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Results of myxomycete experiments could be affected by temperature in "standard" laboratory conditions

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Abstract

Moist chamber cultures are valuable for myxomycete research and can be used for the analysis of ecological patterns with implications in ecosystem conservation programs. However, to make comparisons between datasets valid, the method should be redesigned considering potential biases affecting the generation of results. In the present study, both the effect of the general climate of the laboratory and two microclimatic variables within the moist chamber were studied in relation with the obtained data. Of all the recorded variables, temperature was observed to affect the results, both at the level of the general laboratory climate and in relation with the microclimate of the moist chamber. Increments in laboratory and moist chamber temperature increased the probability, three or four-fold, associated with a higher number of records or species within a group of equivalent samples. Such probabilistic differences are significant enough to suggest that using the moist chamber technique in "standard" laboratory conditions is not enough for cross-dataset comparisons that increase the potential of myxomycete data for applications outside of the biological sciences.

Introduction

The moist chamber technique, since its formal introduction almost 90 years ago (Gilbert & Martin 1933), has been widely used to obtain data on myxomycete biodiversity and ecology (Wrigley de Basanta & Estrada-Torres 2022). This approach has had a very positive impact on the documentation of myxomycetes worldwide (e.g., Härkönen 1977), mainly because it represents a robust secondary method of recording such organisms, after field collecting. Over time, however, researchers have documented some limitations of the method (Alexopoulos 1969, Schnittler et al. 2002), and recently, based on Neotropical research (Rojas et al. 2021a, c) few experimental shortcomings have been addressed as well. One of the main issues is that the technique calls for its application in "standard" laboratory conditions which are never controlled in real life situations and are most likely different every time the method is used, even in the same laboratory.

There is no doubt that the moist chamber technique is a valuable method to characterize myxomycete assemblages associated with different substrates or ecological conditions (Stephenson & Stempen 1994). A clear understanding of its limitations, though, is necessary to understand its potential for scientific applications. For instance, ecological research on myxomycetes has been carried out in the past 50 years or so (e.g., Keller et al. 2017) with few experimental considerations on the biases imposed by the moist chamber technique. As such, most studies are fundamentally incomparable with other similar research in true empirical terms, and at most, can only be used for simple, yet incomplete, evaluations of species-habitat associations. This is a very important issue to discuss because the development of applications based on myxomycete data relies on the true comparability among datasets.

Modern techniques of biological detection such as environmental DNA seem to lead the direction of biodiversity-based assessments (Shchepin et al. 2022), but moist chamber-based surveys, albeit taxonomically biased and incomplete for biodiversity purposes, are cheap and easy to implement. In this manner, the technique is a constraint for the detection of biodiversity units, but certainly represents an asset for bioliteracy and biomonitoring initiatives worldwide (McDonald 2022), with direct documented consequences on human wellbeing (Randers et al. 2019). In other words, myxomycete data based on the moist-chamber method is valuable for pattern assessment as long as the technique is more standardized than it is now.

For those reasons, the present study aimed at testing both the effect of the climate (temperature, atmospheric pressure, humidity, and luminosity) in the laboratory and the microclimate (temperature, substrate humidity) within the moist chamber culture on the results obtained with equivalent substrates collected in standard locations, at the same time and with the same techniques. The idea of this investigation is to support initiatives of standardization of the moist chamber technique, as applied in myxomycete research, for the development of a more controlled method that can increase the scientific validity of the data for diverse applications.

Materials and methods

A series of 720 moist chambers were studied. From those, 360 cultures were set up with samples of bark and twigs collected in 2015 and the other 360 were set up with samples of the same materials collected in 2016. In both cases, the same collecting localities were visited, the same collecting and transportation protocol was applied, the same laboratory supplies were used, and the same space in the same laboratory was selected to run the cultures. Also, for field collecting in two discrete years, the same time of the year was selected for the surveys.

In this manner, during July of 2015 and 2016, three localities in Northwestern Costa Rica were visited. These localities corresponded to the Tenorio, the Miravalles and the Palo Verde National Parks. The first location aims the conservation of tropical wet forest, the second location contains seasonal tropical moist forests and the last one encompasses a large section of seasonal tropical dry forest. As such, there is a decreasing moisture gradient in the Tenorio-Miravalles-Palo Verde direction, with clearly associated differences in the myxomycete assemblages among all three of them (Arenas-Taborda et

al. 2021). These three different locations were selected simply to increase the probability of recording a broader group of species.

