Tanzania Journal of Science 46(1): 129-136, 2020 ISSN 0856-1761, e-ISSN 2507-7961

Spore Germination for Three Edible Zimbabwean Mushrooms Using Biostimulants

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Received 28 Oct 2019, Revised 26 Febr 2020, Accepted 27 Febr 2020, Published 31 Mar 2020

Abstract

Spores play critical roles in the population and community development of ectomycorrhizal fungi (EMF), which grow naturally in the presence of host plants. EMFs have not been cultivated commercially in most countries including those in Africa. *In vitro* germination of their spores is refractory and has been witnessed only in the presence of stimulants. In this study, germination of spores for three wild ectomycorrhizal fungi commonly collected in Zimbabwe was investigated under the influence of three biostimulants in Murashige and Skoog (MS) and Potato Dextrose Agar (PDA) media for the first time. The stimulatory capacity of tomato or potato roots or *Saccharomyces cerevisiae* was investigated for *Amanita loosi*, *Cantharellus cibarius* and *C. miomboensis*. MS and PDA were used as basal media and each had 1.5% activated charcoal. The experiment was in a factorial design: 3*3*2 and each treatment had five replicates. Spore germination and germ tube elongation were determined every 14 days until 120 days after inoculation. Overall, in the three fungi the tomato root resulted in a germ tube length of 78 µm compared to 61.4 µm and 51.33 µm from potato root and *S. cerevisiae*, respectively. There was no significant difference in the germ tube lengths from the three ECMs. There was also no significant interaction in germ tube length between the three factors. It was concluded that biostimulants can activate spore germination in the three indigenous mushrooms. However, there is need for more research to optimise the germination of the wild mushrooms using these biostimulants.

Keywords: Edible fungi, Murashige and Skoog medium, Potato Dextrose Agar, bio-stimulant, ectomycorrhizal fungi, germination

Introduction

Ectomycorrhizal fungi (ECF) are naturally found in a symbiotic relationship with plants. The fungi hyphae penetrate roots of some plants especially trees of the Zimbabwean miombo forest like *Brachystegia* genus (Degreef et al. 2016).The association of the ECF and the tree benefits both organisms. The tree enhances its nutrients uptake through the ECF while the ECF benefits from the plant through acquiring nutrients from the plant and it also gets a safe habitat (Brundrett 2002). Hence the researcher used plant roots to enhance spore germination.

In Zimbabwe, wild edible mushrooms are regarded as a delicacy because they are only picked in the forest during the rainy season. The mushroom picking season is not more than three months long, hence are not available throughout the year (Mlambo and Maphosa 2017). The commonly gathered mushroom species in Zimbabwe are from orders Cantharellales, Amanitales and Termitomycetes (Mlambo and Maphosa 2017). Chanterelles are among the highly priced

mushrooms in the world (Rühl and Kües 2007) and are also a delicacy in Zimbabwe. The dry mass of mushrooms consists of proteins (25.71- 36.51%), lipids (1.4 – 2.79%), carbohydrates (37.38 – 48.63%), minerals (4.45 – 10.29%) and fiber (3.77 – 11.44%) (Chittaragi et al. 2014). They also have antioxidant, anticancer, and low calories (Chittaragi et al. 2014, Muhammad and Suleiman 2015). Chanterelles have been used in the cosmetics and food industry to produce coloured products. The molecule which gives Chanterelles colour is canthaxanthin (Saidi et al. 2016). There are also considerable antimicrobial activities in the *Cantharellus sp* against *Bacillus, Staphylococcus, Micrococcus* and *Streptococcus.* This makes them nutraceuticals as well (Redhead et al. 2016). Therefore, if these three mushrooms could be grown *in vitro*, it makes them available all year round for culinary and industrial uses.

