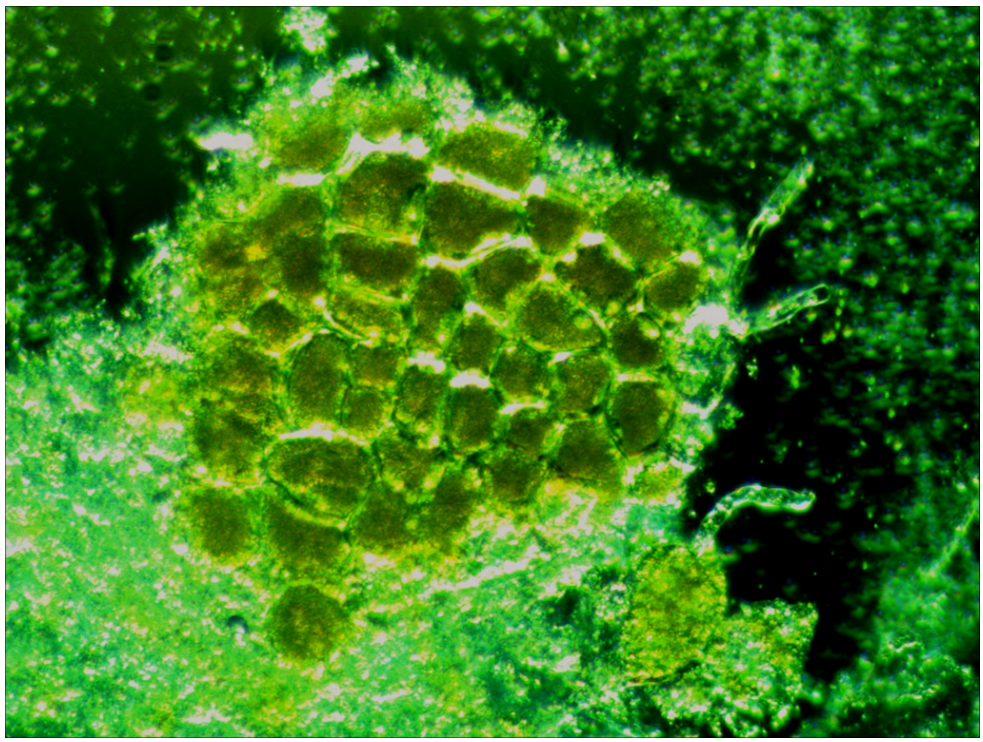
Nutritional requirements, chemical sensitivity, and in *vitro* growth of *Rhizina undulata*



"Structures of *Rhizina undulata* under microscope" Photo: Iryna Semashko

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Swedish University of Agricultural Sciences

Master Thesis no. 226

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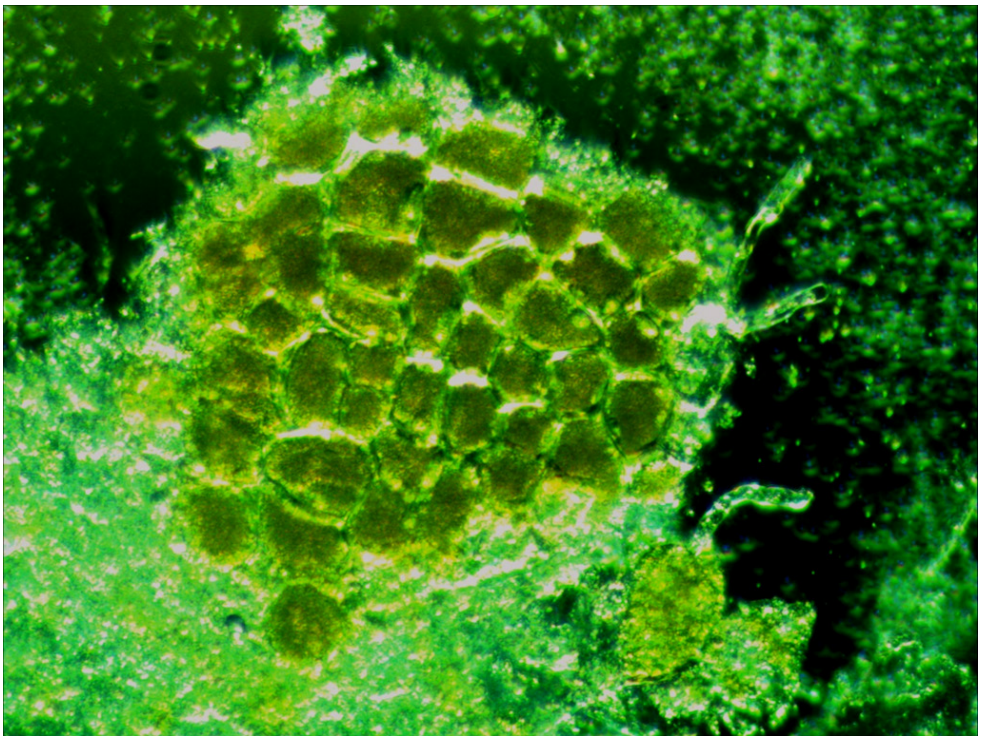

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ABSTRACT

Fire is an important factor for biological diversity in boreal forests. According to the FSC requirements, annually around 5% of the clear-cut forest territory in Sweden should be burned. On the other hand, fire is the triggering factor for the development of the pathogenic fungus *Rhizina undulata*, which causes considerable losses in planted coniferous seedlings on previously burned sites. The damage caused by the fungus can lead to the death of up to 80 % of coniferous seedlings. The aim of this study was to investigate nutritional and chemical preferences of the fungus. In the first phase the production of fungal material (mycelium, ascospores and conidia spores) in different conditions was examined. Only mycelium was found to be produced in amounts sufficient for further experiments. The preferred media was malt extract broth (MEB) maintained at room temperature without agitation. In the second phase, the ability of a fast-growing *R. undulata* strain to utilize more than 285 different chemical substrates within carbon, nitrogen, phosphorus or sulfur groups was tested using Phenotype MicroArray technology. The utilization rates of carboxylic acids and nitrogen compounds were the highest among the tested groups. From a qualitative point of view, the group of carbon-dominated substances had the highest number of compounds utilized by the fungus. The approach used in this study can help to evaluate the qualitative and quantitative chemical traits that increase or decrease the risk for *R. undulata* damages in plant materials. In the long term, the approach could yield information that could potentially be used to formulate guidelines in tree-breeding for increased resistance to the fungus.

Key words: *Rhizina undulata*, pine fire fungus, filamentous fungi, prescribed burning, phenotype microarray technique.

1. INTRODUCTION

Prescribed burning used to be a common practice in Swedish silviculture during the early 1950 – 1970's, but with increased labor costs it was not economic anymore (Niklasson and Granström, 2004; Lazaro, 2009). Currently, fire is acknowledged as an important factor for biodiversity in boreal forest ecosystems. Therefore, according to the Forest Stewardship Council, annually around 5% of the clear cut forest territory in Sweden should be burned (FSC, 2011). Prescribed burning is also used as a tool in fire prevention. Low intensity fires are applied to reduce the fuel load and prevent the risk of high intensity fires (EFI, 2010). One problem associated with this method is the root-rot fungus *Rhizina undulata*, which causes a considerable amount of damage to coniferous seedlings and in particular Scots pine planted on burned sites (Callan, 1993; Vasiliauskas et al. 2005).

Rhizina undulata is a post-fire ascomycete which commonly occurs throughout the world in temperate and boreal regions. It can cause the death of coniferous trees, such as *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Thuja* and *Tsuga* species (Callan, 1993). One of the most susceptible species among conifers is Austrian pine (*Pinus nigra*). There are records of the fungus causing the death of 70 % of the 50-60 year old plantation of Austrian pine (Gremmen, 1971). According to Jalaluddin (1967, b), Gremmen (1971), Callan (1993) and Vasiliauskas et al. (2005), areas colonized by the fungus after prescribed burnings can suffer the death of up to 85 % of all planted conifers' seedlings and of groups of standing coniferous trees. Symptoms of the dying seedling will be similar to those caused by drought or other root diseases; foliage becomes yellow, seedlings decline rapidly and die. After additional examination of seedlings root, the infection by *R. undulata* was visible on the inner root surface as browning branching of mycelia strands (Lee et al. 2005). The death of mature trees is following with resin exudation from the base of the tree and presence of yellow fungal mycelium on the roots (FABI, 2012).

The fungus itself is a weak competitor, and fire plays a dominant role in its development. Fire reduces microbial and fungal diversity on the site (Penttilä et al. 1996), which leads to decrease in competition, and thus creates suitable conditions for the development of *R. undulata*. The most preferable sites for colonization are the sites with the presence of conifers' stumps or old standing coniferous trees. The fungus prefers acid and peat soils (Seaby, 1977) with high moisture and low pH level. *Rhizina undulata* is homothallic fungus, meaning that the fungus can produce its sexual forms from a mycelium originating from a single spore (Grous et al. 2009). The germination of the sexual spores, ascospores, is activated by high temperature, which is caused by prescribed burning after clear cut. The fungus is able to spread either by mycelium growth in the soil, from one conifers root to another or with the help of airborne ascospores (Vasiliauskas et al. 2001). From an infected host, the fungus spreads radially at a rate that can reach 1, 5 - 3 meters per year. On the previously colonized sites *R. undulata* can produce fruit bodies for several subsequent years, without recurrence of fire (Phillips & Burdekin, 1982). Limiting factors for fungus growth are presence of broadleaved trees and absence of conifers. Moreover, on fire treated sites with neutral or alkaline soils (pH 7-8) the occurrence of the fungus was not detected (Jalaluddin, 1967 b).