All substrate samples were collected, placed in paper envelopes, and transported by land to the forest research laboratory of the Engineering Research Institute at the University of Costa Rica in San Pedro de Montes de Oca, central Costa Rica. This laboratory was, at the time of the study, located in the main campus of the university, in a heavily urbanized area, at approximately 1200 m of elevation, in a weakly seasonal tropical moist forest area, with a dry season in winter months between December and March and a wet season between April and November.

Upon entry in the laboratory, all samples were placed in a pre-experimental room with diffused light and controlled environmental conditions at 21°C and about 50% humidity. In both years, samples were used to set up the moist chamber cultures in the manner described by Gilbert & Martin (1933), Martin & Alexopoulos (1969) and Stephenson & Stempen (1994), all of which essentially describe the same general protocol. With this method, the material for examination was placed on top of filter paper within standard-sized 90 mm Petri dishes. Distilled water was then added to the cultures, that were left for 24 hours on top of a laboratory table, upon which, the pH of the cultures was measured, and the excess water was discarded.

During each year, each set of 360 moist chambers was arranged in 36 stacks of 10 moist chambers each, and the complete group of cultures was placed on a table located in the inner part of the laboratory away from direct light but subjected to the indirect light coming from windows without curtains. Each culture was examined for the presence of myxomycetes for eight weeks during the months of October and November and extra water was added when necessary to maintain a moist environment within the culture. During this process, cultures were rotated to minimize effects due to their position within the stacks and relative to the entire batch. When fruiting bodies were found, they were extracted with forceps and glued to matchboxes for storage in the myxogastrid repository of the Engineering Research Institute (INII) at the University of Costa Rica. All names used in the identification process followed Lado (2005-2022).

During each year, an Onset HOBO U12-012 datalogger was placed on one side of the culture stacks to measure the temperature, humidity, and luminosity at the exact location where the experimentation with the moist chambers was taking place. All these data were obtained at one-hour intervals for the entire period of culture examination. An Ambient Weather WS1173A microstation was used to cross-check the latter and to obtain barometric information of the laboratory. In this manner, the obtained data for all four variables was used to determine the general climate of the laboratory and was associated with all cultures examined.

Also, each year, for the examination of microclimates within the moist chambers, a selection of 90 cultures (25% sample of the 360 cultures per year) was carried out using a random-number generator. These cultures, with equal representation from all three collecting locations, were placed in separate stacks, labeled 1 through 9, and kept together with the rest of the stacks during examination. With these 90 moist chambers, the internal temperature was determined using a Etekcity Lasergrip 1080 Infrared Thermometer, and the moisture of the substrates was measured with a General Tools MMD4E Digital Moisture Meter. With the last one, the scale of determination ranges from 5-50% for woody substrates, and the highest value is reached in full water saturation, when half of the woody material is considered to be composed of water and the other half would be the lignocellulose or wood. In both cases, these variables were determined right before the examination of the moist chamber, during each one of the eight weeks of study.

After the experimentation, all the information was gathered and used for analysis. In this manner, the two experimental responses studied herein were the number of records and the number of species observed. These values were associated, during each year, with all four variables used to characterize the general climate of the laboratory and with the two microclimate variables for the 90-moist chamber subsets. For this step, the Pearson Coefficient of Determination (r^2), which establishes the degree of the response explained by the independent variable, was used. Similarly, potential climatic differences between years were examined using a Student´s t-test, with an alpha value of 0.05 for the rejection

Table 1. Abundance of records by species and year associated with the 720-moist chambers studied in the present study. The numbers in parentheses denote the records and species present in the random subset of 90-moist chambers per year for which the internal temperature of the culture and the substrate moisture were recorded during eight weeks.

