# Variability in growth and reproduction of the marine fungus, *Lulworthia floridana*

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KURZFASSUNG: **Variabilität von Wachstum und Reproduktion bei dem marinen Pilz *Lulworthia floridana*.** Nahrungsphysiologische Untersuchungen an 10 Lulworthien der mittleren Sporenbereichgruppe, welche durch *Lulworthia floridana* MEYERS charakterisiert ist, demonstrieren die Variabilität der Isolate hinsichtlich ihres Wachstums und ihrer Reproduktion (Perithecien-Entwicklung) bei Verabreichung verschiedener Kohlenhydratverbindungen. Eintritt und Intensität der Perithecien-Entwicklung werden durch Inkubationstemperaturen von 25° bis 30° C begünstigt. Unterschiedliche Reaktionen der Isolate bezüglich ihrer vegetativen und reproduktiven Entwicklung illustrieren die Heterogenität der Population. Die Untersuchungsergebnisse zeigen, daß zukünftige taxonomische Bearbeitungen der Gattung sowohl morphologische als auch physiologische Eigenarten berücksichtigen müssen. Gegenwärtig ist *Lulworthia* reduziert zu einer kompositären Art – *L. medusa* (ELL. & EV.) CRIBB & CRIBB; in der Zukunft erscheint jedoch eine Trennung von Isolaten dieser heterogenen Gruppe auf Grund physiologischer Kriterien möglich.

## INTRODUCTION

The widespread occurrence of representatives of the marine fungus *Lulworthia* is well documented in marine mycological literature (JOHNSON & SPARROW 1961). Species of this scolecosporous genus comprise a significant part of the mycota active in early infestation of submerged wood and other cellulosic substrates. Until recently, the genus, with a total of 13 species and two varieties, constituted the largest taxon of marine Ascomycetes. However, in 1966, CAVALIERE & JOHNSON reduced the genus to a single composite species *L. medusa* (ELL. & EV.) CRIBB & CRIBB. Nevertheless, these workers conjectured possible existence of physiological differences between two, or perhaps three, spore groups. The need for further work to determine whether major differences in spore size could be used to separate *Lulworthia* into physiological strains was suggested.

Morphological variability of *Lulworthia*, including spore and perithecial characters, i. e., spore and appendage length, perithecial size and gross features, and venter wall structure, has been noted repeatedly. In contrast, little attention has been given to the comparative physiological characters of the taxa of this genus.

In recent unpublished studies of selected isolates within the composite medial spore range group ($> 100$ to $< 500$) of *Lulworthia*, a striking spectrum of physio-

logical dissimilarities has been noted among apparently morphologically similar organisms. This work supported earlier observations based on statistical analysis (MEYERS et al. 1964) wherein physiological tests were proposed to discern significant characters for use in taxonomic separation. In other investigations from this laboratory of two, morphologically similar, isolates of the marine ascomycete, *Corollospora maritima* WERDERM, differences in vegetative growth, utilization of carbohydrates, and aspects of reproduction have been noted. Based on these and other observations, there appears to be a definite need for proper blending of methodology to discern the significance of morphological and physiological properties in the systematics of the higher marine fungi.

Presented below are results of further examinations of the physiology of reproduction of selected isolates of *Lulworthia*. Attempts are made to assess variability in terms of the salient ecological characteristics of these organisms.

## METHODS

The Lulworthias are designated by their Institute of Marine Science (IMS) accession number and include the following organisms within the *L. floridana* (IMS 190) group: IMS 190, IMS 384, IMS 400, IMS 490, IMS 503, IMS 525, IMS 543, IMS 548, IMS 600 A, and IMS 605. The species *L. floridana*, was isolated from submerged wood in Biscayne Bay, Florida and other areas and has been studied extensively in this laboratory (MEYERS 1957, MEYERS & SIMMS 1965). Isolates IMS 384 through 548 are from manila cordage submerged at the IMS pier from July to November, 1960. IMS 600 A and 605 also were collected in Biscayne Bay, the former from cellulose tape exposed off the IMS pier (April, 1964) and the latter from balsa wood test panels anchored within a turtle grass community (December, 1964). Ascospores of these isolates are all in the size range $> 200$ to $< 450$, a population designated the "*L. floridana* group". Variations in colonial and perithecial characteristics are not uncommon among isolates of *L. floridana*. It is doubtful if these variabilities have taxonomic significance.