There are several aspects of the eco-physiology of the fungus that the potential control methods can target. One of these is that the period of activity of the fungus lasts for about 2 to 4 years (Murray & Young, 1961; Vasiliauskas et al. 1999). Delaying planting or replacing it with sowing on previously burned areas is therefore recommended (Gremmen, 1971). Avoiding direct planting

on the area where the fruit bodies are abundant is a way to not provide food base for fungus and try to "starve" it. Infection usually develops from margins of the burned sites, as only there the fire temperature is optimal for ascospores germination, 35 - 45 ^{o}C degree (Jalaluddin, 1967 a, b). Segregation of the burned patches from surrounding territory with trenches can also be used to stop the radial spread of the fungus. In this case, the presence of conifers' roots within the burned area would be cut off and saprophytic colonization and vegetative increase by the fungus reduced (Uotila & Levula, 2011). The intensity of bonfires also has an influence on the fungus' activity. Higher mortality of seedlings occurs on sites, where the fire was more intense and piles were larger, than on medium or small ones (Seaby, 1977; Murray & Young, 1961; Lee et al. 2005). The fire clearing should be carried out from November until May, to decrease the infection of soil by the fungus.

The efficiency of the above mentioned preventive measures strongly depends on the climatic and site conditions and the results vary. In the last decade chemical treatments have been applied on *Pinus patula* and *Pinus elliottii* plantations infected by *R. undulata* in South Africa. Fungicides such as benomyl and prochloraz were used to decrease mortality of seedling after planting in infected areas, as in South Africa the use of these fungicides is not prohibited. After fungicide application the rate of seedlings survival was reaching almost 87 % at 12 months after planting (Rolando, 2006). The use of broad spectrum fungicides is, however, not a prioritized option for sustainable forestry, and for example benomyl is withdrawal of authorization for plant protection in EU (2002/928/EC) and the use of prochloraz cannot be recommended because its potential effects on non-target organisms (Ohlsson, 2009). Therefore, development of new control solutions, based on environmentally acceptable functions, is of interest.

Development of environmentally friendly control methods could be assisted by better understanding of the nutritional requirements of the fungus. The main scientific goal of this thesis was to investigate the substrate utilization profile of *R. undulata* in order to find out which substrates the fungus prefers and which it dislikes. Using Phenotype MicroArray technology, the ability of *R. undulata* to utilize more than 280 different chemical substrates was examined.

A technical goal of the work was to optimize the experimental system for *R. undulata* studies, in particular to define conditions for the ability of *R. undulata* to produce extracellular proteins, spores, conidia and to develop apothecia. First, I examined whether the extracellular enzyme fraction, instead of whole cells, could be used in the assay. Since the enzymes are the key to the pattern of substrate utilization (Archer and Wood, 1994; Have et al. 2002), therefore, to observe the possible available set of *R.undulata* enzymes would be of interest. Second, I investigated whether by manipulation of fungal mycelium through growth conditions, fruit bodies of *R. undulata* could be obtained in in *vitro* conditions.

The obtained knowledge of the nutritional preferences of the fungus can help to estimate the magnitude of risk for *R. undulata* damages on plant materials that differ in their chemical quality. In the long term, this information could be used as guideline for tree breeding for increased resistance to the fungus.

2. MATERIAL AND METHODS

The *Rhizina undulata* isolates (300.56 and 301.56) used in the studies were purchased from MycoBank (http://www.mycobank.org). According to the supplier the strains were collected from the soil under *Picea pungens* trees in the Netherlands (CBS, 2011). Initial cultures of the fungus were established on 3 % malt extract agar (MEA).

2.1 Optimization of growing conditions for spores, conidia and apothecia development

After placing the strain 300.56 on MEA, it was subcultured on the corresponding media in 90 mm diameter Petri dishes, and stored for 7-10 days at room temperature in darkness. The strains were multiplied under different sets of conditions (13 permutations of six factors) in order to observe possible conidia or apothecia development (Table 1). The morphological traits of the cultures were examined under microscope.

Table 1Examination of R. undulata strain (300.56) during periods of 14 and 21 days for possible conidia or apothecia development under 13 permutations of four factors

	Medium[1]	**Agitation[2]**	**Light/Dark[3]**	**Temperature[4]**	**Time, days**	**Replicates**
1	MEA	-	Dark	RT	14	5
2	MEA	-	Light	RT	14	5
3	MEA	-	Dark	I	14	5
4	MEB	-	Light	RT	21	4
5	MEB	-	Dark	I	21	4
6	MEB	-	Dark	RT	21	4
7	MEB	80 rmp	Light	RT	21	4
8	MEB	160 rmp	Light	RT	21	4
9	OMA	-	Light	RT	14	5
10	OMA	-	Dark	I	14	5
11	OMB	160 rmp	Light	RT	21	4
12	OMB	-	Light	RT	21	4
13	OMB	-	Dark	I	21	4

[1] Media: Malt extract agar (MEA) or oatmeal agar (OMA); malt extract broth (MEB) or oat meal broth (OMB); [2] Agitation: no agitation or agitation on an orbital shaker at speed of 80 or 160 rmp per min.; [3] Light/Dark – natural light and dark cycle of laboratory; [4]Temperature: room temperature (RT), about 22 ^{o}C; incubator (I) adjusted to 25 ^{o}C; Replicates; technical replicates, i.e. the number of Petri dishes or bottles.

2.1.1. Cultures of isolates on solid media

In order to obtain enough replicate plates for the comparisons, pieces with size of 0,5 cm^2 with actively growing mycelium were cut off from the margins of a growing colony and placed each on 20 Petri dishes containing 3 % MEA, and 20 Petri dishes with oat meal agar (OMA) under sterile conditions. Groups of five plates were stored under different light (day-light /darkness) and temperature (incubator/room temperature) conditions to observe the radial growth rate, aerial mycelium growth and possible conidia or apothecia development.

2.1.2. Cultures of isolates in liquid media

To examine the possibility to stimulate mycelium growth or conidia production, liquid cultures were established. From MEA and OMA plates, which were vegetatively colonized by the fungus, 0, 5 cm^2 pieces with actively growing mycelium were cut, and transferred into sterile Erlenmeyer flasks containing 50 ml of liquid media corresponding to the media from which they were isolated. The flasks with liquid media were placed on an orbital shaker at 80 rmp and 160 rmp at room temperature. Observation of mycelium growth, radial growth rate, apothecia or conidia cells development was carried out every third day during a three week period.

2.1.3. Records of fungal growth and development

Observation of the growth and development parameters (Table 4, page 10) was carried out during 21 and 14 days of the experiment. There was a set of four conditions under which the fungus was examined, e.g., light, darkness; agitation, no agitation on different solid and liquid growing media e.g., MEA, OMA, MEB, OMB. Each culture was observed for tangential (aerial, and overall biomass development) mycelium growth, radial mycelium growth rate, development of apothecia or conidia cells, and additional physiological and identity characteristics (coloration of media, hyphal size, etc.). In order to obtain information about the most preferable condition for fungus development, the growth response of the fungus in each condition was examined visually and summarized as: "-"- no development; "+"- modest; "++"- intermediate, and "+++"- high.

2.2 Establishing the protocol for measuring extracellular proteins in liquid culture, and optimizing the conditions for high protein yield

The substrate utilization by the fungus is strongly dependent on the extracellular enzymes that fungal cells excrete to their surroundings (Norkrans and Hammarstrom, 1963). In order to obtain information about the ability of *R. undulata* to produce extracellular enzymes that would allow substrate utilization, the production of extracellular proteins by the strains 300.56 and 301.56 of *R. undulata* was examined. The spectrophotometric method for the protein assay was based on previous studies of Bridge (2004) and Simonian (2002), in which the protocol was successfully used for the measurement of protein concentration of filamentous fungi.

The two strains of the fungus were growing on MEA in Petri dishes for 7 days at room temperature and in continuous darkness. Small pieces of agar were cut off from the marginal parts of colonies, where active growth of mycelium was present. Each of the cut 0,5 cm^2 agar pieces were inoculated into 50 ml of MEB medium in a 100 ml sterile Erlenmeyer flask, sealed with aluminum foil, and stored in different conditions according to Table 2.

Table 2 Set of different growing conditions for examination of extracellular proteins production by R. undulata strains (300.56 and 301.56)

Isolate	Agitation/stable condition	Time, days
300.56	80 rpm/min	5
	No agitation	5
301.56	80 rpm/min	5
	No agitation	5

Further treatment of the liquid cultures was carried out as follows. One flask of each strain was kept at room temperature under rotary shaking (80 rpm) and another in steady condition on the laboratory bench during 5 days. Each 24 hours three replicate measurements of extracellular protein concentration were made from each flask using spectrophotometer (SPECTROstar Nano spectrophotometer, BMG LABTECH GmbH, Germany).

Under the fume hood 2 ml of malt extract with fungal cells was collected from each flask using a disposable sterile syringe. In order to get rid of large cell aggregates, the liquid media with fungal strain was filtered through a 0.45 mm filter (Millipore) into labeled Eppendorf vials. Filtered pure MEB was used as a blank.

The absorption was measured at 280 nm and with 5 nm resolution (Simonian, 2002) in an l.00-cm light path, using the pre-programmed "Protein" assay option of the equipment. To determine protein concentration, a standard curve was built up using Bovine Serum Albumin (BSA) as a reference. For the standard curve, 3 mg/ml of BSA was dissolved in water and diluted to the concentrations: 0,001; 0, 01; 0, 1; 0, 5; 1; 1, 5; 2; 2, 5; 3; 3, 5; 4; 4.5; 5 mg/ml. To reset the spectrophotometer before measurements, sterile deionized water was used as a blank.

The absorbance value of proteins in liquid cultures is high due to the presence of amino acids, mainly tyrosine and tryptophan (Grimsley and Pace, 2004). To obtain a proper scale for measurement of protein concentration the malt extract and malt extract with fungal inoculums were dissolved 1:6 dilution with sterile deionized water. The diluted sample was pipetted into 1ml UV quartz cuvette, and processed on the spectrophotometer using the "cuvette measurement" option. The protein concentration was calculated by comparison with a BSA standard curve which was constructed using Data Analysis Software (MARS) of the equipment. In order to obtain accurate results of protein concentration in liquid, the linear regression [R^2] of BSA curve was estimated with R^2=0,999. The equation for standard line was (Equation 1).

$$\mathbf{y = mx + b_2,} \quad (1)$$

where **y** - absorbance at 280 nm; **x** - protein concentration; **m** and $\mathbf{b_2}$ slope and intersection. With this equation the concentration was calculated by entering the absorbance value of each unknown into the equation as the y value and then solved for x.

