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Biological peculiarities of selected species of Ericoid Mycorrhizal Fungi associated with blueberry (*Vaccinium corymbosum* L.)

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Abstract

Ericoid Mycorrhizal Fungi (EMF) play a crucial role in the mutualistic symbiotic interaction with blueberry plants (*Vaccinium corymbosum* L.), aiding in nutrient uptake and resilience to various stresses. Despite their importance, EMF remains among the least studied fungal groups worldwide. This study investigates the biological peculiarities of selected EMF species, namely *Hyaloscypha hepaticicola* and *Oidiodendron maius*, associated with blueberry plants. Laboratory experiments were conducted to evaluate the optimal growth conditions for EMF strains, focusing on media type, temperature, acidity (pH) levels, and humidity. It studied the interaction between two species of EMF as well by co-cultivation *in vitro*. Results revealed Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) as optimal media for EMF growth, with varying temperature preferences observed for each strain (for *Hyaloscypha hepaticicola* it was 25℃ (degrees Celsius) and for *Oidiodendron maius* 30 ℃ (degrees Celsius)). Acidity (pH, *Hyaloscypha hepaticicola* showed better growth when pH was 5 and 7, *Oidiodendron maius* showed better development when pH was 6 and 9) and humidity were found significantly influence EMF growth, with optimal levels differing between strains (for *Hyaloscypha hepaticicola* it was 95% and for *Oidiodendron maius* 95% and 100%). Furthermore, a co-cultivation experiment highlighted an antagonistic interaction between EMF strains, emphasizing the complexity of microbial interactions in soil ecosystems. These findings contribute to our understanding of EMF biology and offer insights for their practical application of mycorrhizal fungi in agriculture. By elucidating optimal growth conditions and interactions between EMF strains, this study lays the groundwork for further research aimed at harnessing the potential benefits of EMF in sustainable crop production systems.

Keywords

Mutualistic symbiosis, Ecology, Laboratory, Enhancing uptake of nutrients.

Introduction

The group of ericoid mycorrhizal fungi (EMF) is among the least studied worldwide within the fungi kingdom (Vohník, 2020). Interest in their significance and application in sustainable and regenerative agriculture is increasing, with commercially available strains of EMF already accessible in some countries (Albornoz *et al*., 2021).

EMF forms mutualistic symbiosis associations with the roots of blueberry plants (*Vaccinium corymbosum* L.) (Jacobs *et al*., 1982; Coville, 1910; Retamales and Hancock, 2018; Scagel *et al*., 2005). Members of the Ericaceae family, to which blueberries belong, have limited nitrogen and mineral absorption capabilities due to their growth in acidic soils (Mu *et al*., 2021). Consequently, the absorption of minerals from the soil poses a significant challenge for these plants (Eccher *et al*., 2006; Apse and Karklins, 2014; Apše and Karkliņš, 2012). Hence, blueberries and other Ericaceae family plants heavily rely on EMF to facilitate the uptake of nitrogen, phosphorus, iron, and other essential minerals from the soil, aiding in their resilience to various biotic and abiotic stresses (Read and Stribley, 1973; Shaw *et al*., 1990; Sadowsky *et al*., 2012; Scagel *et al*., 2005).

'Certain general conditions are essential for all fungi development, while some require a special set of conditions and under the changing conditions of nature, the period of time during which a factor is active is of utmost importance' (Greene and Barnett, 1951) (Greene and Barnett, 1951). In Georgia, research on ericoid mycorrhizal fungi (EMF) is absent, with studies primarily focusing on the positive effects of arbuscular mycorrhizal fungi on plant growth (Bitsadze and Shanidze, 2015; მინდიაშვილი, 1979; ნოზაძე, 1979). Consequently, investigating the biological characteristics of EMF emerges as a crucial priority, offering significant interest worldwide (Perotto *et al*., 2002).