Stock cultures are maintained on seawater agar slants of 1 % glucose and 0.25 % Difco yeast extract (GY/SW) in the Microbiology Section, IMS.

For development in broth culture, fungi were grown in triplicate in 125-ml Erlenmeyer flasks with 25 ml of a seawater medium (designated *F-1003*) containing 0.5 % glucose and, 0.1 % yeast extract, 0.24 % $NH_4NO_3$, 0.246 % $MgSO_4$, 7 $H_2O$, and 0.121 % tris. Glucose was delected for the carbohydrate tests discussed subsequently. The pH of the medium was adjusted to 7.5. Incubation was at 25° C on a reciprocating shaker at 55 strokes/min. Preparation of mycelial inocula and other procedural methodology have been described (MEYERS & SIMMS 1965, SGUROS, MEYERS & SIMMS 1962). Final growth was determined gravimetrically, in triplicate, with mycelia filtered through tared glass filter paper (Whatman GF/C) and brought to constant weight in vacuo.

Carbohydrate supplements were filter-sterilized, usually in 25 % concentrations, through 0.45 $\mu$ porosity membranes (Millipore Filter Corp.) and added aseptically at

0.5 % concentration to the broth in flasks or to melted and cooled (45° C) agar immediately prior to pouring into Petri dishes. Cellulose paper discs, 12.5 mm diameter (S & S No. 740-E assay discs), placed on the agar surface, were used as the cellulosic substrate.

The inoculum for agar tests consisted of ascospore suspensions obtained from mature perithecia in stock cultures. Agar tests were done in quadruplet.

Spore concentration and viability were checked within 24 hours of inoculation, following which dishes were sealed with rubber petri dish closures and incubated at appropriate temperatures. The spore harvest from each fungus was examined carefully to ascertain both general concentration and viability for the longevity/viability characteristics of ascospores of the various fungi differ markedly. *Lulworthia* spores usually "deliquesce" within the perithecium within 4 to 6 months, or less, in artificial culture. In contrast, spores of the ascomycete, *Halosphaeria mediosetigera* CRIBB & CRIBB readily germinate when taken from perithecia in cultures over a year old.

Both concentration and maturity of perithecia (i. e., presence of asci and spores, or both, and comparative density of spore production) were recorded at regular intervals throughout the test. The terms "reproduction", and "perithecial development" are used interchangeably.

## EXPERIMENTAL RESULTS

Response of the Lulworthias to selected carbohydrates is noted in Table 1. The figure given is the average milligram weight for the mycelia of the three flasks of each test. Mycelia were harvested at optimal growth of each organism, usually 14 to 18 days, based on previously run growth determinations. No significant differences was noted between individual growth curves of the various isolates.

The figures, or gravimetric measurements, given in Table 1 do not represent absolute values, but rather show, the range of comparative physiological variability. It can be demonstrated that the growth of any particular isolate varies with different experimental conditions or with its physiological stage at the time of inoculation. Nevertheless, in view of the uniform conditions of these tests, the observed response to different carbohydrates does reflect the heterogeneity of this fungal population.

Differences between isolates in utilization of specific carbohydrates are apparent. In general, negligible growth occurs in raffinose and sucrose and poor growth in glucosamine, (except for IMS 400), sorbose (except for IMS 543), and ribose (except for IMS 400). IMS 400 differed from other isolates in its negligible response to galactose and arabinose, whereas the majority of the isolates grew well on these two sugars. There is no direct correlation between maximal utilization of a particular carbohydrate by an organism and its response to other compounds. Cultures IMS 503, 600 A, and 605 all grew well on galactose; IMS 503 did well on maltose and rhamnose while IMS 600 A failed to develop on these two substrates. Converse relationships, i. e., IMS 543 grows on sorbose but not on maltose while IMS 190 utilizes maltose but not sorbose, may be of considerable significance, as has been postulated for strains of actinomyces (D. GOTTLIEB, personal communication). In general, the Lulworthias

