RESEARCH | Egyptian Journal of Agricultural Research | Mycology

Effects of manganese, sodium nitrate, and ammonium nitrate on the growth rate of *Ganoderma lucidum* mycelium

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Received: 02-01-2023; **Accepted**: 25-02-2023; **Published:** 03-05-2023 **DOI**: 10.21608/ejar.2023.114228.1191

ABSTRACT

Ganoderma lucidum is a mushroom with numerous medicinal properties. It is usually grown on substrates based on hardwood sawdust that is supplemented with costly additives, such as wheat or rice bran. It is well-established that substrate composition significantly affects the mycelial growth of G. lucidum. In our previous review study, we proposed that substances that stimulate extracellular enzyme secretion can replace expensive additives. Among such substances, inorganic compounds are cost-effective and are appropriate candidates. This study determined the effect of manganese, sodium nitrate, and ammonium nitrate on the mycelial growth of G. lucidum. Wheat straw was used as a growing substrate, and the supplements included manganese, sodium nitrate, ammonium nitrate, wheat bran or flour, and sucrose. These additives had concentration-dependent effects on mycelial growth. Higher concentrations of sucrose, sodium nitrate, and ammonium nitrate retarded mycelium growth significantly. Comparing the mycelium growth rate, manganese could replace wheat bran or flour as a more cost-effective additive.

Keywords: *Ganoderma lucidum*, Growth rate, Manganese, Mycelium, Nitrogen

INTRODUCTION

Ganoderma lucidum is an economically important traditional herbal medicine, which is gaining popularity with increasing demand in the global market (Bulam *et al.,* 2019; Bijalwan *et al.,* 2020). This edible basidiomycetous fungus has shown several medical efficacies including anti-HIV, hepatoprotection, immunomodulating, anticancer, anti-diabetic, and anti-oxidant activities, and inhibition of histamine secretion due to different types of biocompounds (Pattanayak *et al.,* 2020). Considering the growing application of G. lucidum in complementary and alternative medicine, the traditional collection does not meet the market demand, and studies for improving the artificial mushroom cultivation are ongoing worldwide (Hapuarachchi *et al.,* 2018).

Active compounds of the mushrooms have been extracted from the fruiting bodies (basidiomes), mycelia, and spores of G. lucidum (Heleno *et al.,* 2012). Efforts for solving the problem of long cultivation time of basidiome have directed researchers to the invention and improvement of the mushroom cultivation methods such as submerged cultivation of the mycelium (Jayasinghe *et al.,* 2008; Saltarelli *et al.,* 2009; Nhung *et al.,* 2018; Peng *et al.,* 2019). However, the basidiome production on the substrates that are usually locally-available agro-waste materials is still interesting for producers. As the primary experiments in the domestication of Ganoderma had used the hardwood-derived substrates, for a long time, the main constituent of these substrates was composed of different sorts of sawdust or wood chips. Therefore, alternative ones such as using some sorts of straws have been recently introduced (Ćilerdžić *et al.,* 2018). Moreover, another convenient exertion in preparing the substrates is to use wheat, rice, and corn bran to supplement the substrates. Although supplementing with these materials is very effective, they are very costly. For this reason, replacing these materials with less-costly ones or other treatments are favored. Using the materials reported to stimulate the production of extracellular enzymes of laccase (Lac) and/or manganese peroxidase (MnP) may be a considerable alternative to these expensive supplements (Amiri-Sadeghan *et al.,* 2022).

Metals including manganese (Mn) have important roles in fungal biology, especially in mycelial growth and fruiting body production and fungal biomass through being adsorbed and accumulated in different concentrations (Schroeder, 1965; Favero *et al.,* 1991; Sanglimsuwan *et al.,* 1993; Gabriel *et al.,* 1996; Yang *et al.,* 2017). However, the effect of metals on the mycelium growth of G. lucidum has rarely been studied, and there are just a few reports (Yang *et al.,* 2017; Zhang *et al.,* 2019).