2.3 Assay of fungal nutrient utilization using a Phenotype MicroArrays technique

To examine nutritional requirements of the fungus, BIOLOG micro-well plates, also known as Phenotype MicroArrays (PM) technique, were used (www.biolog.com, Biolog, Inc. Hayward, CA). The PM technique is based on the examination of cellular phenotypes, e.g. the nutritional pathways for carbon, nitrogen, phosphorus and sulfur metabolism, sensitivity to chemical compounds and wide range of pH (Borglin et al. 2011; Garland and Mills, 1991). One Biolog plate consists of 96 wells, containing 95 different compounds (stressors or nutrients) and one well without any substrate as a control. To indicate the substrate utilization by the cells added into a well, each well contains a redox dye (0.01% tetrazolium violet) that acts as a color indicator (Bochner et al. 2001). If the substrate is utilized by the fungal cells, the reduction of tetrazolium violet influences the color intensity of the well and the intensity can be read at a certain absorbance wavelength. The PM technique is used for drug discovery, identification and characteristics of fungi, mammal and microbial cells.

Isolate of *R. undulata* (300.56) was tested with Phenotype Microarray technique using PM1, PM3B, and PM4A plates, with carbon, nitrogen, phosphor and sulfur sources correspondingly. The experiment was based on the protocol for filamentous fungi, provided by the manufacturer. In order to gather mycelial cells, fragments of actively growing mycelium from previously growing liquid cultures were cut off and placed in Erlenmeyer's flasks under the sterile hood. The flasks were filled with 60 ml of 3% malt extract broth and kept at room temperature in natural light and dark cycle of laboratory during 7 days. Pieces of mycelium were then collected and placed into a sterile tube where the mycelial mass of the fungus was homogenized for 20 seconds (IKA T-18 Basic Homogenizer 115 VAC). In order to separate the malt extract liquid from the fungal cells, the tube with the material was placed into a centrifuge and centrifuged for 2 min at 5 000 rpm speed. The malt extract liquid was removed with a pipette, and the tube was filled with 1 ml of sterile water. The centrifugation was repeated for 1 min at 3000 rpm speed. The water remnant was also removed from the tube. The presence of approximately 70% of intact cells was confirmed under microscope. Following the Biolog protocol, the fungal material was then transferred into a sterile glass tube with 12 ml of inoculating fluid. By adding mycelium cells or inoculating fluid, the transmittance of the inoculums was adjusted to 62% of cell concentration.

Three types of PM plates and three replicates for each of them were used. The plates PM1, PM3B, and PM4A contained carbon, nitrogen, phosphor and sulfur sources, in total 285 different substrates (see a detailed list with the substrates per plate in Appendix 3). According to the Biolog protocol for filamentous fungi, the inoculation suspension for each type of plate was prepared in the same way, adjusting the cell suspension and inoculating fluid volume so that a comparable cell concentration was obtained (Table 3). The procedure was carried out in following steps: preparation procedure for PM1 plate with carbon source was conducted under the fume hood, inoculating fluid was poured into a sterile plastic vial and mycelium suspension was added. The components were mixed in vial with help of a sterile plastic stick for 2 minutes. Using a multichannel pipette, 100 ml of suspension was transferred into each well of 96 wells of the PM1 plate. All plates were kept under the sterile hood until the inoculums suspension in wells was gelatinized.

Table 3 Protocol for inoculation of Phenotype MicroArray plates.

	PM1 (Carbon sources)	PM3B (Nitrogen sources)	PM4A (Phosphor& Sulfur sources)
Inoculating fluid, ml	23,95	59,875	11,975
Cell suspension, ml	0,05	0,125	0, 025
Replicates, plates	3	3	3

After inoculation, the initial absorbance at 590 nm was measured in all plates (time point zero). Next after measurement all plates were covered with a lid and sealed with film tape. The plates were stored in an incubator at 26 ^{0}C degree until next measurement. The measurement was done after each 24 hours. The period of measurement was limited to 13 days, due to the saturation of the reaction and the possibility of plate's contamination. The groups of compounds were separated according to Garland & Mills (1991), and Bohner et al. (2001) according to similarity of substrate's chemical characteristics (see Appendix 3).

In order to qualitatively analyze compounds utilization, the values which were obtained after calculation of average well color development (AWCD, n=3) were separated by setting the threshold of 0,002. The values which were less than 0,002 were treated as not utilized substrates, and the values which were higher than this point as utilized substrates. To segregate utilized compounds from non-utilized, the coding with "+" and "-" was used. Since most of the compounds in all groups had a lag phase up to 6th day and the response showed saturation after the 13th day, the data collected between 6th and 13th day (active reaction) is considered.

The data obtained from Spectrophotometric analysis were accumulated in Data Analysis Software - MARS, and further processed in Microsoft Office Excel.

The obtained data from protein's assay was analyzed by factorial ANOVA using software MiniTab (Minitab 16.2.4., LEAD Technologies, Inc., Charlotte, NC, USA.). One –way ANOVA (unstucked) test was used to test the difference between mean values of produced proteins by 301.56 vs. 300.56 strains, and in between the different experimental conditions.

3. RESULTS

3.1 Optimization of growing conditions for spores, conidia and apothecia development

Following the given methodology, results about appropriate conditions for fungus development were acquired. The given strain of *R. undulata* was examined for six different characteristics according to Table 4. During the observation period of 14 and 21 days optimal conditions for growing fungal material for further use in experiments were selected.

There were several criteria for examination of fungus development on solid and in liquid media under given conditions (Table 1 and Table 4). The main distinctive feature in fungus growth was development of "apothecia" on OMA media (Table 4. # 9 and 10), (Appendix D), on MEA it was not present during all observation time.

Table 4 The results of examination of R. undulata (300.56) development based on six different characteristics on solid (MEA, OMA) and in liquid media (MEB, OMB).

	Composition of media	Mycelium growth ratio (tangential growth)	Radial mycelium growth ratio	Development of apothecia	Development of cells (conidia)	Time, days	Replicates
1	MEA	+	+	-	-	14	5
2	MEA	+	+	-	-	14	5
3	MEA	++	++	-	-	14	5
4	MEB	+++	+++	-	-	21	4
(4)	MEB	Without agitation, pure liquid media, stored at room temperature, in laboratory conditions					
5	MEB	++	++	-	-	21	4
6	MEB	++	++	-	-	21	4
7	MEB	++	+	-	-	21	4
8	MEB	++	+	-	-	21	4
9	OMA	++	++	+	-	14	5
10	OMA	++	+	+	-	21	4
11	OMB	+	+	-	-	21	4
12	OMB	+	+	-	-	21	4
13	OMB	+	++	-	-	21	4

[1]MEA –malt extract agar; [2]OMA- oatmeal agar; RT[4]- room temperature; Incubator[5] at 25 ^{o}C;MEB[3]- malt extract broth; OMB[4]- oat meal broth.

The highest mycelium biomass production was observed in MEB which was stored in laboratory condition at room temperature, without agitation under natural light and dark cycle of laboratory (see Table 4, # 4). The aerial mycelium growth was almost absent and the overall fungus growth rate was intermediate on OMA media in contrast to MEA media. Increased temperature did not help to obtain higher aerial mycelium growth in liquid and on solid media.

In further assay of fungal nutrient utilization mycelium material was used. In order to get as much of material as possible the conditions with highest production of it were chosen (see Table 4, # 4).

3.2. Protein assay

Fungal strains of *R. undulata* 300.56 and 301.56 were examined in laboratory conditions under rotary shaking (80 rpm) (fig. 1) and in stable condition (fig. 2, 3) during 5 days. The development of proteins in MEB extract under examined conditions increased proteins in solution in three cases out of four (fig. 1, 2, and 3). The strain 301.56 had rapid exponential phase from 2nd day and reached average protein amount of 1127 μg/μl on the 4th day (fig. 1). However, fungal strains have shown low production rate of proteins under agitation conditions, and strain 300.56 did not have positive values during all observation period (data is not shown)

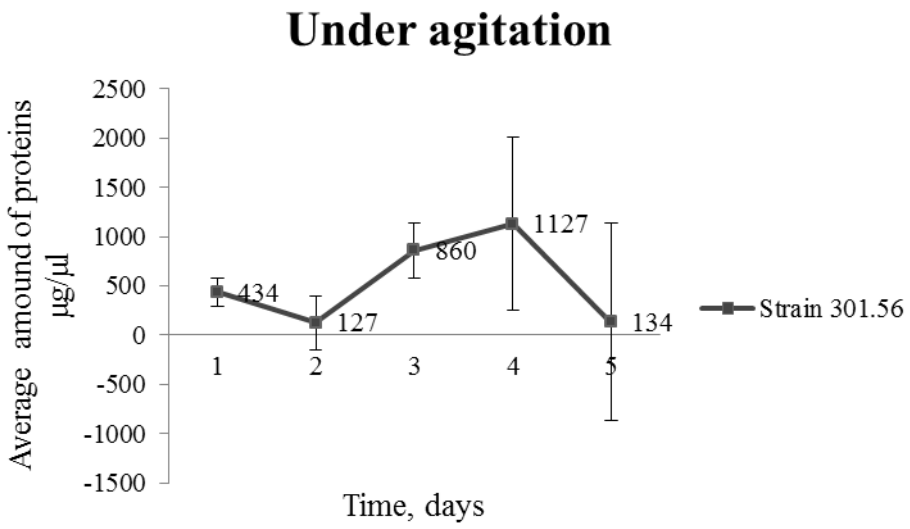

Figure 1 The production of proteins by R. undulata strain 301. 56 in MEB extract during examination period, under agitation. Data presented in average protein amount (μg/μl) with error bars representing standard deviation (SD), each time point is based on three technical replicates.

Adhering to the same preparation procedure, the same strains of fungus (300.56 and 301.56) were tested under stable conditions (figs. 2 and 3). During examination period of the strain 301.56 under stable condition it has started a rapid development at day 3 (fig. 2). The production of proteins was progressing until the end of the experiment by reaching markedly higher concentration of proteins than under agitation condition, i.e. 1127 μg/μl compared with 9767 μg/μl. On the other hand, there was a high variation between the technical measurements, and a decreasing phase in agitation experiment of the strain 300.56 during the same day i.e. 4 day.