Chanterelles exist in a symbiotic relationship with trees mostly of the *Brachystegia* genus such as *B. spiciformis* and *B. utilis*. Another tree which forms a symbiosis with *Cantherellus sp* is *Uapaca kirkiana* which is also found in miombo forests. *Julbernardia globiflora* and *J. panaculata* also partner with *Cantharellus sp* (Clasen et al. 2018). The second genus for this study is the *Amanita. Amanita* genus is diverse and comprises of around 900 - 1000 species (Clasen et al. 2018). It is mainly an ectomycorrhizal genus though there are a few saprophytic species (Halbwachs and Bässler 2015). *A. loosi* is considered as a delicacy by Zimbabweans and it has very good nutritional values and has antibacterial properties. This makes the mushroom a nutraceutical as well (Mlambo and Maphosa 2017). However, it is only available during the rainy season which is mostly not more than three months all year round.

Spores are important in the life cycle of an ECF because they are the 'seeds' and the next generation depends on their survival, germination and ability to colonise territories for their survival Khan 2005). Germination is a very important step in any attempt to grow mushrooms commercially. The progression of a spore from an inactive state to an active form, whereby the spore is committed to growing and the reactions taking place are irreversible is called germination (Paidhungat and Setlow 2002). The successful germination of spores depends on a number of variables which include presence of root exudates, optimum temperature, optimum pH, light intensity, carbon dioxide concentration (Bever et al. 2001) and microflora in the rhizosphere (Rigamonte et al. 2010, Saidi et al. 2016). The successful germination of the indigenous fungi spores in this study will enable their commercialisation.

In vitro spore germination of ectomycorrhizal fungi without a stimulant has always been refractory (Thongklang 2011). Early mycologists like Fries and colleagues tried to germinate some ECF spores without a host and it was in vain. The first microbiologist to germinate ECF spore *in vitro* was Melin when he used *Solanum lycopersicum* (tomato) roots exudates to stimulate spore germination of *Russula* species in 1962. Fries in 1979 described the germination of *Leccinum* species using tomato roots. The roots exude biomolecules which were termed the 'M factors' are hypothesised to stimulate germination of spores (Melin and Rama Das 1954). *Solanum tuberosam* (potatoes) have not been used to stimulate germination of the three fungi in previous investigations. However, they have been used to trigger resting spore germination of *Spongospora subterannea*. *Saccharomyces cerevisiae* has been used in previous investigations to stimulate the germination of *Neocallimastix frontalis* a probiotic for ruminants (Adesogan 2007, Chaucheyras-Durand et al. 1995, Chaucheyras-Durand et al. 2016).

Stimulation of spore germination of ECF has also been attempted by varying the concentration of glucose and nitrogen source in the environment. These two elements affect amino acid metabolism of the fungal spores which contribute to germination (Wang et al. 2015). The Scientific and Industrial Research

Development Centre (SIRDC) tried growing *A. loosi* in PDA without success (Savadye 2018 personal communication).

In vitro cultivation of ECF is mostly hampered by contamination because of the commensal microflora found on the mushroom tissues which include spores. Antibiotics like streptomycin are used to reduce the contamination rates (Santiago 2014). Activated charcoal is used to counter effect inhibitory substances produced by agar during autoclaving. Danell (1994) was able to grow mycelium using antibiotics, activated charcoal and modified Fries medium. In an attempt to increase the successful germination of the spores in this study, the researcher used antibiotics and activated charcoal.

If ECF spores germinate there is hope that they can develop to form mycelium. The mycelium can then be further treated to produce commercial spawn for mushroom cultivation. The spawn can be injected into natural forests so that they continually produce the desired mushroom in the correct environment. This has been successfully done in truffle production (Reyna and Garcia-Barreda 2014, Yamada et al. 2017). Growth of mycorrhizal mushrooms has been tried since early 19th century with mixed successes. However, some workers in China and Japan have been able to grow *Lyophyllum sp, Tricholomamatsutake,* and *Rhizopogan roseolus* which are mainly grown artificially in tree plantations through inoculation of tree seedlings with the desired fungi mycelium (Jie 2009, Yamada et al. 2017). This study aimed to use the three bio-stimulants, tomato roots, potato roots and *Saccharomyces cerevisiae* on the three mushroom species, *A. loosi, C. cibarius* and *C. miomboensis* which are indigenous to Zimbabwe.