The source of nitrogen (N) and its concentration were found to influence MnP and Lac production (D'Souza *et al.,* 1999; Martínez *et al.,* 1996). For example, high N concentration suppressed MnP production by Phanerochaete chrysosporium (Kirk and Farrell, 1987) and Lentinus edodes (Buswell *et al.,* 1995) In contrast, MnP titers in cultures of Bjerkandera sp. BOS55 and Phanerochaete flavido-alba were stimulated by a high N

medium (Mester & Field, 1997). Also, it is shown that high concentrations of N increased up to five times the production of the Lac enzyme in G. lucidum (D'Souza *et al.,* 1999). The effects of nitrogen sources on G. lucidum mycelial growth (Jayasinghe *et al.,* 2008) and enzymes (Malarvizhi *et al.,* 2003) have been studied previously. However, various nitrogen sources have different effects on the mycelia biomass yield of G. lucidum (Suberu *et al.,* 2013). This study evaluated the effects of different substrates containing Mn, sodium nitrate, and ammonium nitrate on the mycelium growth rate of G. lucidum in comparison with convenient bran or flour supplements. It is worth mentioning that the higher mycelium growth rate is not necessarily correlated with a higher yield of basidiome (Amiri-Sadeghan *et al.,* 2022). Hence, the translation of the results of this study on basidiome production needs to be evaluated.

MATERIAL AND METHODS

The growth rates of mycelia in 24 substrates were examined in 15-cm glass tubes. Table1 indicates the contents of each substrate that were prepared by mixing solid and liquid portions. The solid portion in all substrates contained 1.5 g of 0.5-cm-cut dry wheat straw (WS), which was supplemented with wheat flour or bran in four treatments. The liquid portion was a solution (15ml) that was soaked completely into the solid portion before filling into the glass tubes. The composition of the solution was different for each substrate and was prepared by mixing different volumes of water and the stock solutions, as indicated in Table1. The stock solutions included sucrose (20% w/v), MnSO4 (4mM), NH4NO3 (1M), and NaNO3 (2M). Each substrate was prepared in triplicate in plastic cups and was placed into both-end cotton-capped glass tubes and autoclaved for 1 hour. The substrates were inoculated by placing two micellized wheat grains at one end of the glass tube under aseptic conditions. The tubes were placed in a dark chamber with 90% humidity at a controlled temperature of 25°C. The mycelium growth was recorded once a day by measuring the length of the white -covered area from a marked line as the origin of coordinates up to 300 h.. The slope of the regression line (length (mm)/ time (hours)) as the growth rate and its corresponding coefficient of determination (R2) were calculated by MS Excel (2013) for each repeat. A single repeat with R2≤96% was omitted from the further statistical analysis. The comparison of the means was carried out by One-way ANOVA using SPSS (2016).

Table1. The composition of the substrates.

Treatment number	Label	Volume from deionized water	Volume from $MnSO_4$ Stock (ml)(final conc.)	Volume from $NaNO_3$ Stock (ml) (final conc.)	Volume from NH_4NO_3 Stock (ml) (final conc.)	Volume from Sucrose Stock (ml) (final conc.)
T1	WS	15	0	0	0	0
T2	WS+Mn	11.25	3.75 (1mM)	0	0	0
T3	C1	10.5	3.75 (1mM)	0	0	0.75 (1% w/v)
T4	C2	7.5	3.75 (1mM)	0	0	3.75 (5% w/v)
T5	C3	3.75	3.75 (1mM)	0	0	7.5 (10% w/v)
T6	NO_3-1	10.875	3.75 (1mM)	0.375 (0.05M)	0	0
T7	NO_3-2	9.75	3.75 (1mM)	1.5 (0.2M)	0	0
T8	NO_3-3	7.5	3.75 (1mM)	3.75 (0.5M)	0	0
T9	NH_4-1	10.875	3.75 (1mM)	0	0.375 (25mM)	0
T10	NH_4-2	9.75	3.75 (1mM)	0	1.5 (100mM)	0
T11	NH_4-3	7.5	3.75 (1mM)	0	3.75 (250 mM)	0
T12	C1N1	10.125	3.75 (1mM)	0.375 (0.05M)	0	0.75 (1% w/v)
T13	C1N2	9	3.75 (1mM)	1.5 (0.2M)	0	0.75 (1% w/v)
T14	C1N3	6.75	3.75 (1mM)	3.75 (0.5M)	0	0.75 (1% w/v)
T15	C2N1	7.125	3.75 (1mM)	0.375 (0.05M)	0	3.75 (5% w/v)
T16	C2N2	6	3.75 (1mM)	1.5 (0.2M)	0	3.75 (5% w/v)
T17	C2N3	3.75	3.75 (1mM)	3.75 (0.5M)	0	3.75 (5% w/v)
T18	C3N1	3.375	3.75 (1mM)	0.375 (0.05M)	0	7.5 (10% w/v)
T19	C3N2	2.25	3.75 (1mM)	1.5 (0.2M)	0	7.5 (10% w/v)
T20	C3N3	0	3.75 (1mM)	3.75 (0.5M)	0	7.5 (10% w/v)
T21	Flour 1%	15	0	0	0	0
T22	Flour10%	15	0	0	0	0
T23	Bran 1%	15	0	0	0	0
T24	Bran 10%	15	0	0	0	0