The aim of the study was investigation the biological peculiarities of two strains of EMF, *Hyaloscypha hepaticicola* (D.J.Read) and *Oidiodendron maius* (G.L.Barron). It evaluated optimal growth media type, acidity of media (pH), temperature, and humidity, to understand their impact on growth and development of strains. Additionally, experiments were conducted to determine inhibition rates through the cocultivation of two different fungal species (Scheer *et al*., 2001; Ezz-Eldin and Sharabash, 1959).

Materials and Methods

Fungal strains: *Hyaloscypha hepaticicola* (UAMH number: 5828) and *Oidiodendron maius* (UAMH number 8507) were used for the experiments obtained from University of TORONTO – Centre for Global Microfungal Biodiversity MycoBank collections.

Influence of different media on fungal growth: Potato Dextrose Agar (PDA), Melin-Norkrans Agar (MNA), and Malt Extract Agar (MEA) were prepared as fungal growing media on Petri dishes for the experiment [21]. Subsequently, Petri dishes were inoculated with fungal strains of both *H. hepaticicola* and *O. maius*. The radial growth rate of the EMF strains was measured on the tenth and twentieth days after inoculation. Four replicates were done per variant.

Influence of different temperatures on fungal growth: For the experiment, Petri dishes with PDA were inoculated with fungal strains of both *H. hepaticicola* and *O. maius*. [20] The Petri dishes were then subjected to different temperature regimes: +4°C, +7°C, +20°C, +23°C, +25°C, and +30°C. Four replicates were done per variant. The growth rate of the EMF strain's mycelium was measured on the twentieth day after culturing (Ali *et al*., 2017).

Influence of different acidity (pH) on fungal growth: To investigate the impact of acidity (pH) on the growth and development of EMF strains, a Potato Dextrose Agar (PDA) broth medium was used (Scheer *et al*., 2001). The acidity of the (PDA) broth was pre-measured, and acidity levels ranging from 3 to 10 were achieved using hydrogen chloride (HCl) and sodium hydroxide (NaOH). Fifty milligrams of the broth medium were dispensed into 100-milligram flasks and sterilized. After media sterilization, each variant was inoculated with fungal strains of both *H. hepaticicola* and *O. maius*. After inoculation, the flasks were incubated in a shaker thermostat at 25 ℃ (degrees Celsius). Four replicates were used per treatment.

On the 25th day after inoculation, the mycelium of the fungi was harvested on predried and weighed filter papers and dried in a drying cabinet at 60 ℃ for one day. The pure weight of the fungus of the mycelium was determined, to identify the optimum acidity (pH) for fungal growth (Sajili *et al*., 2017).

The acidity of the growing media was measured after the experiment to assess any changes in acidity following EMF growth.

Influence of different humidity on fungal growth: An experiment was conducted to determine the optimal humidity for the growth and development of the EMF (Sokolová and Ryparová, 2019; Ezz-Eldin and Sharabash, 1959).

The influence of varying humidity levels on EMF growth and development was investigated using the Ez-Eldin and Sharabash method (Sokolová and Ryparová, 2019; Ezz-Eldin and Sharabash, 1959). Petri dishes with PDA were cultured with 7-day-old EMF - both *H. hepaticicola* and *O. maius* strains and sterilized salt solutions of different concentrations were dispensed in specific amounts into smaller parts of the empty Petri dishes. Two small parts of the Petri dishes were combined together (plate with liquid and plate with fungi cultured on PDA) and parafilm was wrapped around each Petri dish to ensure airtightness. The different concentrations of salt solutions were prepared with varying air humidity levels: 80%, 85%, 90%, 95%, and 100%. Daily observations were made to monitor the growth and development of the EMF. The diameter of the mycelium was periodically measured to determine the humidity level at which the fungus exhibited maximum growth. Tree replicates were used per concentration and conducted in a thermostat at an optimal temperature of 23˚C.

Co-cultivation of two strains: Fungal strains of the *H. hepaticicola* and *O. maius* on Petri dishes with PDA. The petri dish was incubated in a darkness at a temperature of 23˚C.