Materials and Methods

Cantherellus mushrooms were picked from a forest 14 km from Marondera, Zimbabwe (18.1930° S, 31.5427° E) and *A. loosi* was picked in Charlote Brooke, 30 km from Harare Central Business District, Zimbabwe (17°39'6"S 31°11'58"E). All laboratory works were done at Scientific and Industrial Research and Development Centre (SIRDC) in Harare and the spore prints were done in Marondera, Zimbabwe.

Fresh mushroom of each species was placed on a sterilised bond paper then covered with a sterilised beaker overnight to minimize contamination. The next day the bond paper was then packed in a plastic zip bag and closed to keep spores dry and to maintain viability. The bags were kept at room temperature in a dark environment. Only fresh mushroom caps which had recently 'opened' were picked and they were all used within 60 days.

Using a top pan analytical balance, one gram of the spores scrapped from the spore print was mixed with one millilitre of distilled water. The whole mixture was mixed with five millilitres of 0.01% Tween 20 and two millimetres of ethanol and 0.1 g streptomycin for 10 minutes.

Three bio-stimulants were used, namely, tomato roots, potato roots and *S. cerevisiae* on each of the three mushrooms. *S. cerevisiae* was bought from the market as dried brewer's yeast (Anchor yeast). The plant roots were obtained from Scientific and Industrial Research and Development Centre SIRDC axenic plant cultures which were grown on MS medium. The biostimulants were soaked in 70% ethanol for 30 minutes then rinsed in distilled water until the smell was removed. The biostimulants were then soaked in sodium hypochlorite for ten minutes and then rinsed three times.

A 1 ml portion of spores' suspension of each species was inoculated in a 100 ml sterile tissue bottle which contained 30 ml of either MS or PDA basal medium with 1.5% of activated charcoal. The bottles were autoclaved at 121 °C for 30 minutes at 15 psi. The biostimulants were then soaked in 70% ethanol for 30 minutes then rinsed in distilled water until the smell was completely removed. They were then soaked in 10% sodium hypochlorite for ten minutes and then rinsed three times. Five pieces of 1.5 cm of potato or tomato roots were placed on set media. Half a millilitre of

0.5% w/v *S. cerevisiae* was flooded on the medium. Tissue culture bottles were closed with lids and sealed with shrink wrap to prevent drying out and contamination. The treatments are as shown in Table 1.

Table 1: Treatments of the three fungi with stimulant and medium

Fungi	Tomato	Potato	S. *cerevisiae*	Control	Media
C. *miomboensis*	CMTMPDA	CMPTPDA	CMSCPDA	CMPDA	PDA
C. miomboensis	CMTMMS	CMPTMS	CMSCMS	CMMS	MS
C. cibarius	CCTMPDA	CCPTPDA	CCSCPDA	CCPDA	PDA
C. cibarius	CCTMMS	CCPTMS	CCSCMS	CCMS	MS
A. loosi	ALTMPDA	ALPTPDA	ALSCPDA	ALPDA	PDA
A. loosi	ALTMMS	ALPTMS	ALSCMS	ALMS	MS

Key: CM = *C. miomboensis;* CC= *C. cibarius;* AL = *A. loosi;* PT = Potato; SC = *S.cerevisiae;* TM = Tomato; MS = Murashige and Skoog; PDA = Potato Dextrose Agar.

A 3*3*2 factorial experimental design was used. Each treatment had 5 replicates. There were two basal media, three biostimulants and a control which had the basal media and *C. miomboensis, C. cibarius* and *A. loosi* spores but no biostimulant. Analysis of variance using GenStat Copyright 2013, VSN International Ltd software was used.

Number of days taken to spore germination were recorded. Bottles were inspected once per fortnight up to 120 days for germinating spore or young mycelium. Congo red and methylene blue dyes were used to stain the spores before observations on the microscope. Sampled bottles were discarded and were not replaced. Germ tube formation observation and measuring its length were done once per fortnight. A digital microscope, Motic picture (F series, 2004) was used to capture pictures.