WS: Wheat straw

RESULTS

The mycelium growth of G. lucidum in different substrates containing Mn, nitrate, and ammonium as extracellular enzyme stimulators was examined. The mycelium growth was recorded every day by measuring the white covered area in the glass tubes **Fig. (1).** The linear regression of growth (mm) against time (hours) was calculated for each repeat. Besides, its coefficient of determination (R2) was used to eliminate unsuitable estimation if R2< 97%. The slope of this line is considered as the growth rate. Comparing the means of the growth rates by One-way-ANOVA showed that there are significant differences among the substrates ($F_{(50.89,23)}$, ($p<0.001$)). As indicated in Fig. 2, Sodium nitrate with a concentration of 0.5M in treatment T8 was toxic and stunted the growth completely. However, when sucrose as a carbon source was applied, the toxicity of nitrate decreased, and the growth was not completely harnessed (T14, T17, T20). When considering the treatments of T12-T20, in which nitrate is applied besides sucrose, although not statistically different, there is a trend showing that a lower concentration of sucrose provided a higher mycelial growth rate. This is also true when the treatments contain only sucrose (T3-T5). The concentrations of ammonium nitrate and nitrate were adjusted so that similar nitrogen molarities existed among T6-T11, and ammonium nitrate was less toxic than sodium nitrate. We used these stimulators to replace with costly supplements such as bran and flour. Among these stimulators, Mn seems to act more effectively. However, there was no significant difference between WS alone with those supplemented with bran, flour, Mn (1mM), sucrose, ammonium nitrate (25mM), and sodium nitrate (50mM).

Fig. 1. Mycelia growth in 15-cm glass tubes. Showing a photograph of the glass tubes. The length of the white covered area of mycelium in each tube was measured considering the marked line as the origin of coordinates.