On the 25th day after the transfer of strains, the inhibition rate of EMF strains was measured using the formula developed by

Kowalik and Krechnik: I=(C-T)/C x 100. where I - represents the inhibition rate, C - is the diameter of the colony on the control plate, and T - is the diameter of the colony on the plate with a preparation (Ali *et al.*, 2017).

Statistics: Data from the experiment was analyzed and ANOVA and Tukey's Honestly Significant Difference has been calculated. Graph Pad Prism 5 software was used for the data analyses.

Results

Influence of different media on fungal growth: After the experiment it was determined that ten days after inoculation the optimal growth medium for both the *O. maius* and *H. hepaticicola* strains is Potato Dextrose Agar (PDA). However, by the twentieth day of the experiment, it was found that the growth of *H. hepaticicola* was optimal for the development of Malt Extract Agar (MEA), while for *Oidiodendron maius*, the optimal medium remained Potato Dextrose Agar (PDA), as observed in the ten-day result. Statistical analysis revealed no significant difference in the obtained results, ($P>0.05$) (Pic. 1).

Influence of different temperatures on fungal growth: Based on the study of the influence of temperature on the growth and development of EMF strains, it was found that the optimal temperature for the *Oidiodendron maius* strain is 25˚C, while for the *Hyaloscypha hepaticicola* strain, it is 30˚ C. However, it's noteworthy that the latter strain also exhibits good growth at temperatures of +20, +23, and +25 degrees Celsius, as indicated in (Pic 2). Statistical analysis revealed significant differences in the obtained results at +20, +23, +25, and +39 degrees Celsius ($P< 0.05$) (Pic. 2)

Influence of different acidity (pH) on fungal growth: Based on the results obtained after the experiment, the optimal growth acidity for the *Oidiodendron maius* strain was determined to be 9, while for the *Hyaloscypha hepaticicola* strain, it was 7 (Pic 3). It's essential to note that these acidity levels reflect the initial conditions of the experiment, as depicted in the (Pic 3). However, after the experiment, the acidity of the media has been changed, as illustrated in Table 1.

The results indicate that both fungal strains have the ability to alter the acidity level of the media, particularly in instances where the media is alkaline, as it tends to undergo acidification. Additionally, the results show that fungi are showing best growth in a more acidic and more alkaline environment.

Influence of different humidity on fungal growth: Based on the results of the humidity experiment, as depicted in (Pic. 4), it is evident that 100% humidity is optimal for the growth of the *H. hepaticicola* strain, as observed in both the 10-day and 20-day trials. For the *O. maius* strain, the optimal humidity level differs slightly between the 10-day and 20-day results. At 10 days, 95% humidity yields the best growth, whereas at 20 days, 100% humidity is optimal, albeit with a minor variance. Notably, a statistically significant difference is observed only at 85% humidity in the 10-day results, as illustrated in (Pic. 4).

Co-cultivation of two strains: Based on the results of the co-cultivation experiment, the inhibition rate of the *Hyaloscypha hepaticicola* strain is 41, while for the *Oidiodendron maius* strain, it is 18. Consequently, the interaction between the fungi can be classified as antagonistic.

Discussion

Influence of different media on fungal growth: From the research, it is identified that (PDA) and (MEA) as optimal media for EMF growth. This finding aligns with previous research suggesting that nutrient-rich media support better fungal growth (Sajili *et al.*, 2017). However, it is worth noting that different EMF species may exhibit preferences for specific media types, as observed in research.

Influence of different temperatures on fungal growth: The varying optimal temperatures for different EMF strains are consistent with studies indicating that temperature influences fungal growth rates and metabolic activity and the ability of EMF strains to grow across a range of temperatures suggests their potential adaptability to different environmental conditions (Ali *et al.*, 2017).

Influence of different acidity (pH) on fungal growth: The research demonstrated the significant impact of acidity (pH) on EMF growth with optimal acidity levels varying between strains. This is supported by previous research showing that pH affects nutrient availability and microbial community composition in soil and the ability of EMF strains to modulate pH levels in the growth medium highlights their potential role in soil ecosystem processes [1]. It has to be mentioned that *O. maius* showed better development when pH was 6 and 9; *H. hepetiticola* showed better growth when pH was 5 and 7. Better development of the fungi in both acidic and alkaline conditions can be explained by the amphoteric nature of the fungal wall (Sajili *et al.*, 2017; Ali *et al.*, 2017).