Results and Discussion

Using the Bonferroni test analysis, the stimulants and media had significant effects on the germ tube length, while there was no significant effect on the germ tube growth from each mushroom species at 5% significance level. There was no significant effect on the interactions of the mushrooms and the stimulant or medium. Medium and stimulant interaction did not result from a significant difference in the germ tube length.

Germ tube length on the three mushrooms

A germ tube was observed in all the spores of the three mushrooms within 90 days after inoculation. However, there was no germination in all the controls. In *A. loosi* the longest germ tube was observed where spores were stimulated by the tomato roots in MS medium, Figure 1.

In *C. miomboensis,* the longest germ tube came from roots in PDA medium. In *C. cibarius,* potato roots in PDA stimulated the growth of the longest germ tube. Overall, *C. cibarius* had a longer germ tube of 64.63 µm compared to *A. loosi* and *C. miomboensis* which had 63.67 µm and 62.43 µm, respectively. However, there was no significant difference on the germ tube length amongst the three fungi at 5% significance level as shown in Table 2. The three fungi produced germ tube lengths which are insignificantly different which suggested that the germination capacity of the spores of the three mushrooms is almost the same. All the three fungi exhibit the same features as ECM. Failure of the control to germinate in all the three mushrooms is explained by absence of biostimulants in the control setups. All the three biostimulants had positive effects on the stimulation of spore germination as shown in Table 2.

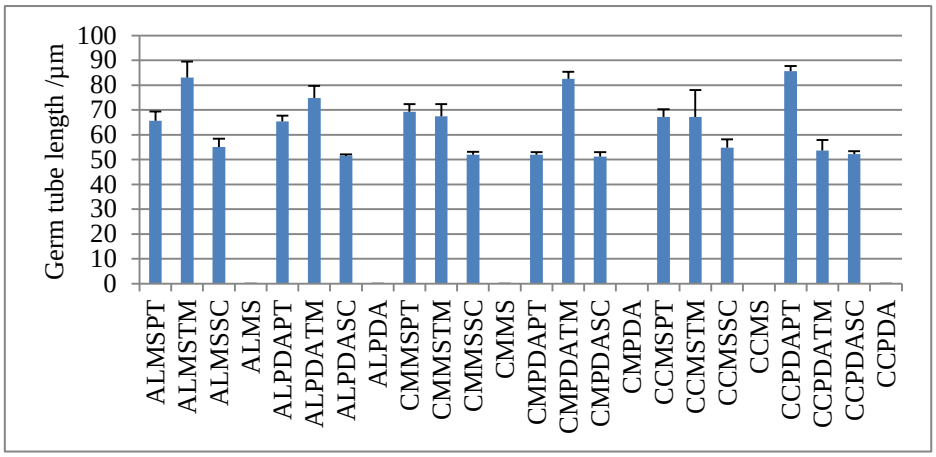

Figure 1: Average length of germ tubes in all treatments after 120 days.

Table 2: Comparison of the average germ tube length of the three mushrooms after 120 days

Type of mushroom	Average germ tube length[a] (µm)
C. miomboensis	62.43a
A. loosi	63.67a
C. cibarius	64.63a

[a]Germ tube length means followed by letters. If letters are different there is a significant difference (P = 0.05) according to the Bonferroni test. There was no germination in all controls without a biostimulant.

Effects of the biostimulants

In this investigation, the tomato as a biostimulant initiated spore germination and germ tube formation for all the fungi. In culture bottles where there was no tomato there was no germination, which indicated that there were exudates from the tomato that were enhancing germination. The effect of the tomato resulted in a germ tube of 78 µm and is significantly different from the other two stimulants as shown in Table 3. These results are in agreement with Melin who in 1962 used tomato and pine seedling roots to stimulate the germination of spores *in vitro* by Foltz (2011). Melin (1962) established that there was a factor which was then called the 'M' factor which stimulated mycelia growth and spore germination in fungi. Recent advances have shown that specific molecules like amino acids, organic acids, sugars, vitamins, purines, nucleosides, enzymes are produced in large quantities and there are other molecules which are produced in small quantities like terpenoids and flavonoids in biostimulants (Tahat and Sijam 2012). Strigolactones are small lipophilic molecules that are found widely in root exudates but they are not easy to analyse hence the little literature (Barman et al. 2016, Wang et al. 2015). The results observed in this investigation are in agreement with Steinkellner et al. (2005), where they positively stimulated the germination of microconidia of *Fusarrium oxysporum* Fusarium. *lycopersici* (Fol) and *Fusarium. oxysporum* sp. *radicis-lycopersici* which are tomato pathogens.