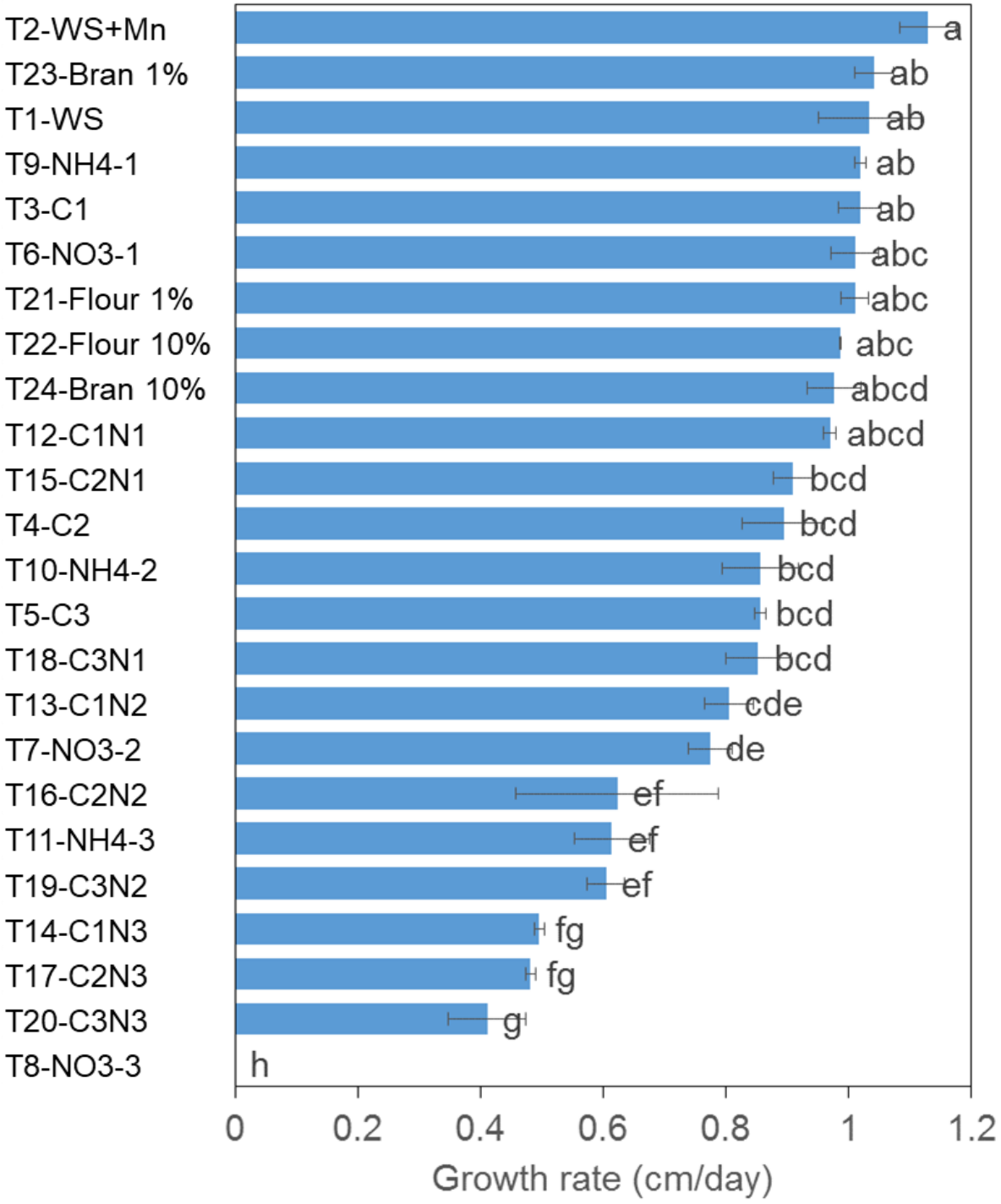

Fig. 2. Comparison of mycelia growth rates in different substrates by one-way ANOVA. As depicted here mycelium growth rates were not significantly different in substrates with common letters. Among the substrates, T8, which was supplemented with 0.5M sodium nitrate showed the most inhibiting effect on mycelium growth. However, adding a carbon source (Sucrose) lowered this inhibitory effect. The highest growth rate was observed for Mn, however, there was no significant difference with the non-supplemented condition.

DISCUSSION

Here, we examined the mycelium growth rates of G. lucidum on different substrates. The main purpose of this study was to determine whether mycelium growth is retarded or enhanced by some inorganic supplements. The lignocellulose portion of the substrates was selected to be wheat straw as the locally available agro-waste as an alternative for wood-derived substances. Four treatments (21-24) were supplemented by wheat bran and flour as ordinary supplements. The benefit of these types of supplements is well-demonstrated. However, they are

costly and finding appropriate substitutes are favored. Hence, here, we examined the effect of other supplements on the mycelium growth rate, as a basic marker of the suitability of a substrate.