Influence of different humidity on fungal growth: The influence of humidity on EMF growth is consistent with previous research indicating that moisture levels affect fungal physiology and metabolism and understanding the humidity requirements of EMF strains is essential for optimizing their growth in both natural and controlled conditions (Greene and Barnett, 1951).

Co-cultivation of two strains: The anta-gonistic interaction observed between EMF strains in research is consistent with findings from other co-cultivation studies, which have documented competitive interactions between fungal species and this highlights the complexity of microbial interactions in soil ecosystems and underscores the importance of considering interspecies dynamics in mycorrhizal inoculation strategies (Reyes *et al.*, 2022).

Conclusion

In conclusion, this study sheds light on the biological peculiarities of selected species of Ericoid Mycorrhizal Fungi (EMF) associated with blueberry (*Vaccinium corymbosum* L.). Through a series of laboratory experiments, we investigated the optimal growth conditions for two EMF strains, *Hyaloscypha hepaticicola* and *Oidiodendron maius*, focusing on the effects of different media, temperatures, pH levels, humidity, and co-cultivation.

Our results revealed the best medium for fungal cultivation on artificial substrate. It was learned that Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) are optimal media for EMF growth. It was observed as well varying temperature preferences for each EMF strain, emphasizing the need for temperature control to optimize growth conditions.

Furthermore, our study demonstrated the significant impact of pH and humidity on EMF growth, with optimal levels differing between strains. These findings underscore the importance of environmental factors in shaping EMF physiology and growth dynamics. Moreover, our co-cultivation experiment revealed an antagonistic interaction between EMF strains, highlighting the complexity of microbial interactions in soil ecosystems.

Overall, our research contributes to the understanding of EMF biology and offers valuable insights for their practical application in the field. By elucidating optimal growth conditions and interactions between EMF strains, our findings pave the way for further research aimed at harnessing the potential benefits of EMF in sustainable crop production systems.

In conclusion, this study provides a foundation for future studies aimed at optimizing EMF inoculation strategies and enhancing their role in promoting plant growth and resilience in diverse agricultural settings.

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References

Vohník, M. (2020). Ericoid mycorrhizal symbiosis: Theoretical background and methods for its comprehensive investi-gation. *Mycorrhiza, 30*(6), 671–695. https://doi.org/10.1007/s00572-020-00989-1

Albornoz, F. E., Dixon, K. W., & Lambers, H. (2021). Revisiting mycorrhizal dogmas: Are mycorrhizas really functioning as they are widely believed to do? *Soil Ecology Letters, 3*(1), 73–82. https://doi.org/10.1007/s42832-020-0070-2

Jacobs, L. A., Davies, F. S., & Kimbrough, J. M. (1982). Mycorrhizal distribution in Florida rabbiteye blueberries. *HortScience, 17*(6), 951–953. https://doi.org/10.21273/hortsci.17.6.951

Coville, F. V. (1910). Experiments in blueberry culture. *U.S. Department of Agriculture, Bureau of Plant Industry Bulletin, N*, 2588–2593.

Retamales, J. B., & Hancock, J. F. (2018). *Blueberries*.

Scagel, C. F., Wagner, A., & Winiarski, P. (2005). Frequency and intensity of root colonization by ericoid mycorrhizal fungi in nursery production of blueberry plants. *Small Fruits Review, 4*(4), 95–112. https://doi.org/10.1300/J301v04n04_10

Mu, D., Du, N., & Zwiazek, J. J. (2021). Inoculation with ericoid mycorrhizal associations alleviates drought stress in lowland and upland velvetleaf blueberry (Vaccinium myrtilloides) seedlings. *Plants, 10*(12). https://doi.org/10.3390/plants10122-786

Eccher, T., Noé, N., & Bacchetta, D. M. (2006). The influence of ericoid endomycorrhizae and mineral nutrition on the growth of micropropagated plants of *Vaccinium corymbosum* L. *Acta Horticulturae, 715*.