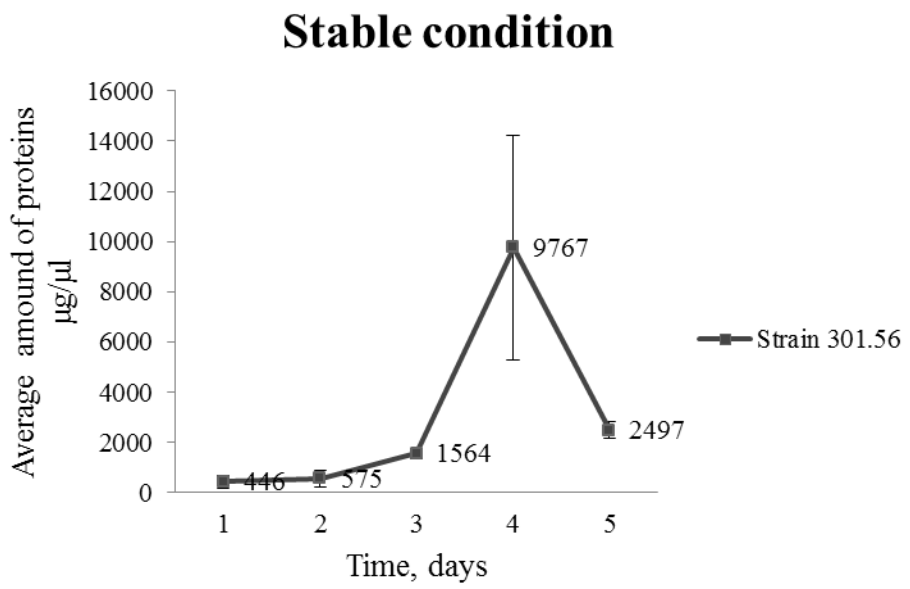

Figure 2 The production of proteins by R. undulata strain 301. 56 in MEB extract during examination period in stable condition. Data presented in average protein amount (µg/µl) with error bars representing SD, each time point is based on three technical replicates.

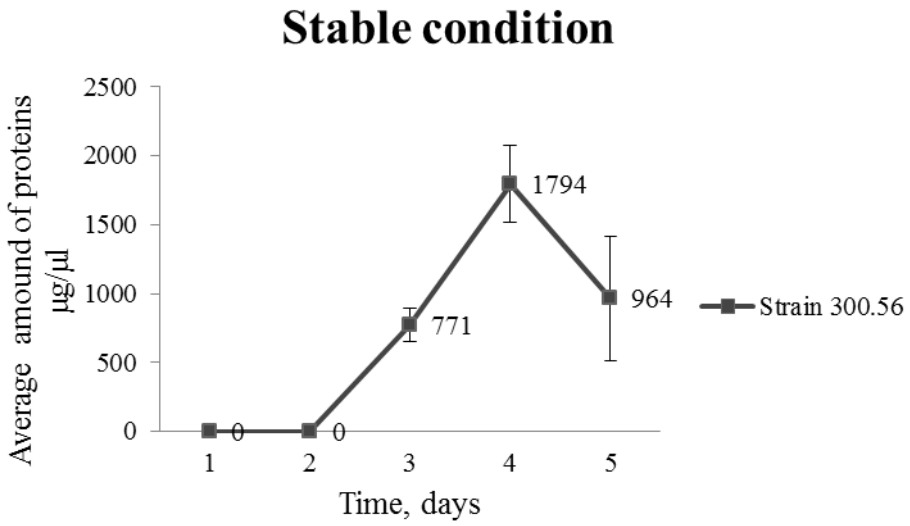

Figure 3 The production of proteins by R. undulata strain 300. 56 in MEB extract during examination period in stable condition. Data presented in average protein amount (µg/µl) with error bars representing SD, each time point is based on three technical replicates.

3.3 Assay of fungal nutrient utilization using a Phenotype MicroArrays technique

The utilization of 285 different nutritional sources in BIOLOG plates was analyzed. The given compounds were distributed according to chemical groups (Table 5). Groups of compounds such as nitrogen, sulfur, phosphor and carbon were located on different plates from the beginning. In case of carbon group, all compounds were manually arranged into three sub-groups: carboxylic acids, carbohydrates and "other carbons", according to Garland and Mills (1991).

To compare the substrate utilization within the groups and in between the groups, the AWCD and the percentage of the utilized compounds were used. The most utilized compounds are shown in separated figures (fig. 5, 7, 9, 11, 13 and 15). Overall substrates utilization in three tested PM plates PM1, PM3B, and PM4A is presented as average AWCD (fig. 6, 8, 10, 12, 14 and 16) deviation in measurements from the three technical replicates is represented by standard deviation error bars.

Table 5 Qualitative and quantitative results of nutrient utilization with list of most and least utilized compounds among examined groups. The results is shown in separate groups, where utilization rate was measured in average wells color development (AWCD) and amount of utilized compounds in percentage equivalent (amount of compounds in the group/amount of utilized compounds), where utilized compounds were those which overcome threshold of AWCD +0,002.

	Carbohydrates	Carboxylic acids	Other carbons	Sulfur	Phosphor	Nitrogen
Average utilization (AWCD)	0,009	0,016	0,005	0,005	0,007	0,019
Amount of utilized compounds (%)	82% (23/28)	75% (22/29)	63% (24/38)	61% (21/34)	47% (28/59)	27% (26/95)
The most utilized compounds in the group	L-Arabinose (0,016) D-Galactose (0,018) D-Ribose (0,019) M-Inositol (0,016) D-Xylose (0,019) α-D-Glucose (0,016) Maltotriose (0,018) L-Lyxose (0,018) D-Psicose (0,015)	D-Glucuronic Acid (0,112) Formic Acid (0,033) D,L-Malic Acid (0,119) Fumaric Acid (0,025) α-Keto-Glutaric Acid (0,043) M-Tartaric Acid (0,015)	L-Aspartic Acid (0,008) L-Glutamic Acid (0,009) L-Asparagine (0,009) Tween40 (0,033) Adonitol (0,011) L-Serine (0,009) L-Alanyl–Glycine (0,019) Phenylethylamine (0,021)	Cystathionin (0.016) Taurocholic Acid (0.051) D,L-Lipoamide (0.017)	Triethyl Phosphate (0.025) Adenosine-5'–monophosphate (0.155) Cytidine-2'–monophosphate (0.037) Cytidine-3'–monophosphate (0.018) Methylene Diphosphonic Acid (0.048)	L-Cysteine (0.019) L-Tyrosine (0.133) Guanine (0,265) Glucuronamide (0,571) Ethylenediamine (0,215)
The compounds with the lowest utilization rate	β-Methyl-Dglucoside (-0,001) α-Methyl–Dgalactoside (-0,002) Maltose (-0,001) D-Trehalose (0,001) D-Mannose (0,002) D-Mannitol (0,001)	D-Galactonic Acid-γ–Lactone (-0,004) D-Glucosaminic Acid (-0,004) Tricarballylic Acid (-0,004) m-Hydroxy Phenyl Acetic Acid (-0,004) Pyruvic Acid (-0,003)	Glycyl-L–Proline (-0,005) L-Alanine (-0,001) Adenosine (-0,002) 2-Deoxy Adenosine (-0,002) Tween 80 (-0,020)	Glycyl-L–Methionine (-0,001) D-Cysteine (-0,002) Tetramethylene Sulfone (0) L-Djenkolic Acid (0)	Phosphate (-0,001) Trimetaphosphate (-0,002) Adenosine - 2' – monophosphate (-0,002) Phospho-Glycolic Acid (-0,003) Cytidine - 3' ,5' – cyclicmonophosphate (-0,004)	L-Alanine (-0,005) Glycine (-0,002) L-Valine (-0,002) L-Citrulline (-0,004) L-Homoserine (-0,004)

The groups which had highest utilization rate were carboxylic acids and nitrogen compounds with AWCD rates of 0,016 and 0,019 respectively. From qualitative point of view carbon group has the highest amount of elements which were utilized by fungus, allthough at the low utilization rate (Table 5).

The average utilization of each investigated group is present in Fig. 4. There was a steadily low utilization rate of most compounds in the carbohydrate, phosphorous groups on the contrary to nitrogen group with steadily high utilization rate from the first day. In the groups of carboxylic acids progressive utilization of compounds was observed with reaching the peak of utilization on 10th day. The steep development in first two days of average utilization in the group of "other carbons", was caused by high level of utilization of a few compounds e.g, Tween 80 (0,104), 2-deoxy adenosine (0,145), L-asparagine (0,303), phenylethylamine (0,418), which have influenced the overall tendency of average line in the group.

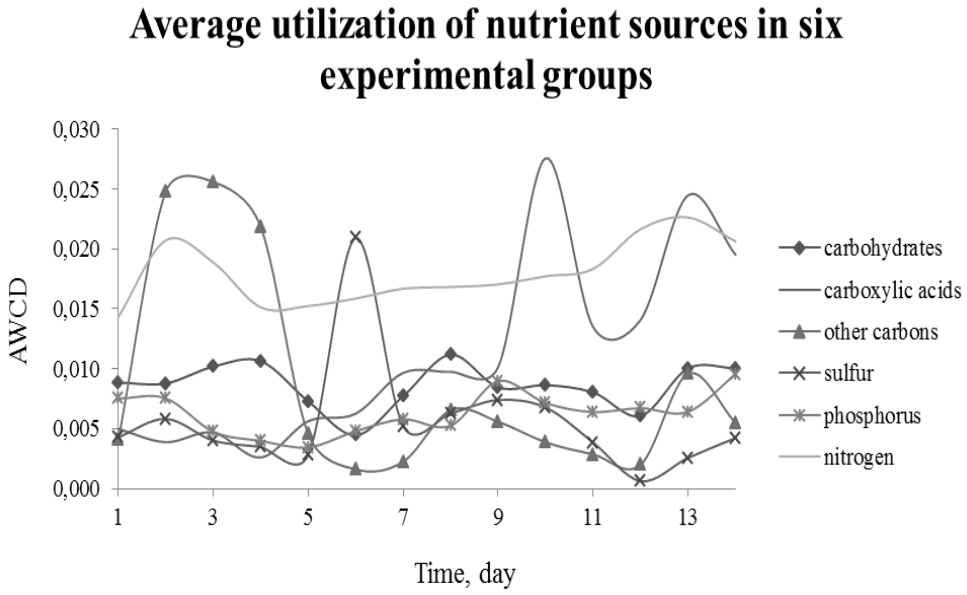

Figure 4 Average substrate utilization by R. undulata in six examined groups e.g. carbohydrates, carboxylic acids, other carbons, sulfur, phosphorus, nitrogen. Data points are presented during 14 days of experiment.