Table 1

Growth response of *Lulworthia floridana* isolates to various carbohydrates.
Table body: Mycelial growth in milligrams

| Substrate | Fungus | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | IMS 190 | IMS 384 | IMS 400 | IMS 490 | IMS 503 | IMS 525 | IMS 543 | IMS 600A | IMS 605 |
| Xylose | 118 | 103 | 91 | 73 | 56 | 53 | 139 | 48 | 24 |
| Arabinose | 88 | 95 | 7 | 64 | 50 | 58 | 97 | 50 | 26 |
| Ribose | 0 | 13 | 43 | 1 | 3 | 13 | 7 | 2 | 9 |
| Rhamnose | 0 | 77 | 72 | 34 | 31 | 13 | 88 | 28 | 46 |
| Fructose | 150 | 113 | 73 | 59 | 57 | 51 | 147 | 47 | 34 |
| Sorbose | 0 | 7 | 15 | 0 | – | 7 | 59 | 0 | 5 |
| Mannose | 101 | 107 | 62 | 67 | 57 | 41 | 104 | 53 | 36 |
| Galactose | 62 | 76 | 2 | 76 | 69 | 25 | 116 | 65 | 89 |
| Glucose | 113 | 117 | 105 | 84 | 47 | 70 | 150 | 39 | 27 |
| Glucosamine | 6 | 9 | 60 | 0 | 11 | 3 | 37 | 0 | 2 |
| Mannitol | 0 | 97 | 80 | 91 | 43 | 76 | 158 | 49 | 25 |
| Cellobiose | 138 | 113 | 141 | 99 | 47 | 50 | 143 | 43 | 30 |
| Maltose | 94 | 87 | 100 | 81 | 49 | 0 | 2 | 1 | 64 |
| Trehalose | 107 | 102 | 75 | 60 | 49 | 59 | 85 | 37 | 34 |
| Sucrose | 0 | 0 | 0 | 0 | 0 | 9 | 3 | 0 | 6 |
| Raffinose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Starch | 39 | 92 | 70 | 63 | 43 | 41 | 100 | 50 | 23 |
| *Thalassia* extract | 0 | 10 | 3 | 2 | 7 | 6 | 15 | 4 | 4 |

showed good growth on glucose, cellobiose, mannose, arabinose, xylose and fructose, and with the exception of IMS 190, on mannitol. In other tests discussed subsequently, it is shown that IMS 190 cannot utilize mannitol for reproduction. Moderate vegetative development of this fungus occurs in agar with this carbohydrate, in contrast with its lack of growth in the mannitol liquid medium.

Certain isolates, IMS 190, IMS 384, IMS 548, and IMS 605, were selected for examination of the effect of various carbohydrates on reproduction. Differences were observed earlier (unpublished data) wherein IMS 190 utilized 0.5 and 1.0 % cellobiose and cellulose for reproduction, while mannitol and $\beta$-methyl-*D*-glucoside were ineffective. In contrast, IMS 384 and 490 had excellent reproduction in glucose, xylose, $\beta$-methyl-*D*-glucoside, 0.5 and 1.0 % cellobiose and cellulose.

Reproduction of IMS 190 and three other isolates at 25° C is given in Table 2. The effect of temperatures above 25° C is discussed subsequently. Except for noteworthy reproduction on arabinose at 20 days and on trehalose by 28 days, IMS 190 showed scant to heavy (asci few or immature spores) reproduction on the various carbohydrates, and none at all on fructose and mannitol. This isolate exhibited excellent vegetative growth on fructose (Table 1) in contrast to its failure to reproduce on this sugar. IMS 384 also responds favorably to arabinose and trehalose. Reproduction by IMS 548 is particularly interesting in that the fungus developed heavy crops of mature perithecia uniformly on all of the sugars by 28 days. Culture IMS 548 and the other three isolated developed only immature perithecia, or perithecia with very few spores, on arabinose at room temperature (20° to 22° C) after 20 days in contrast to their excellent reproduction on this sugar at 25° C.