Apse, J., & Karklins, A. (2014). Modification of soil properties for blueberry (*Vaccinium corymbosum* L.) cultivation. *Proceedings of the Latvian University of Agriculture, 30*(1), 1–10. https://doi.org/10.2478/plua-20130009

Apše, J., & Karkliņš, A. (2012). Influence of soil modification on change in its properties and mineral nutrition of highbush blueberries. *Research for Rural Development, 1*, 61–66.

Read, D. J., & Stribley, D. P. (1973). Effect of mycorrhizal infection on nitrogen and phosphorus nutrition of ericaceous plants. *Nature, 241*, 20.

Shaw, G., Leake, J. R., Baker, A. J. M., & Read, D. J. (1990). The biology of mycorrhiza in the Ericaceae XVII. The role of mycorrhizal infection in the regulation of iron uptake by ericaceous plants.

Sadowsky, J. J., Hanson, E. J., & Schilder, A. M. C. (2012). Root colonization by ericoid mycorrhizae and dark septate endophytes in organic and conventional blueberry fields in Michigan. *International Journal of Fruit Science, 12*(1–3), 169–187. https://doi.org-/10.1080/15538362.2011.619346

Scagel, C. F., Wagner, A., & Winiarski, P. (2005). Inoculation with ericoid mycorrhizal fungi alters root colonization and growth in nursery production of blueberry plants from tissue culture and cuttings. *Small Fruits Review, 4*(4), 113–135. https://doi.org/10.-1300/J301v04n04_11

Greene, V. L., & Barnett, H. L. (1951). *Physiology of the fungi*.

Bitsadze, N., & Shanidze, S. (2015). Effect of arbuscular-mycorrhizal fungi (AMF) on growth and development of different Georgian wheat genotypes. *Conference Papers, July*.

მინდიაშვილი ქ. (1979). კარტოფილის მიკოტროფულობა. *მეცნიერება.*

ნოზაძე ლ. (1979). რთულყვავილოვანთა მიკოტროფულობა. *ბოტანიკა.*

Perotto, S., Girlanda, M., & Martino, E. (2002). Ericoid mycorrhizal fungi: Some new perspectives on old acquaintances.

Scheer, A., Kruppke, H., & Heib, R. (2001). *Root methods: A handbook*. https://doi.-org/10.1007/978-3-642-56680-6

Sajili, M. H., Wahida, W. N., Badaluddin, A., Khandaker, M., Mohammed, S., & Kadir, J.

(2017). Influence of culture media, temperature and pH on *Colletotrichum gloeosporioides*, isolated from *Carica papaya* in Besut, Terengganu, Malaysia. *Journal of Agrobiotechnology, 8*(2), 49–55.

Ali, S. R. M., Fradi, A. J., & Al-Aaraji, A. M. (2017). Effect of some physical factors on growth of five fungal species. *European Academic Research, 5*(2), 1069–1078.