3.3.1 Utilization of carbohydrates

Average utilization of the carbohydrates occurred at the intermediate level within the test (Fig.5). The AWCD reached a value of 0,009 during the 13 days period. The proportion of utilized compounds in the carbohydrates group was the highest among all investigated groups. The fungus utilized nearly 82 % of tested compounds in this group (23/28) (Table 5). Most of carbohydrate compounds had a lag phase up to the 6th day, almost without any color development in the wells.

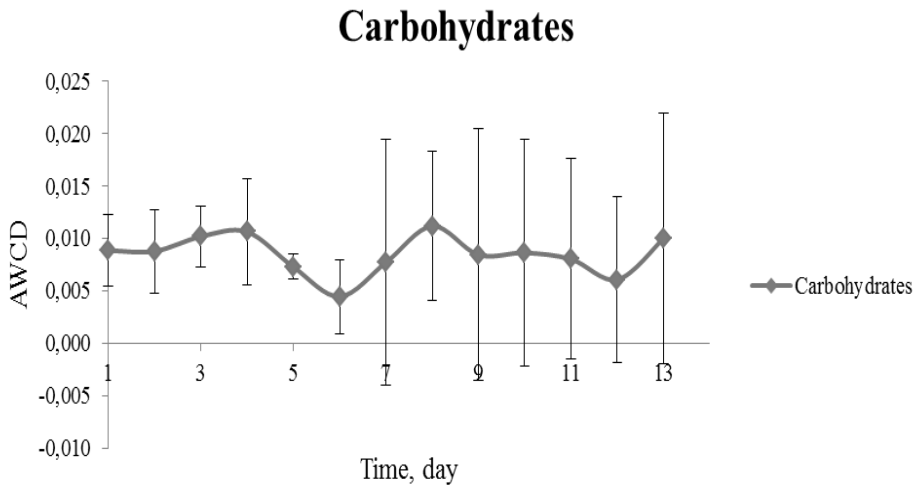

Figure 5 The utilization of all carbohydrates by R. undulata, with average development of all compound are presented in AWCD and standard deviation of each point among three replicates of plates (PM1, PM2, PM3) is shown during 13 days of examination. Vertical bars represent SD (n=3).

There were several chemical compounds which had a relatively high utilization rate in the whole group (Fig.6). Those that started at the 6th day and exhibited an exponential growth phase, are: α-D-Glucose (0,016) with maximum value (MV) of 0,025; maltotriose (0,018) with MV (0,028); m-Inositol (0,016) with MV (0,025).Those that showed rapid increase of compound utilization in the end of experiment : D-xylose (0,022) with MV of 0,025; D-ribose (0,021) with the highest peak on the 13 day with a MV of 0,029; L-lyxose (0,019) with MV of 0,026. The compounds with lowest utilization rates had a limit in between 0 and -0,002 values, those were : β-methyl-Dglucoside (0); α-methyl –D-galactoside (-0,002); Maltose (-0,001); D-trehalose (0).

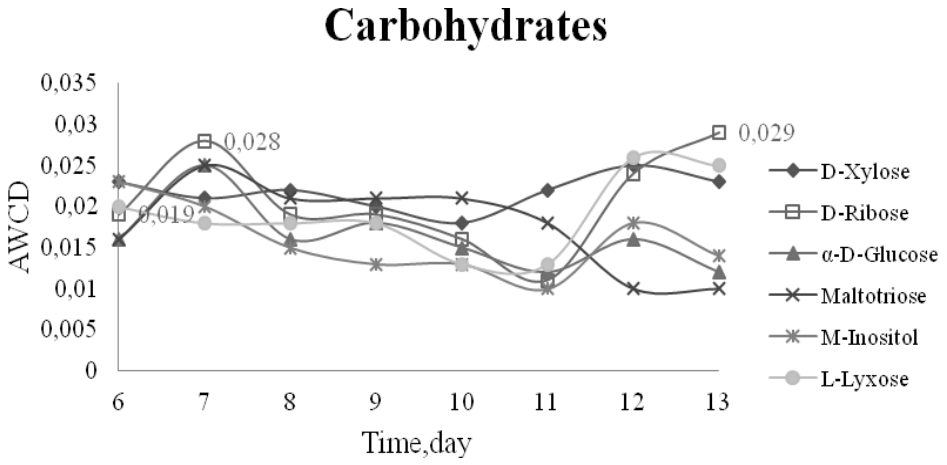

Figure 6 Most utilized compounds in carbohydrate group, data points are presented in AWCD.

3.3.2 Utilization of carboxylic acids

Rhizina undulata also showed a relatively high rate of utilization of several carbon sources belonging to the carboxylic acids. The average utilization rate (0,016) was one of the highest among groups. However the fungus utilized 22 out of 29 substrates but only a few of them

actively. As in previous group, most of carboxylic acids exhibited a lag phase until the 6th day, started with slow utilization and reached a stationary phase on the 10th day (fig.7).

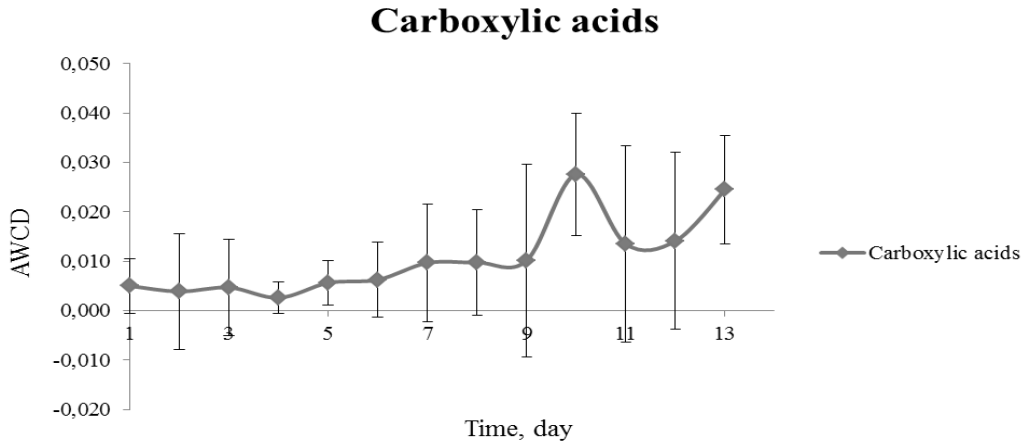

Figure 7The utilization of alcohols, amines, amides, amino acids, aromatic chemicals, phosphorylate chemicals and polymers substrates by R. undulata in three plates (PM1, PM2, PM3) and in average (AWCD) during 13 days of examination. Vertical bars represent SD (n=3).

Figure 8 shows the AWCD of the most utilized compounds. The majority had the higest value of 0,01, except D,L-Malic Acid and α-Keto -Glutaric Acid. The D,L-Malic Acid showed negligible development during the first week of observation. Exponential development begun only on the 8th day and reached the maximum AWCD value of 0,454 followed by a rapid decline in values on 10th day .The two compounds that had the second highest utilization values in the group were D-Glucuronic Acid and Furamic acid. D-Glucuronic Acid and Furamic acid had exponential increase in utilization on the 4th day, with reaching maximum value of 0,158 on 11th day (264 hours), second compound had the value of 0,101 on 12th day.

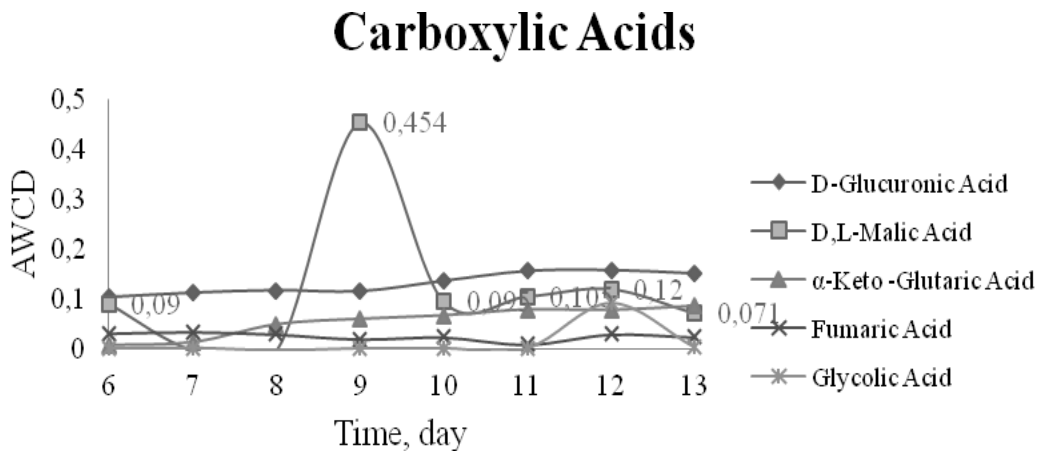

Figure 8 The most utilized compounds in carboxylic acids group, data points are presented in AWCD.

3.3.3 Utilization of other carbon sources (including alcohols, amines, amides, amino acids, aromatic chemicals, phosphorylate chemicals and polymers)

The utilization of carbon compounds in the last group differs from the prevoius two by the low AWCD values. In the group the majority of compounds were utilized at a level below AWCD 0,001, and only a few compounds had utilization rate above 0,01. In contrast to carboxylic acids, the group peaked of Tween 40 as highest utilized value of 0,125 in comparison with 0,454 value of D,L-Malic Acid.

The overal utilization of compouds was of 63 % (24/38), and reached an AWCD value of 0,005.

A distinctive feature of the data from this compound group is the absence of kinetics (phases) in utilization of substrates. Among all compounds for which the AWCD-value exceeded the treshold of 0,001, and which thus were considered to be utilized by the fungus, exponential development took place in first days of experiment (fig.9) The development of AWCD-value for reached a stationary phase on 3^{d} day with no marked increase observed for most of the compounds, except for Tween 40 and Phenylethylamine (fig.10).