Table 3: Comparison of the mean germ tube length of the three biostimulants

Type of mushroom	Mean germ tube length[a] (µm)
S.cerevisiae	51.33[a]
Potato roots	61.40[b]
Tomato roots	78.00[c]

[a]Germ tube length means followed by letters. If letters are different there is a significant difference (P = 0.05) according to the

Bonferroni test. There was no germination in all controls without a biostimulant.

Potatoes resulted in a germ tube of 61.4 µm which was shorter than the tomato but longer than the effect of *S. cerevisiae* as shown in Table 3. Potatoes are able to initiate spore germination because they exude molecules like pentose sugars, phenolics and organic acids (Steinkellner et al. 2007. Molecular characterisation of these biomolecules has shown that there are glucose molecules, galactose, arabinose, galactorunic acid and other biomolecules in the potato root exudates (Koroney et al. 2016). Balendres and his colleagues in 2016 carried out an investigation to find out the effect of potato root exudates on the resting spores of *S. subterranean* which is a pathogen found in the soil. Their results confirmed that the exudates increase spore germination (Balendres et al. 2016). However, there is no prior similar investigation which compared the effect of the potato and the other two biostimulants.

S. cerevisiae had a positive stimulatory effect which resulted in the growth of a germ tube in all the treatments. However, it had the least mean length of 51.33 µm. Its effect is significantly different from that of the two other biostimulants. The stimulatory effect of the yeast which were found from this investigation are in agreement with the findings from Chaucheyras and colleagues (1995). They used the yeast to stimulate spore germination of an anaerobic fungus *N. frontalis*, which is found in the rumen. In that investigation, *S. cerevisiae* positively induced spore germination in the probiotic. However, there is no similar investigation which compared the effect of the three biostimulants.

In terms of interactions between the three factors, there was no significant effect on the interaction between the biostimulant*media, mushroom*media, mushroom*biostimulant. This is shown in Table 4 which shows an F probability larger than 0.05.

Table 4: Germ tube length due to interactions between factors

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr
Mushroom	2	72.96	36.48	0.47	0.629
Medium	1	960.4	96.4	12.27	< 0.01
Stimulant	2	10880.09	5440.4	69.49	< 0.01
Mushroom*medium	2	105.0	52.50	0.67	0.514
Mushroom*stimulant	4	556.31	139.08	1.78	0.142
Medium*stimulant	2	327.20	163.60	20.9	0.131
Residual	76	5950.0	78.29		
Total	89	18851.96			

There is need to investigate the effects of potato and tomato plant roots of different ages. Twenty-one day old *Pinus* seedlings were more effective in inducing spore germination of *Rhizopogon luteolus* spores than four-day old seedlings (Theodorou and Bowen 1987) probably indicating increased leakage of exudates from older seedlings. The seedlings used in the current study were of the same age.

Conclusions

The results herein show that the three biostimulants have stimulatory effects on the germination of the three edible Zimbabwean indigenous mushrooms. The tomato has a higher stimulatory potential. However, further work is essential to optimise the germination of the wild edible mushrooms spores by the biostimulants and to establish the exact metabolites and the stages when they are produced in the tomato and the other

biostimulants. There is need to investigate the effects of plant roots of different ages as the concentrations of root exudates vary with age since only one age group was used in this study.

Acknowledgement

We acknowledge assistance from SIRDC with the tomato and potato plant roots and permission for the laboratory work to be carried in the Mycology laboratory of the same institute in Harare.

Conflict of Interest

The authors declare no conflict of interest.

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