Table 2

Reproduction of *Lulworthia floridana* isolates on various carbohydrates at 25° C. Table body: perithecial development; 0: perithecia absent; 1: perithecia immature, scant to heavy production; 2: perithecia mature, asci few or immature spores, scant to heavy production; 3: perithecia mature, heavy yield of spores, scant to moderate production; 3a: perithecia mature, heavy yield of spores, dense production

| Substrate | Fungus | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IMS 190 | | IMS 384 | | IMS 548 | | IMS 605 | |
| No. days | 20 | 28 | 20 | 28 | 20 | 28 | 20 | 28 |
| Xylose | 0 | 1 | 2 | 2 | 2 | 3a | 1 | 3 |
| Arabinose | 3a | – | 2 | 3a | 2 | 3a | 2 | 3 |
| Fructose | 0 | 0 | 2 | 2 | 2 | 3a | 2 | 3 |
| Mannose | 0 | 2 | 2 | 2 | 2 | 3a | 1 | 2 |
| Galactose | 1 | 1 | 2 | 2 | 2 | 3a | 1 | 2 |
| Glucose | 1 | 2 | 2 | 2 | 2 | 3a | 1 | 3 |
| Mannitol | 0 | 0 | 2 | 2 | 2 | 3a | 2 | 2 |
| Cellobiose | 1 | 2 | 2 | 2 | 1 | 3a | 2 | 3 |
| Trehalose | 1 | 3 | 3a | – | 3a | – | 2 | 3a |
| Starch | 1 | 2 | 2 | 2 | 2 | 3a | 2a | 2a |
| Cellulose | 1 | 2 | 2 | 3 | 2 | 3a | 2 | 3 |
| Control (No added carbohydrate) | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |

Perithecial development of IMS 605 also differs in substrate response and intensity from the other Lulworthias. Apparently, this variability in reproductive response is commonplace within *Lulworthia,* and may be indicative of small, but important, physiological differences among isolates of this genus. In addition to dissimilarities seen in Table 2, characteristics of the perithecia differ on the various media. These differences have been observed by other workers, and include variability in perithecial color, size, mode of production, and especially in number and length of the neck. The

Table 3

Reproduction of IMS 190 on various carbohydrates at 25° and 30° C. Table body: perithecal development (see text to Table 2)

| Substrate | Temperature | | | | | | |
|---|---|---|---|---|---|---|---|
| | 25° C | | | | 30° C | | |
| | Period of Growth (days) | | | | | | |
| | 15 | 19 | 25 | 36 | 15 | 19 | 25 |
| Control (No added carbohydrate) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Xylose | 0 | 0 | 1 | 2 | 1 | 3a | – |
| Arabinose | 1 | 3 | 3a | – | 3a | – | – |
| Fructose | 0 | 0 | 0 | 2 | 1 | 2 | 3a |
| Mannose | 0 | 0 | 2 | 2 | 1 | 2 | 3 |
| Galactose | 1 | 1 | 1 | 2 | 1 | 2 | 2 |
| Glucose | 0 | 1 | 2 | 3 | 1 | 2 | 3 |
| Mannitol | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cellobiose | 0 | 1 | 2 | 3 | 1 | 2 | 3 |
| Trehalose | 0 | 1 | 3 | 3 | 1 | 3a | – |
| Starch | 0 | 1 | 2 | 3 | 3a | – | – |
| Cellulose | 1 | 1 | 2 | 2 | 3a | – | – |

effect of incubation temperature on reproduction in IMS 190, IMS 384 and IMS 490 has been examined. Perithecia were absent or, if present, were immature after 18 days at 15° and 20° C, but were produced in heavy concentrations at 25° and 30° C. Colonies of these three Lulworthias held at 25° C for one week and subsequently transferred to 15° and 20° C failed to sporulate within 38 days. While IMS 384 and 490 produced mature perithecia at 20° C after prolonged incubation, IMS 190 still showed only immature structures at 60 days.

Table 3 shows reproduction of IMS 190 on various sugars at two incubation temperatures. IMS 384 was run for comparative purposes and is discussed below. The effect of incubation at 30° C on IMS 190 is marked in onset of reproduction, especially on cellulose and starch where dense crops of mature perithecia were present by 15 days. Characteristics of reproduction by IMS 190 on fructose, noted in Table 2, apparently are temperature dependent (Table 3). IMS 384 showed vigorous mature reproduction on trehalose, starch and cellulose by 15 days at 30° C. Comparable perithecial development occurred by 20 days on all of the other carbohydrates tested, except galactose, mannitol, and mannose. Only galactose failed to support heavy development of perithecia of IMS 384 even after 25 days at 30° C. At 25° C, IMS 384 showed heavy mature perithecial development on all media by 36 days except on fructose and starch where 42 days were required for mature reproduction.