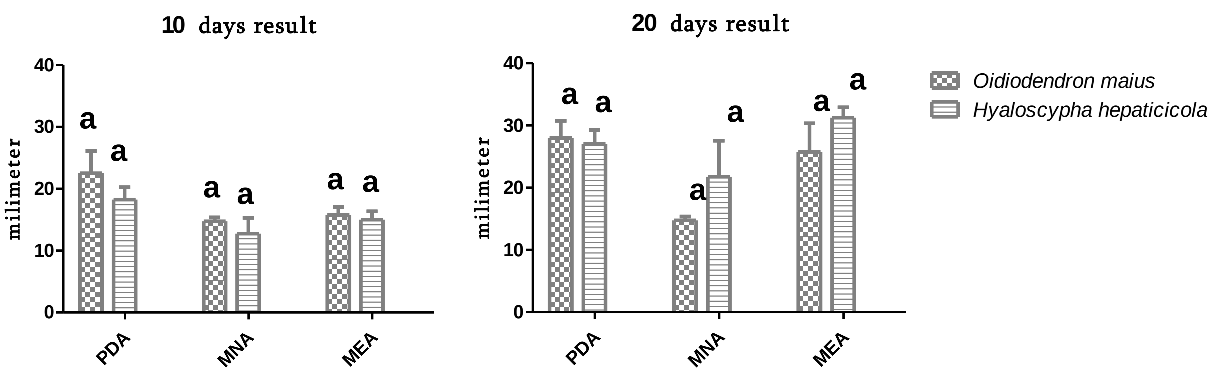
Sokolová, M., & Ryparová, P. (2019). Biological susceptibility of earth plasters: The influence of relative humidity on fungal growth. *International Review of Applied Sciences and Engineering, 10*(1), 9–14. https://doi.org/10.1556/1848.2019.0002

Ezz-Eldin, M. T., & Sharabash, M. M. (1959). A simple method for studying the effect of relative humidity on fungal growth. *Egyptian Journal of Botany*.

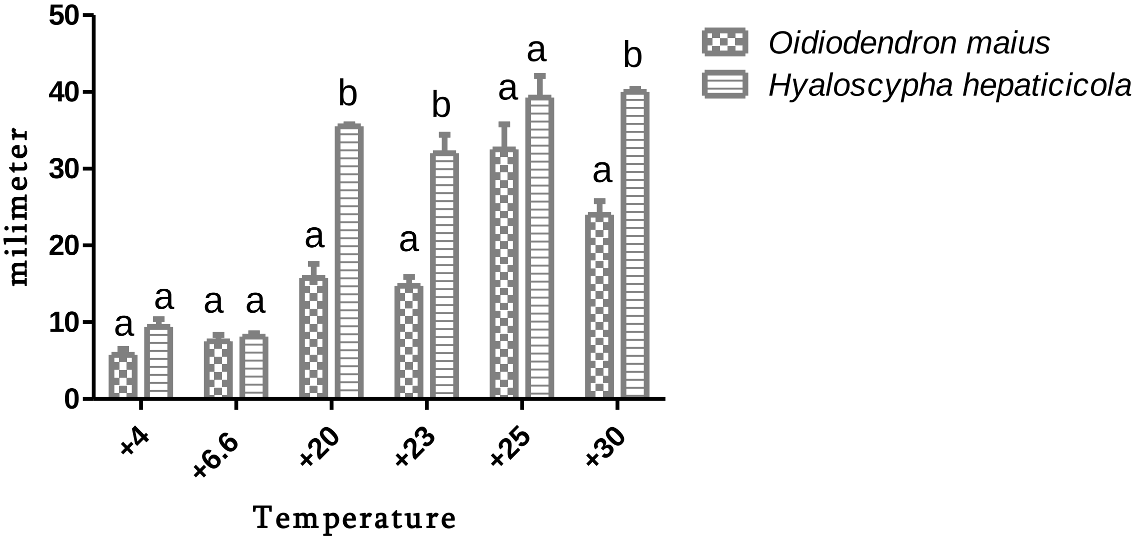
Reyes, C., Sajo, Z., Susanna, M. K., Sinha, A., Schwarze, W. M. R. F., Ribera, J., & Nystrom, G. (2022). Cocultivation of white-rot fungi and microalgae in the presence of nanocellulose. *Microbiology Spectrum, 10*(5), 1–12. https://doi.org/10.1128/spe-ctrum.03041-22.

Table 1. Influence of different acidity (pH) on fungal growth.