As a metal-enriched edible fungus, *G. lucidum* is capable of adsorbing Mn effectively. Mn ion is demonstrated to play an important role in the synthesis of MnP and other physiological activities during *G. lucidum* growth (Zhang *et al.,* 2019). However, these effects are concentration-dependent; for example, it has been reported that the mycelial biomass of *G. lucidum* was higher in cultures supplemented with 200 or 400 mg/L Mn, and it was significantly lower with concentrations of 800 mg/L Mn or higher ones (Cai & P, 2011). It is shown that Mn promoted MnP activities, mycelial growth, and the dry yield of *G. lucidum*. The metabolites such as triterpenoids, chavicol, and palmitoylethanolamide were increased by Mn addition, suggesting the promotion of Mn on the nutritional value of *G. lucidum* (Zhang *et al.,* 2019). MnP is one of the main extracellular enzymes produced by *G. lucidum* for lignin decomposition and cellulose degradation (Bolaños *et al.,* 2018) by the production of oxidant Mn^{3+}, which results in decomposing the lignin polymer (Hatakka, 1994). Lac is another degrading extracellular enzyme and its production is also enhanced by Mn ion in fungi (Galhaup & Haltrich, 2001). At higher Mn^{2+} concentrations, production of MnP increased and that of Lac decreased, but the rate or the number of decolorizations was unaffected (Swamy and Ramsay, 1999). In this study, the fastest growth was gained by wheat straw supplemented with 1mM Mn, and no retardation of growth took place. However, caution should be taken, as Mn accumulates in basidiomes.

It has been reported that different nitrogen sources affect the mycelial growth of *G. lucidum* differently, and ammonium or nitrate-containing compounds are among the most suitable sources (Jayasinghe *et al.,* 2008). It has been reported that *G. lucidum* is unable to decolorize rubidium bromide in a low nitrogen medium (Eichlerová *et al.,* 2005), suggesting that the medium component has a great influence on the decolorization of dye by Lac. Nitrogen sources can regulate Lac gene transcription, which results in different transcriptional profiles in different species and within the same strain (Piscitelli *et al.,* 2011; Pezzella *et al.,* 2013). In *G. boninense* treated with different nitrogen sources, including ammonium nitrate and sodium nitrate, expression of manganese peroxidase GbMnP_U6011 was up-regulated while GbMnP_35959 was up-regulated by ammonium nitrate, and suppressed by sodium nitrate. Also, the Lac coding genes GbLac and GbLac_U90667 were up-regulated by ammonium nitrate, while GbLac_U30636 was suppressed by sodium nitrate (Ho *et al.,* 2020). On the other hand, the sensitivity of fungi to ammonium nitrate was studied and results showed that ammonium nitrate inhibited the growth of fungi only in higher concentrations. In contrast, the growth of *Gaeumannomyces graminis* was stimulated by even the highest concentration of 0.6M ammonium nitrate (Veverka *et al.,* 2007). Ammonium nitrate was used for enriching experimental substrates in different cultivation techniques of *G. lucidum* (Kurd-Anjaraki *et al.,* 2021). Ammonium nitrate repressed MnP formation and effluent remediation capacity of *G. lucidum* (Asgher *et al.,* 2010). Ammonium nitrate was noticeably found to be one of the best nitrogen sources for the mycelial growth of *G. boninense* (Peng *et al.,* 2019). In this study, we tested the effect of these inorganic compounds on the mycelium growth rate, and their retarding concentrations were elucidated. The future purpose of using these molecules is using them as stimulators rather than as nutrition sources. Hence, due to the results of this investigation, using minute amounts of these substances will be examined on basidiome production.

CONCLUSION

In conclusion, a high concentration of nitrate was toxic for the mycelial growth of *G. lucidum*, which could be eliminated to some extent in the presence of sucrose. Increasing the concentrations of supplements of sucrose, as a simple carbon source, ammonium nitrate or sodium nitrate, as a simple nitrogen source reduced the growth rate, and only in lower concentrations were similar to non-supplemented ones. Therefore, supplementing by these substances may be non-beneficial and further examinations are required. On the other hand, applying Mn in this concentration did not affect the growth negatively and maybe a better choice for further evaluations.

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