## DISCUSSION AND CONCLUSION

In this present study of ten Lulworthias of the medial spore range, designated the "*L. floridana* group", it is not possible or feasible to establish arbitrary physiological groups or even to delimit the characteristics of such grouping. This investigator recognizes that slight dissimilarities in ascospore size, color and gross morphology of perithecia, and colonial characteristics are of questionable taxonomic significance. However, comparative studies of reproduction and carbohydrate metabolism are quite useful in discerning the range of physiological variability of this heterogeneous population. Furthermore, information on factors and conditions that directly affect fungal sexual reproduction should facilitate a better understanding of the tremendous variabilities in perithecial development noted among the marine Ascomycetes. A broad spectrum of biological diversity within the genus *Lulworthia* may explain the widespread abundance of this taxon in the sea and its ability to vigorously colonize submerged lignocellulose substrates.

Differences observed in utilization of carbohydrates may be part of the general assimilatory characteristics of this *Lulworthia* group in that some are more efficient assimilators than others. Investigations are planned to examine individual isolates for presence or absence of particular distinguishing enzymes. Dissimilarities in utilization of cellulose and cellulose-degradation products may indicate differences in ability of these fungi to develop and reproduce on submerged cellulosic substrates. Response to various sugars may permit one isolate to establish itself in a specific ecological niche, or reproduce in advance of other associated fungi. Similarly, physiological properties of ascospores may be significant in the intensity and periodicity of colonization by

these organisms, for variation in length, especially velocity of elongation, of the spore germ tube of different isolates are not uncommon.

The temperature study data suggest that the *Lulworthia* isolates from subtropical marine waters in all likelihood are different from those prevalent in northern and arctic marine regions. In earlier investigations of these latter areas (MEYERS & REYNOLDS 1960), wood panels were actively attacked by species of *Lulworthia* during periods of water temperature of 0° to 15° C. Perithecia were present on wood exposed during periods of water temperature of less than 5° C, although in most instances, submergence periods of 100 to as long as 200 days were required for fungal reproduction to appear. In contrast, in Biscayne Bay, Florida, where water temperatures approach 30° C, *Lulworthia* reproduction on test panels may occur within 10 days following exposure. In view of these field observations, the stimulation of reproduction by isolates of *Lulworthia* under cultural conditions at temperatures above 25° C is quite significant. Studies are needed of *Lulworthia* from northern and arctic regions before the properties of this genus can be evaluated thoroughly. Similarly, it is necessary to compare physiological properties of Lulworthias with ascospores $< 100$ to $> 500$ in length with those of the medial spore range group. Are there discrete physiological features, characterized by genetic stability, that can be utilized to differentiate isolates with marked differences in overall ascospore length?

At this time, treatment of the *Lulworthia* complex as a single composite species is impractical. A more feasible approach would be to recognize three broad physiological groups (ascospores $< 100\ \mu$, $> 100$ to $< 500\ \mu$, $> 500\ \mu$) and to assign strain numbers to these *Lulworthia* isolates until a thorough evaluation of the physiological spectrum of the genus is achieved. This current work is further evidence of the interesting biological variability of *Lulworthia* and indicates that final separation of this widespread marine ascomycete genus may indeed be along physiological lines.

## SUMMARY

1. Dissimilar response in both vegetative and reproductive development of separate isolates of the marine ascomycete, *Lulworthia floridana*, demonstrate the heterogeneity of this fungal population. Particular differences are noted in the carbohydrate utilization spectra of the various isolates.
2. Striking stimulation of onset and intensity of perithecial development is seen at incubation temperatures of 25° and 30° C. Extrapolation of these experimental observations to field studies of infestation of lignocellulose substrates by *Lulworthia* suggests that *Lulworthia* isolates from subtropical marine waters are different from those prevalent in northern and arctic marine regions.
3. While the genus *Lulworthia* currently has been reduced to a single composite species, in all likelihood, subsequent breakdown of this heterogeneous group will be along physiological lines.

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