Species	**Year**		
	2015	**2016**	**Total**
Arcyria cinerea (Bull.) Pers.	90 (14)	48 (13)	138
Arcyria denudata (L.) Wettst.	7	3	10
Clastoderma debaryanum A. Blytt	2		2
Comatricha laxa Rostaf.	1 (1)		1
Comatricha nigra (Pers. ex J.F. Gmel.) J. Schröt.	7	1	8
Comatricha pulchella (C. Bab.) Rostaf.		4	4
Comatricha tenerrima (M.A. Curtis) G. Lister	11 (2)	15 (2)	26
Cribraria microcarpa (Schrad.) Pers.	4		4
Cribraria tenella Schrad.	14 (1)		14
Cribraria violacea Rex	24 (5)	15 (2)	39
Diachea leucopodia (Bull.) Rostaf.	1		1
Diderma hemisphaericum (Bull.) Hornem.	3 (1)		3
Diderma rugosum (Rex) T. Macbr.	2		2
Didymium anellus Morgan	3	1	4
Didymium bahiense Gottsb.	4 (1)	9 (2)	13
Didymium clavus (Alb. & Schwein.) Rabenh.		5 (1)	5
Didymium difforme (Pers.) Gray	3 (1)	3	6
Didymium dubium Rostaf.	1		1
Didymium minus (Lister) Morgan	1	12	13
Didymium squamulosum (Alb. & Schwein.) Fr. & Palmquist	1	15 (2)	16
Hemitrichia calyculata (Speg.) M.L. Farr	1 (1)		1
Hemitrichia minor G. Lister		2	2
Hemitrichia serpula Fr.		1	1
Lamproderma scintillans (Berk. & Broome) Morgan	4 (1)	15 (2)	19
Licea pusilla Schrad.	1		1
Lycogala conicum Pers.		1	1
Perichaena chrysosperma (Curr.) Lister	19 (3)	9 (1)	28
Perichaena depressa Lib.	14 (3)	9 (2)	23
Perichaena pedata (Lister & G. Lister) G. Lister ex E. Jahn	3		3
Perichaena vermicularis (Schwein.) Rostaf.	1		1
Physarum album (Bull.) Chevall.	6 (2)	7	13
Physarum bivalve Pers.	16 (1)	1	17

Species	Year		
	2015	2016	Total
Physarum cinereum (Batsch) Pers.		3	3
Physarum citrinum Schumach.		1	1
Physarum compressum Alb. & Schwein.	10 (3)	12 (4)	22
Physarum crateriforme Petch		2	2
Physarum decipiens M.A. Curtis	18 (3)	6 (2)	24
Physarum didermoides (Pers.) Rostaf.	1 (1)		1
Physarum javanicum Racib.	2		2
Physarum leucopus Link	1		1
Physarum pusillum (Berk. & M.A. Curtis) G. Lister	14 (1)	1	15
Physarum sp.	7 (2)		7
Physarum viride (Bull.) Pers.	1		1
Stemonitis fusca Roth	10 (3)	6	16
Stemonitopsis aequalis (Peck) Y. Yamam.	7 (2)		7
Trichia decipiens (Pers.) T. Macbr.	1		1
Total	**316**	**210**	**526**

of the null hypothesis and differences in diversity between years were calculated using the same test on the Shannon´s Diversity Index. The overlap of species assemblages between years was calculated using the Bray-Curtis Index. All these calculations were performed in JMP, v 10.0.

Results

A total of 526 myxomycetes arranged in 47 different morphospecies were recorded in the 720 studied moist chambers. From those, 316 records in 38 species were observed in 2015, whereas 210 observations in 28 species were recorded in 2016. These results represented a decrease of 33.5% (106) records and 26.5% (10) species from 2015 to 2016. No differences in species diversity were observed (t=0.4, df=510, p=0.6) and the Bray-Curtis Index showed an intermediate overlap value of 0.53 between yearly assemblages.

From all samples, only 265 (36.8%) yielded any myxomycetes. Results were not different between years with 135 positive samples (37%) in 2015 and 130 (36%) in 2016. The most recorded species in 2015 were *Arcyria cinerea* (Bull.) Pers., *Cribraria violacea* Rex and *Perichaena chrysosperma* (Curr.) Lister, whereas for 2016 these species were *A. cinerea*, *C. violacea* and *Comatricha tenerrima* (M.A. Curtis) G. Lister (**Table 1**). All these species were present in the subset of moist chambers (1/4 of the total effort) that corresponded to the cultures for which the internal temperature and substrate moisture were recorded.