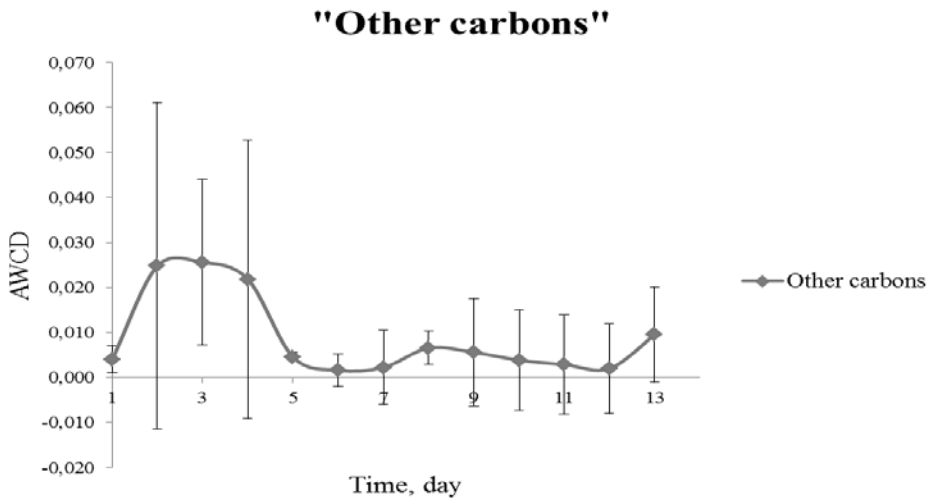

Figure 9 The utilization of alcohols, amines, amides, amino acids, aromatic chemicals, phosphorylate chemicals and polymers substrates by R. udulata in three plates (PM1, PM2, PM3) and in average (AWCD) during 13 days of examination. Vertical bars represent SD (n=3).

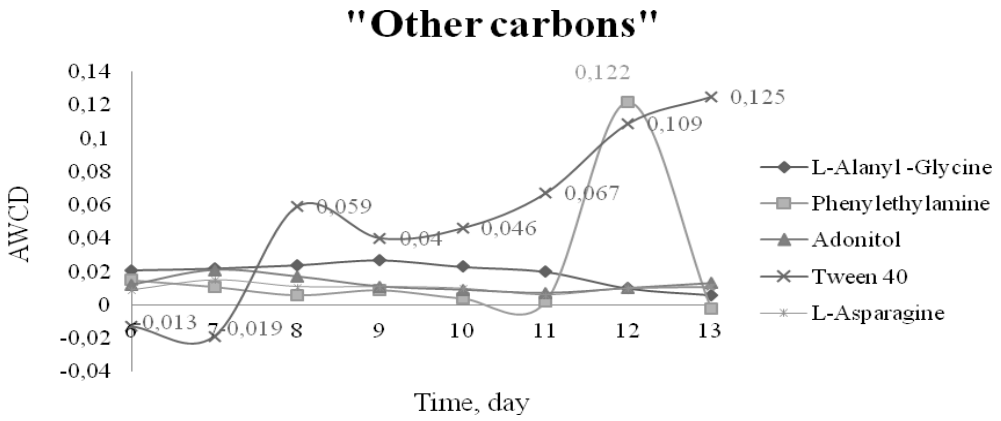

Figure 10 Most utilized compounds among Alcohols, Amines, Amides, Amino acids, Aromatic chemicals, phosphorylate chemicals and polymers substrates in the group of "other carbons". Data points are presented in AWCD.

3.3.4 Utilization of nitrogen sources

The highest average utilization rate of compouds (AWCD) was found for nitrogen compounds. On the other hand, the fungus was most selective in utilizing these compounds, as indcated by the lowest percentage of substrate utilization (26 utilized substrates out of 96, which coresponds to 27%; Table 5). Alike the last carbon group of "other carbones", the nitrogen groups did not have a lag phase, but exhibited an exponential phase from first day, reaching a plateau already on 3rd day and after that decreasing to a stationary phase that was slightly raised during the last days of the experimental period (Fig.11).

Several substrates were, however, actively utilized by fungus. Among those were: L-Tyrosine (0.133); Guanine (0,265); Glucuronamide (0,571); Ethylenediamine (0,215) (see Fig. 12). Negative utilization values were recorded for substrates such as: L-Alanine (-0,005); Glycine (-0,002); L-Citrulline (-0,004); L-Homoserine (-0,004); Ethanolamine (-0,005); Acetamide (-0,005); Thymine (-0,008).

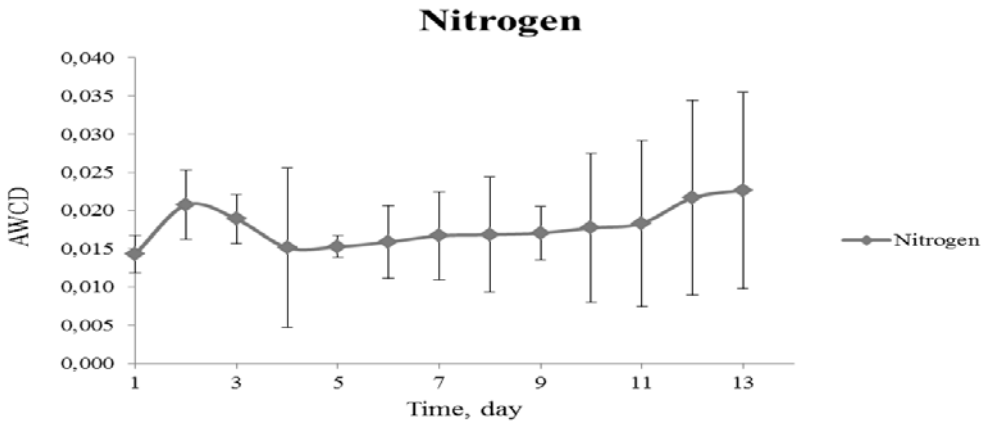

Figure 11 The utilization of Nitrogen compounds by R. udulata, with average development of all substrates in three plates , data points are presented in AWCD, during 13 days of examination. Vertical bars represent SD (n=3).

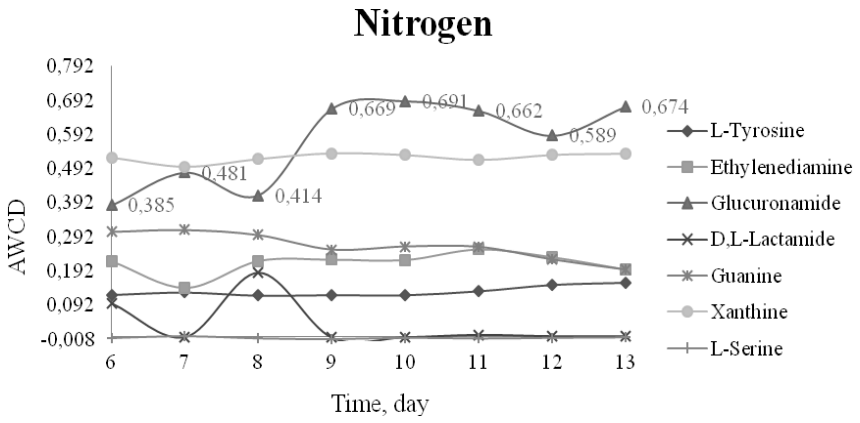

Figure 12 Most utilized compounds in Nitrogen group. Data points are presented in AWCD.

3.3.5 Utilization of phosphorus sources

The substrates in phosphorus group were utilized by the fungus at an intermediate with AWCD of 0,007, and overall compounds utilization of 47% (28/59). The active utilization started on the 6th day with rapid utilization of substrates during the next three days (7-9th day), and followed by a slow decline and fluctuation in absorbance afterwards (Fig.13).

In comparison to other substrate groups, only a few compounds in the phosphorus group were actively utilized. Those were : Triethyl Phosphate (0.025); Adenosine - 5' –monophosphate (0.155); Cytidine - 2' –monophosphate (0.037); Methylene Diphosphonic Acid (0.048). Some compounds showed deviating kinetics, for instance Adenosine - 5' –monophosphate had a negligeable rate of utilization up to the 7th day, reaching AWCD of 0,274 on 8th day (Fig. 14).

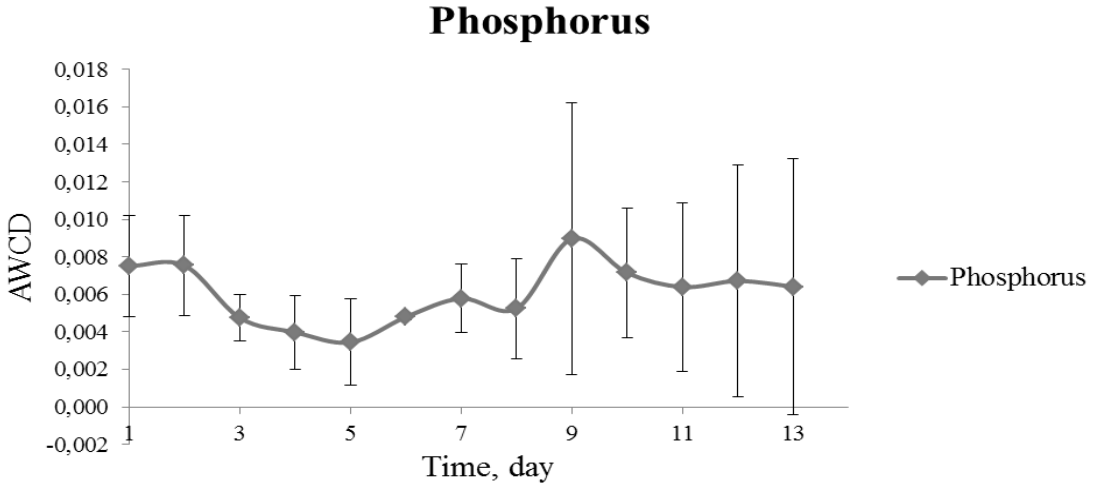

Figure 13 The utilization of phosphorus compounds by R. undulata, with average development of all substrates in three plates , data points are presented in AWCD, during 13 days of examination. Vertical bars represent SD (n=3).

