor extent as shown in Figure 1a–d, suggesting that they are intrinsically adapted to the same natural habitats. This was not surprising as, indeed, the source sporocarps were harvested from the same miombo woodlands. The optimum pH for growth found was to be a pH of 6 with *C. miomboensis* exhibiting a wider optimum range. In general, mycelium growth rate patterns were similar for *A. loosii* and *C. miomboensis*, but they differed from that of *C. heinemannianus*. This is because the latter was also significantly influenced by medium concentration, while the other two were not significantly influenced by medium concentration. However, regression analysis for growth rates of the three MM species gave varying growth performance under varying pH, while leaf extract concentration (*d*)



**Figure 2:** Contrasting appearance of leaf extract agar (LEA) and potato dextrose agar (PDA) in flask and Petri dish.



**Figure 3:** *Cantharellus miomboensis* in leaf extract agar (LEA) (a) and in potato dextrose agar (PDA) (c) and its sporocarps (b); *Amanita loosii* in LEA (d) and in PDA (f) and sporocarp (e); *C. heinemannianus* in LEA (g) and PDA (i) with sporocarps (h).

only influenced the mycelium growth rate of *C. heinemannianus* ( $d > 0$  g/L) (Equation 3) for our novel media. We found  $d$  to have no influence ( $d > 0$  g/L) on mycelium growth rates of *A. loosii* and *C. miomboensis* (Equations 2 and 4). However, mycelium growth rates were higher in unmodified standard PDA (pH 5.4) for all MM mycelia, except for *C. miomboensis* which gave similar growth rates in both media, suggesting that LEA was a good alternative for *C. miomboensis* (Table 3). The high mycelium growth rates found in LEA media were attributed to the availability of all required nutrients as our analyses showed (Tables 1 and 2). The LEA content of monosaccharide-reducing sugar as a carbon source (Table 2), albeit in low concentration, could have greatly promoted the observed mycelium growth.

For MM species from elsewhere in temperate woodland biomes, several in-vitro culture successes were reported for *Tuber* sp.<sup>6</sup>, *Tricholoma* sp.<sup>41</sup>, *Cantharellus cibarius*<sup>42</sup>, *Laccaria bicolor*<sup>43</sup>, *Cantharellus tropicalis*<sup>44</sup> and *Boletus* sp.<sup>45</sup> using standard media under pH ranges of 4 to 7, suggesting that MM mycelium growth rates show strong pH dependence. We found optimum mycelium growth rates for *A. loosii*, *C. miomboensis* and *C. heinemannianus* to range between pH 4 and pH 6, which closely matched soil pH of 5.0 to 5.7 measured in their natural habitat soils in central Zimbabwe. The optimum pH of 4–6 that we found was similar to the findings of Li et al.<sup>25</sup>, Adeoyo et al.<sup>33</sup>, Obi et al.<sup>46</sup>, Bedade et al.<sup>47</sup> and Daza et al.<sup>48</sup>

Mycorrhizal mushrooms thrive in phosphorus- and nitrogen-poor soils where they assist their host species to extract these nutrients from

the soil.<sup>49</sup> MMs are able to meet their own phosphorus requirements, particularly from organic sources such as phosphate monoesters and diesters, with the largest amount in phytate form.<sup>49–51</sup> This is due to their ability to use released and/or surface-bound acid phosphatases of varying molecular species in the acidic pH range to mobilise organic phosphorus and carbon.<sup>52,53</sup> In particular, the optimum pH for acid phosphomonoesterase was reported to range from 4 to 6<sup>54</sup>, that of phytase from pH 1.3 to 5.5<sup>55</sup>, while that for phosphodiesterase was pH 3<sup>54</sup>, which explains our pH optima observed for MM mycelium growth. In addition, the MM genera *Amanita* and *Suillus* were reported to have high efficiency in mobilising metabolic carbon and nitrogen from organic sources.<sup>53</sup> As the enzymes required for mobilising carbon from organic media, namely cellulase, xylanase and cellobiohydrolase, also have pH optima between 4 and 7<sup>54</sup>, this further explains the mycelium growth optima we found.

Successful MM mycelium growth in LEA was also attributed to adequate iron, magnesium and calcium content (Table 1), which positively complement conditions for high activity of phosphatase and endoglucanase. Furthermore, the moderate content of phosphorus and nitrogen (Table 1) to support MM mycelium growth also accounted for the highly successful result we found for miombo MM species. Consistent with pH optima for phosphatase, phytase and carbon-liberating enzymes, therefore, under acidic pH 2 and 3, the studied MM species grew slowly (Table 3), irrespective of  $d$  – a finding also in agreement with the findings of Obi et al.<sup>46</sup> Failure of *C. miomboensis* to grow at a pH of 2 suggests its inability to liberate its phosphorus and carbon requirements under such a

low pH; for example, phosphorus mobilisation from phytate.<sup>55</sup> However, *A. loosii* and *C. heinemannianus* managed to grow under such a low pH, suggesting their possible reliance on phosphodiesterase, which has a lower pH optimum than phosphomonoesterase, in phosphorus liberation from the media.<sup>54</sup> We hypothesise that these two MM species possess different metabolic enzyme systems as they were also found to develop sporocarps at different times of the season in their woodland habitats.<sup>56</sup> Hence further research, particularly for miombo MM species, needs to be conducted to test our hypothesis, also considering conditions other than pH and leaf extract concentrations used in the current study.