The laboratory environment

The average atmospheric pressure of the laboratory was 88.01±0.12 kPa. No differences were observed between years with an average of 87.98±0.13 kPa for 2015 and 88.04±0.11 kPa for 2016. These values corresponded with the elevation (~1180 m) of the

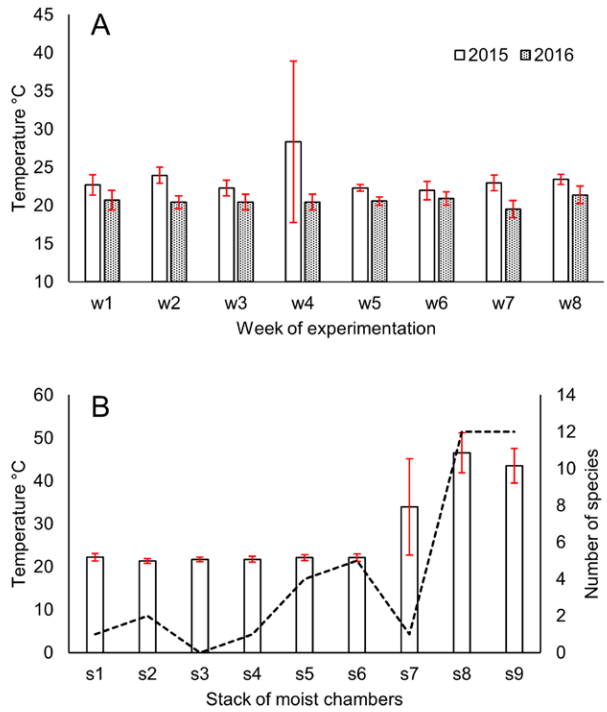

Figure 1. Average temperatures (and standard deviations as red lines) of the 90-moist chamber subsets studied in the present investigation. **A.** Data arranged by week (w1–w8) showing the anomaly on week 4 of 2015. **B.** Data arranged by stacks (s1–s9) showing the effect of such anomaly only on stacks 7–9 and the effect on the recorded number of species in those stacks as well (dotted black line).

laboratory. The difference between the lowest and the highest recorded pressures was only 1.28 kPa, equivalent to about 150 m of elevation, during the entire experiment. The highest values were recorded early in the morning, around the 7:00 hours and the lowest around the 17:00 hours.

The temperature of the laboratory had an average of 23.1±1.9°C and a range between 18.4–30.5°C. Even though the dissimilarity in averages between years was only 0.6°C (23.6±2.1°C for 2015 and 23.0±1.9°C for 2016), the comparison was significantly different (t=-11.2, df=2383, p=0.0001) showing that temperatures in 2015 were higher than in 2016. The range of temperatures for each of the evaluated years was equivalent (always within 18.3–30.5°C). The highest values were recorded in the three-hour period between 10:00–13:00 hours and the night period was associated with the lowest values.

The average humidity of the laboratory was 73.7% and a range between 53.0–84.3%. No differences were observed between years with an average of 73.5%. The highest values were observed overnight, normally between the 19:00–5:00 hours and the lowest values were recorded during the period between 9:00–15:00 hours.

The average illuminance of the laboratory was 179.4±249.6 lux with an average of 161.2±231.3 lux for 2015 and 182.6±252.6 for 2016. The overall range oscillated between 11.8–1375 lux with significant differences between years (t=3.6, df=2637, p=0.0003)

due to the maximum levels in 2016 (1375 lux) that were higher than in 2015 (1273 lux). The highest values were recorded in the six-hour period between 10:00–15:00 hours and the lowest were associated with the night period.

No strong direct correlation was found between the number of records, or the number of species observed, overall and by year, and any of the four climatic variables recorded in the laboratory. However, for the 2015 set, weak but significant associations were observed between the number of species (r^2=0.21, p=0.0001) or the number of records (r^2=0.26, p=0.0001) and the temperature in the laboratory. The strength of these correlations was different to the non-significant, non-correlated, relationship between randomized datasets of abundance/species richness and temperature in 2015 (r^2=0.00, p=0.98).