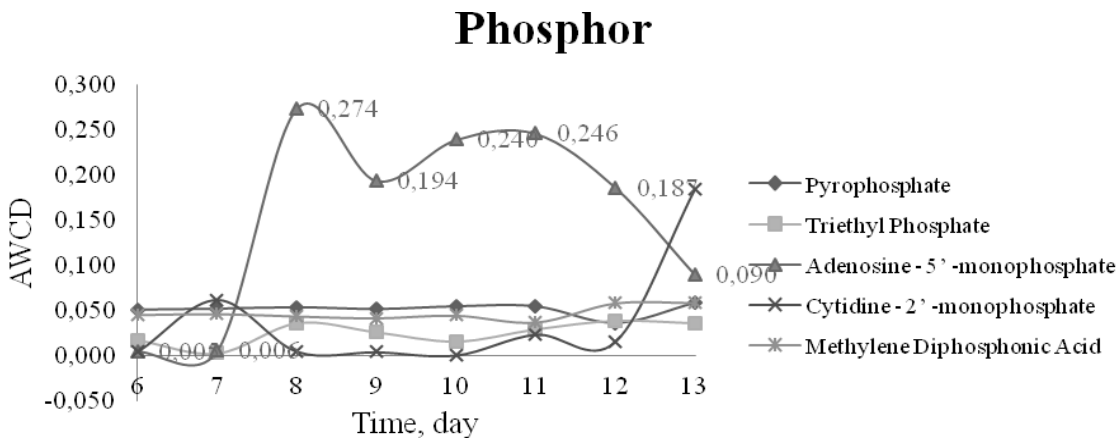

Figure 14 Most utilized compounds in Phosphor group. Data points are presented in AWCD.

3.3.6 Utilization of sulfur sources

The fungus started to utilize compounds belonging to the sulfur group from the beginning up to 4th day, peaking (with high variation) between day 6-7 (Fig.15). The group had an overall low utilization rate with an AWCD of 0,005, and proportion of utilized compounds 61% (21/38). The most actively utilized compounds among the utilized were: Cystathionin (0.016); Taurocholic Acid (0.051); D,L –Lipoamide (0.017) (Fig. 16), which influenced the observed trendin AWCD of this copound group (Fig. 16). The compounds with negative or no utilization, were Glycyl -L – Methionine (-0,001); D-Cysteine (-0,002); Tetramethylene Sulfone (0); L-Djenkolic Acid (0); N-Acetyl-D,L –Methionine (0).

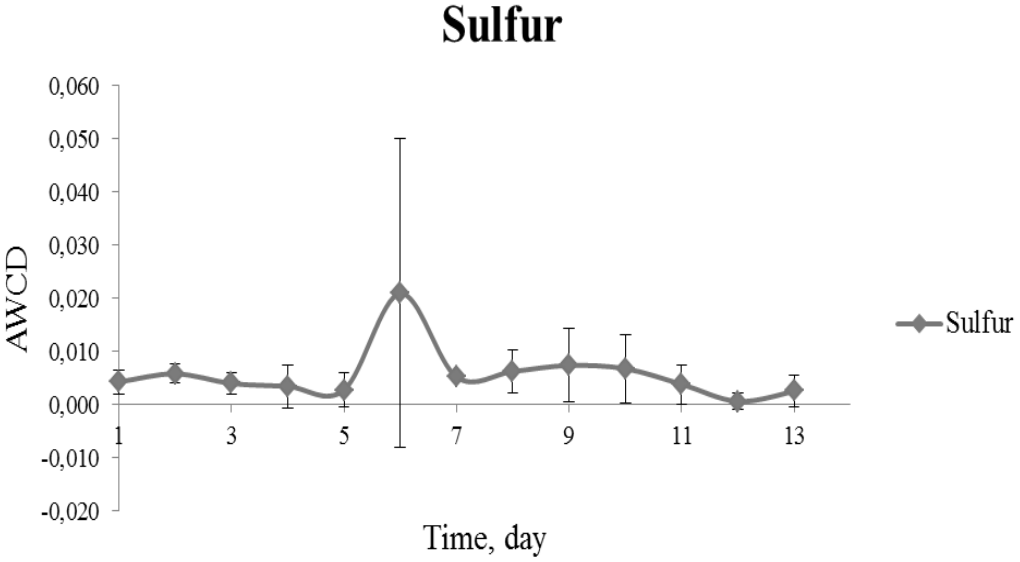

Figure 15 The utilization of Sulfur compounds by R. undulata, with average development of all substrates in three plates , data points are presented in AWCD, during 13 days of examination. Vertical bars represent SD (n=3).

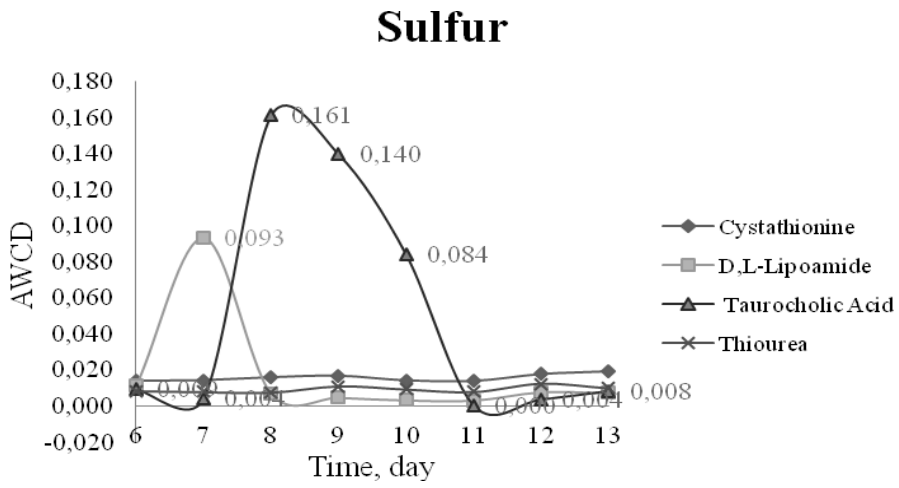

Figure 16 Most utilized compounds in Sulfur group. Data points are presented in AWCD.

4. DISCUSSION

Assay of fungal nutrient utilization using a Phenotype MicroArrays technique

To my knowledge, there have been no studies on the chemical sensitivity of *R. undulata* using the Phenotype Microarrays. Therefore, this study can be regarded as a pilot study against which future analyses of the ability of *R. undulata* strains to utilize nutrient sources can be developed.

The degradation of plant cell walls by filamentous fungi occurs by means of released extracellular proteins (enzymes) from hyphal tips of the fungus (Graem et al. 2011). The production of extracellular enzymes is an energy consuming process which is controlled by mechanism of carbon source dependent regulation of specific genes. For instance, activity of enzymes involved in carbon, nitrogen, phosphorus and sulfur metabolism, is controlled by a defined set of regulatory genes and specific metabolic repressors (Aro et al. 2005). This ensures that the fungal enzymes will be produced under condition when the fungus needs and is able to catabolize plant polymers as an energy source (Aro et al. 2005). In order to induce extracellular enzymes such as hemicellulases, cellulases, lignolases and pectinases it is needed to have preferable compounds which would derive from polymers and provoke enzymes induction (Aro et al. 2005).

The differential intensity of nutrient catabolism observed in my study is likely to reflect the presence or absence of specific degrading enzymes in *R. undulata* cells. For instance, the utilization of D-Galactose (0,021), with a high intensity in carbohydrate group, can indicate the presence of cellulotic enzymes. Norkrans & Hammarström (1963) studied a strain of *R. undulata* on a medium with filter paper cellulose and also found *R. undulata* to be an organism with cellulolytic ability. In the meanwhile, the degradation of hemicellulose is not so certain, as some constituting parts of xylan and glucomannan e.g. B-1,4 linked D-mannose (0,002) were not catabolized, opposite to B-1,4 linked D-xylose (0,022) which are constitute parts in the polymer backbone (Aro, et al. 2005). However, the overall low ability to utilize the substrates within carbohydrate group (see. section 3.4), indicates the absence of carbohydrate polymer degrading enzyme which is playing an essential role in degradation of the plant cell walls (Aro, et al.2005), thus, the low ability of deep penetration into woody tissue.

The root death of conifers does not occur due to the production of toxic substances in the rhizosphere by the fungus, but rather via its invasion of the root cell with the help of enzymes which dissolve the cell walls (Lee et al. 2005). The distinct feature of seedling roots penetrated by mycelium was a brown color (Lee et al. 2005), which support the presence of cellulases and indicate the lack of ligninases. The lack of ligninases can be also supported by the low ability to harm mature trees (Jalaluddin, 1967, b; Gremmen, 1971; Callan, 1993) opposite to seedling due to differences in amount of lignin in young and mature wood tissue. The commercially available PM arrays did not contain lignin or its direct precursor phenolics and thus my results do not further elucidate this aspect.

Most of the nitrogen-containing nutrients which were catabolised by the fungus were chemically related to each other. For instance the high utilization of Guanin (0,265), which is the main nucleobase compound, and Xanthine (0,525), which is the product on the pathway of purine degradation, indicates that the fungus produces Guanine deaminase enzyme which is needed to convert Guanine to Xanthine (Kalckar, 1947). Furthermore, the results suggest that *R. undulata* possesses an incomplete pathway of purine catabolism only up to urea and ammonia. First

compound in this pathway was Adenin which was not utilized by the fungus (0,000), indicating that the fungus lacks adenine deaminase and starts purine degradation from Guanine (0,256) to Xanthine (0,525). The fungus seems to be able to degrade Xanthine (0,525) to Uric acid (0,015) with the help of xanthine oxidase, but as the Allantoin (0,001) was not utilized, it appears that the fungus lacks other degrading enzymes in this chain (Koning & Diallinas, 2000). Furthermore, it can be supported with the neutral reaction on Ammonia (0,001) and Urea (-0,003). The incomplete pathway in purine degradation can lead to, in average, low amino acid utilization. Similarly, we can suppose that *R.undulata* has no transporter genes for allantonin-allantonic acid-urea-ammonia (Koning & Diallinas, 2000).