The MM species growth rate models for *A. loosii* and *C. miomboensis* could not use  $\alpha$  as a predictor where pH was the only important external growth factor, unlike that for *C. heinemannianus* (Equations 2, 3 and 4). Hence, apart from pH as a limiting growth factor, the models for *A. loosii* and *C. miomboensis* suggest that growth-limiting factors are more intrinsic than extrinsic in nature for these two MMs. Compared to growth of the latter two MM species, *C. heinemannianus* was generally more vigorous and not indifferent to substrate concentration, demonstrating its inherent intrinsic voracity to substrate utilisation (Table 3). When grown in PDA, *C. heinemannianus* also showed prolific growth, suggesting its successful adaptation in utilising different organic substrates through possession of more efficient carbon-metabolising enzyme systems.<sup>57</sup> The suppressed early growth for *A. loosii*, irrespective of pH level, indicated that environmental factors other than pH (such as temperature, food content/nutrient balance) and intrinsic factors were more important factors in regulating growth rates of its mycelium, and that the species may have completely lost its saprotrophic ability through co-evolution with woody host species.<sup>58</sup> This generally slow growth was also found when *A. loosii* was cultured in PDA. We found mycelium growth rates to be higher at higher pH values under pH 7 for the three MMs, with that of *C. miomboensis* being the highest (Equations 2 to 4). Growth rate models for *A. loosii* and *C. miomboensis* therefore suggest nutrient concentrations in the LEA medium to be non-limiting in minerals like iron, calcium, manganese and copper in supporting mycelium growth, even at the lowest concentration of 150 g/L (Table 1). The generally high mycelium growth rates of *C. heinemannianus* indicate the ability of this species to use phosphoesterases more efficiently in a wider pH range, which also explains the significant influence of  $\alpha$  observed for this species (Equation 3).

Results of a *t*-test to compare the efficacy of LEA against the standard PDA proved that LEA was generally inferior to PDA for *A. loosii* and *C. heinemannianus* but not for *C. miomboensis* (Table 2). Although the crude potato infusion extract had higher amino acid content (Table 2) than the leaf infusion extract used, the high carbon content in PDA owing to addition of 20 g/L dextrose accounted for PDA's superior performance. The surface hardness observed in LEA, due to resins and tannins, may also have retarded growth through restriction of air supply to the submerged mycelium. Hence, physical characteristics of LEA, including surface hardness, aeration, moisture retention and its organic nutritional content, need to be investigated further in developing these media for culturing MMs, particularly *A. loosii* and *C. heinemannianus*. The brown colour observed in the LEA was characteristic of phenolics, flavonoids and tannins, as found in most leaf infusions,<sup>59</sup> suggesting these compounds may be a contributing carbon source for mycelium growth in the cultures. Although the brown colour hampered visual observation of submerged mycelium, LEA revealed its potential as a discriminating morphological indicator (Figure 1) for MM species in this study.

## Conclusion

Our research widens the frontiers of culturable ectomycorrhizal mushrooms and the scope of MMs usable in in-vitro culture media. These media were able to discriminate mycelium characteristics, particularly between *C. heinemannianus* at varying pH, and complement existing media like PDA to discriminate MM morphological appearances, as demonstrated for *A. loosii* and *C. miomboensis*. Hence, use of both of these media can help future identification of the three species for future morphological studies. Our novel media proved a good substitute for PDA in culturing MM, particularly *C. miomboensis*. MM mycelium growth rate

was also demonstrated to be strongly influenced by pH, with optimum pH being 6, and influenced to a lesser extent by media concentration, although more studies are necessary to establish the critical threshold concentration values. In attempting to in-vitro culture MM species, the extent to which they have lost saprotrophic ability must be understood so as to develop suitable optimum conditions different from those given by conventionally used general media for fungi. It is also clear that new media development without the need for costly additives such as glucose seems promising when materials from the mushroom's habitat are used. In addition to their ease of preparation, such cheaply sourced materials have the potential to replace conventionally used material in favour of those substrates that are more adaptable to the hard-to-culture MM, hence guiding microbiology into a future in which some of the new substrates can be used in identifying mushroom species on account of their different appearances in culture. To better understand the growth characteristics of subtropical mycorrhizal mushroom mycelium in culture, more detailed studies involving leaf or root extracts of their host woody species need to be explored to involve wider pH ranges and other environmental factors that simulate the mushrooms' natural habitats. From such studies, more predictive growth models can be developed.

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## Competing interests

We have no competing interests to declare.

## Authors' contributions

A.M.: Conceptualisation; methodology; data collection; sample analysis; data analysis; validation; data curation; writing – the initial draft. M.M.: Writing – revisions; student supervision; project leadership; project management.

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