The moist chamber microenvironment

The average internal temperature of the moist chambers in the studied subset was 22.2±2.0°C. The average was significantly higher in 2015 (23.7±1.7°C, t=16.07, df=89, p<0.0001) than in 2016 (20.5±0.5°C) and the temperature range was broader in 2015 (13–50°C) than in 2016 (16.6–28.8°C). This was due to an anomaly detected during week 4 that affected only specific groups of Petri dishes, associated with stacks 7 to 10 in 2015 (**Fig. 1**). That year, both the number of records (r^2=0.68, p=0.005) and the number of species (r^2=0.70, p=0.004) observed in those stacks showed non-random strong mathematical relationships with the mean maximum temperature recorded in them.

In the studied subsets, a total of 85 records were obtained, from which only 33 (39%) were recorded in 2016 and 52 (61%) in 2015. From the latter, 36 records (42%) were associated with the moist chambers subjected to the anomaly in temperatures, showing that the probability of obtaining records of myxomycetes in those cultures increased fourfold, to 1.2, from the average of 0.32 observed in the rest of the stacks for both years. Similarly, the probability of recording species increased threefold, from 0.19 for any other group of 30 moist chambers to 0.56 for the 30 moist chambers subjected to the temperature anomaly.

Figure 2 shows the temperature range associated with the records of the 12 most common species in the subdataset. For the year 2015, *Lamproderma scintillans* and *P. chrysosperma* were associated with a lower temperature range than *C. tenerrima*, *Physarum decipiens* and *Ph. compressum*. Such difference in temperature ranges did not take place for most other species recorded in 2015 and for the complete 2016 assemblage.

The average humidity of substrates inside the moist chambers was 36.3±8.3% (about 73% on a 100% scale). The average was significantly higher in 2015 (41.3±8.4%, t=10.3, df=89, p<0.0001) than in 2016 (31.2±8.3%) and the range was also broader in 2015 (5–60%) than in 2016 (8–50%). No correlations were observed between substrate humidity and either the number of records or the species richness. In a similar manner to the temperature case, the substrate humidity of the stacks 7–9 during 2015 was different to any other recorded value, with a decrease of minimum humidity of 35% (equivalent to 24% on a 100% scale) between the average of any stack (34.6±1.1%) and the average of the stacks involved in the temperature anomaly (22.3±0.4%).

Discussion

The results presented herein show that climatic variables perform ecological pressures on myxomycetes developing fruiting bodies in moist chamber cultures. Researchers have already known for quite some time that the moist chamber technique affects myxomycete results (Schnittler & Mitchell 2000, Rollins & Stephenson 2016). However, it is still uncertain to what degree important climatic variables, such as temperature, impose an effect on biological processes taking place within the moist chamber culture.

Historical research shows that even before the publication of the moist chamber method in 1933, there were accounts of the effect of temperature

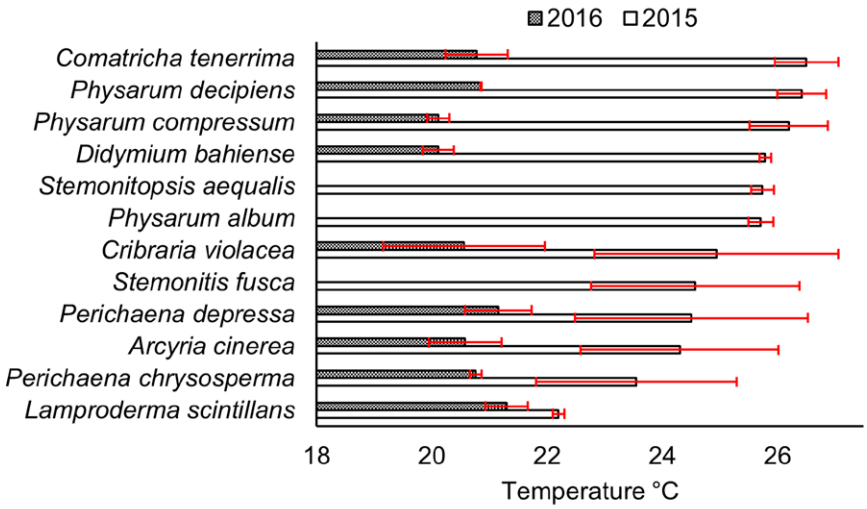

Figure 2. Average internal temperatures (and standard deviations as red lines) of the moist chambers associated with the 12 most common species of myxomycetes recorded during both years of study in the subdatasets studied herein.