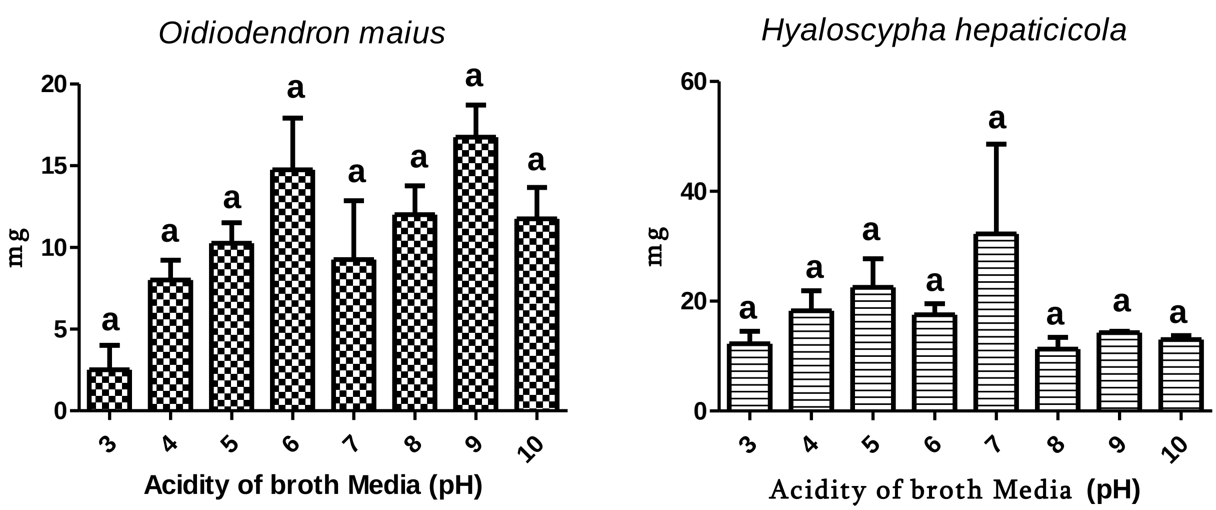
Initial Acidity of the liquid media (pH) / The acidity of the liquid media after the experiment(pH)	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
The acidity of broth media (pH) after the growth of EMF strain (*Oidiodendron maius*)	3	4	5	5	6	6	6	7
The acidity of broth media (pH) after the growth of EMF strain (*Hyaloscypha hepaticicola*)	3	4	5	5	5	6	6	6



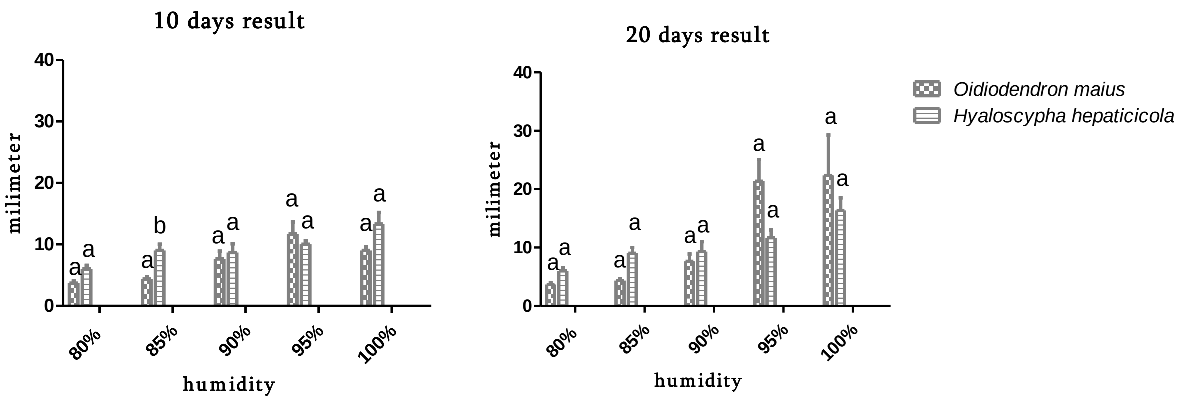
Pic. 1. Influence of different media on fungal growth.



Pic. 2 . Influence of different temperatures on fungal growth.



Pic 3. Influence of different acidity (pH) on fungal growth.



Pic 4 . Influence of different humidity on fungal growth.