on the germination of myxomycete spores (Smith 1927), suggesting a first level of impact that was also later documented by Blackwell & Gilbertson (1984). It has been proposed, though, that most myxomycete propagules on substrates in moist chambers are primarily sclerotia (Alexopoulos 1964) and not spores. However, sclerotia have also been known to form plasmodia and fruiting bodies in response to conditions that are external to the culturing environments (Seifriz & Russell 1936).

In the present study, higher laboratory temperatures in 2015 and an increase in the number of records and species during that year relative to the overall results, could suggest a first level of effect as well. Even though direct links between independent variables and temperature were weak (0.21 and 0.26), there were, first, significantly different than a random association of abundance/species richness and temperature in 2015; and second, significantly different than mathematical associations without contextual meaning (as observed in the probabilities under 0.0001). For climate change research on myxomycetes, these results could delineate potential future investigations (see Kryvomaz et al. 2022). After all, results showed that an increase of 0.6°C in air temperature in laboratory conditions was associated, although not causally, with an increment in the number of myxomycete records and species observed in moist chamber cultures.

Even though most climatic variables were not observed to be associated with the results, the moist chamber technique as generally applied in modern research is an uncontrolled microcosm unable to provide comparable data for empirical ecological or evolutionary analysis. The differences in illuminance between years or the differences in substrate moisture in some stacks, not significantly linked with the results, also demonstrate that the external pressures are constantly changing and that several sets of moist chambers in the same or in different laboratories are likely subjected to different conditions. For biodiversity purposes, the main reason of the utilization of the moist chamber technique (Gilbert & Martin 1933), those variations seem to favor the development of different species, as expected by the ecological theory of niche partitioning.

In this manner, the results from the 90-moist chamber subset showed a more direct association between temperature and either the number of records or the number of species, even though

the anomaly in temperatures during week 4 of the 2015 evaluation was never identified (very likely the moist chambers were moved to a direct light situation). Whatever the source of heat was, it produced a sharp but quick increment in temperature that impacted the moist chambers and was documented at multiple levels (e.g., even the broader range of temperatures during 2015 in comparison with that of 2016). Such quick external stimulus impacted the microcosm and resulted in the generation of 42% of all records (for both years) in only 30 cultures. This means that 4% of the moist chambers yielded a large percentage of the results, also indicating that there could be alternative protocols to induce the generation of myxomycete data in particular experimental conditions. This approach could be similar to the use of "cold moist chambers" as explained by Wrigley de Basanta & Estrada-Torres (2022) for snowbank myxomycetes.

Field research on myxomycetes has shown that quick climatic changes, such as rapid freezing (Schnittler et al. 2015) or sudden precipitation (Rojas et al. 2021b) negatively affect the production of fruiting bodies in field conditions. In a similar manner, the results presented herein suggest that a rapid increment in temperature, albeit for a short period of time, might have the opposite effect but in moist chamber situations. However, the implication of these observations is that for meaningful comparisons of non-biodiversity related data, more control of the external climatic variables should be included in the general method of moist chamber culturing. Such controlled approaches are quite common in general microbiological work, and the historical link of myxomycete research activity with mycological rather than microbiological research, could be also related with less strict guidelines in the protocol.

There is a technical definition of "room temperature" for scientific practice (McNaught & Wilkinson 1997), which in myxomycete research is practically never addressed. Since most references to the use of the moist chamber (e.g., Stephenson & Stempen 1994) simply call for "incubating the cultures at room temperatures", it is very likely that a vast number of investigations have generated data under conditions beyond the accepted limits. In fact, the study presented herein is an example of the latter since the accepted temperature range for "room temperature" conditions is between 20-25°C and the temperature ranges observed herein extended beyond those values in both directions. For myxomycete research, a redefinition of the moist chamber method for different applications is necessary and as observed in the present study, could be beneficial for meaningful comparisons and for increasing the probability of obtaining data in different ecological situations.

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