There was in general lack of catabolism induction by any of the acidic and basic amino acids, suggesting that the fungus does not use most of amino acids as an alternative nitrogen source. The only amino acid which was preferred by the fungus was L-Tyrosine (0,133). This could be a consequence of the inhibition of gluconeogenesis, which results in the production of glucose from non-carbohydrate carbon substrates such as glucogenic amino acids like L-Tyrosine (Thevelein, 1988). In our experiment, L-tryptophan was utilized on a low level, which is consistent with the results of Norkrans & Hammarström (1963) that the addition of malt extract, casein hydrolysate with tryptophan or different B vitamins had a neutral effect on *R. undulata* mycelium growth (App.3).

The last two sets of nutritional sources i.e. phosphorus and sulfur were utilized by the fungus on very low levels, although with some exceptions. The preference of phosphorus compounds Pyrophosphate (0,051), Adenosine-5-monophosphate (0,155), Methylene-diphosponic acid (0,048) indicates presence of specific enzymes ensuring nutrients catabolism of these nutrients, but no references were found for the tentative identity of these enzymes. Of the sulfur group compounds, most actively utilized was Taurocholic acid (0,051) whereas the product of the hydrolysis of this component, taurine (0,002), was not utilized. In addition, the fungus was not able to utilize two main sulfur base amino acids, which can also show the inability to degrade specific sulfur proteins. The lack of utilization of sulfur sources can be explained by the fact that the fungus does not have or have a weak regulatory protein i.e. CYS-3 (Marzluf, 1993), or it lacks evolutionary traces of this element in the environments in which fungus naturally occurs.

Implications of the substrate utilization patterns for *R. undulata* growth in nature are not straight-forward. First of all, more studies with a larger number of isolates are needed to validate these patterns. The growth possibilities of the fungus are limited by the nutrition availability in surrounding environments. If the nutrients are available in limited amount, the growth rate might be reduced, and in the case of *R. undulata* the depth of cell penetration, and thereafter the damage of seedlings will be lowered. The obtained results can be related to availability of macronutrients in soil rhizosphere, which becomes more available with enhanced seedlings root exudation (Meier et al. 2012) and increased supply of substrates for fast-turnover rhizosphere microorganisms (Phillips et al. 2012). The study of Meier et al. (2012) shows that presence of pathogenic fungus trigger exudation of organic compounds (oxalic acids, phytoalexins, proteins, and other yet unknown substances) from the seedling roots as exudates. It can explain the preference of examined carbon and nitrogen compounds, as well as appearance of fungus only in limited area with presence of coniferous roots. The low ability of the fungus to utilize phosphorus and sulfur compounds is in agreement with findings from other studies. Sulfur is a well-known product in plant protection and phosphite, a reduced form of phosphate, is marketed as either a fungicide, fertilizer or a biostimulant that promotes plant health and resistance (Dalio et al. 2014; Hoang Thi

Bich Thao & Takeo Yamakawa, 2009; Lamberth, 2003). Environmentally friendly doses and forms of phosphorus and sulphur might thus have potential in control of *R. undulata*.

Optimization of growing conditions

The present study revealed the difficulties in obtaining sexual or asexual spores from mycelial cultures of *R. undulata*. The findings of the current study are thus consistent with those of Jalaluddin (1967 b), who confirmed that in order to obtain apothecia development, it would be needed to grow the original spores of the fungus on non-specialized media. Similarly, the lack of conidial development agrees with the earlier results (Booth, 1972).

The study showed that the mycelium of *R. undulata* was capable of growing on a simple synthetic media without addition of any essential metabolite. It should be noted, however, that according to Norkrans & Hammarström (1963), the addition of hydrolyzed yeast nucleic acid positively affects mycelium growth rate. Hydrolysis of nucleic acids releases pentose sugars, phosphates and purines or pyrimidines. The latter two groups were, however, not preferred by R. undulata. Thus, the result of Norkrans & Hammarström (1963) could be explained by the high capacity of *R. undulata* to utilize pentose sugars (arabinose, ribose, xylose and lyxose; Table 5) which was found in my study.

The amount of aerial mycelium production on MEA, MEB, and OMA growing media was insufficient to use it in PM assay, but there were slight differences between the media. This feature of the fungus can indicate the lack or weakness of enzymes which help in adaptation to growth in atmospheric conditions (Carlile, 1994). This can be also be one explanation for the statement by Callan (1993), that silviculture measures such as trenching prevents vegetative spread of the fungus.

The observed development of cell aggregations (App. D) on OMA could be related to the selective nature of media. It is possible that these aggregates were primordial of apothecia, although this hypothesis needs to be further studied. OMA itself does not contain large amounts of added sugar and therefore it may promote development of sporulating structures at the expense of vegetative growth (Waller et al. 1998). MEA is a semi-synthetic media consisting of both natural ingredients and certain synthetic components. These include malt extract which contains compounds such as carbohydrates i.e. glucose (dextrose), maltose, and alcohol-glycerol, all of which were included into the Phenotype Microarrays in this study (see section 3.3). The difficulty in catabolism of disaccharides and alcohols and the preference of simple sugars, such as glucose, by *R.undulata* could be related to enzymatic specificity of the fungus and the lack of glycosyl hydrolases to degrade glycosidic bond (Aro, et al. 2005).

Hydrolysis of glycosidic bonds occurs with help of O-glycosyl hydrolases (EC 3.2.1.x). The lack of these enzymes can be identified with lactose intolerance (Gideon & Henrissat, 1995). The lack of lactose splitting enzyme in *R. undulata* has already been detected by Norkrans & Hammarström (1963). This was indicated also by the component utilization pattern in the PM assay, namely the low level of utilization of a-D-L-Lactose (0,002). In addition, the low level of utilization of two nucleophiles/bases i.e. aspartate and glutamate (L-Aspartic acid (0,000), D-aspartic acid (-0,001), L-Glutamic acid (-0,001), D-Glutamic acid (-0,001)), supports the explanation above, which is

based on the lack of glycosyl hydrolases, because only these residues have been found to perform it's catalysis (Dies & Henrissat, 1995).

It has been reported that the addition of simple sugars into growing media results in changes in metabolic pathway on the example of yeast *Saccharomyces cerevisiae* and ascomycete *Aspergillus nidulans* (Thevelein, 1988; Felenbok, et al. 2001). For instance, this can result in inhibition of gluconeogenesis, inhibition of galactose and maltose transport; activation of glycolysis and trehalose mobilization (Gadd, 1988; Thevelein, 1988). Hence, the available set of nutrients in malt extract may affect the ability of *R.undulata* to catabolise preferred substrates i.e. galactose and maltose, and, in addition, activation of non-favorable ones, such as trehalose. A repression of various genes (enzymes) needed for catabolism of carbon sources others than glucose is caused by the presence of easily metabolisable and energetically favorable glucose (Aro, et al. 2005). That could be a reason for the absence of cell aggregates on MEA and MEB media.

In contrast to the growth medium, the light cycle and temperature did not seem to affect the fungal growth patterns. However, during incubation period in steady temperatures of 25 ^{o}C (Norkrans & Hammarström, 1963) the vegetative growth of mycelium tended to increase. A slightly better growth of mycelium on MEB as compared to MEA may be explained by easier accessibility of nutrients from the liquid media. The tested conditions were not favorable for sexual or asexual sporulation of *R. undulata*. To optimize the culturing conditions, i.e. temperature, acidity of media, UV-light, different initial media, further studies are needed using a broad set of preferred/ not-preferred substrates and conditions, which should be tested with several different strains of *R.undulata*. The mix and variation of substrates in selective media and conditions can provide us with information about their effects on pathogenesis in filamentous fungi, as well as can give a possibility to test the ability of fungus to develop cell aggregations into apothecia in artificial conditions.

Protein assay

The current study aimed at screening the production of extracellular proteins of *R. undulata* under different conditions. This was of interest for two reasons. Firstly, majority of pathogenic microorganisms secrete proteinases which are involved in the infection process (Moore, 1998). Hence, the level of pathogenicity of the fungi is correlated with their extracellular proteinases activity (Archer and Wood, 1994; Have et al. 2002). Secondly, it was of interest from the experimental point of view: if the extracellular protein fraction could be used in Phenotype Microarrays instead of mycelial cells or spores, technical problems related to the heterogeneity of mycelial cells and spores could be avoided. The results indicate significant difference in ability to produce extracellular proteins (enzymes) by the two studied *R.undulata* strains (300.56; 301.56) ($p<0,05$). The distinction could be seen between the different and within similar tested conditions (fig.1,2,3). The excessive production of proteins in stable condition by slow growing strain 301.56 suggests that the more vigorous or fast growing strain of *R.undulata* 300.56 deposit more energy into other components of vegetative mycelium than proteins (Fig.3), while the less vigorous strain i.e. slow growing *R. undulata* strain 301.56 produce more proteins on the expense of mycelium growth.

In both conditions slow growing strain 301.56 demonstrated higher productions of proteins (see fig. 1, 2), while strain 300.56 failed to produce any proteins under agitation condition. This difference could be explained in part by the specificity of the tested condition, when under

agitation, mycelium aggregate into spherical balls, which could trap some proteins inside of them, and not release them into media. In stable condition mycelium did not aggregate into spherical balls but spread itself evenly in the medium. In the case of tested condition under agitation, the area of mycelium which was in direct contact with the liquid could be reduced. Thus, the initial amount of measured proteins would decrease. On other hand, this can be also explained by the fact that fast growing isolates are not often very effective catabolisers i.e. enzyme producers (Klepzig 1998).

The results may suggest that the strain 301.56 has greater pathogenity than the strain 300.56. That could be due to the slow growth of strain 301.56, thus, possibly lower mechanical pressure on woody tissue i.e. penetration of plant cuticle, but stronger enzymatic attack. To prove this, inoculation studies with a larger sample size would be necessary. Further studies should also be conducted to examine a higher number of *R. undulata* strains for their protein production, to characterize which fungal proteins can be found in the medium outside cells, and to test whether the protein fraction could replace the more heterogenous spores or mycelial cells in substrate-use experiments, such as Phenotype Microarrays. In general, high protein production may be suppressed by MEA. This medium is regarded as a neutral media for *R. undulata* growth, and therefore it might not support production of extracellular enzymes (Archer et al. 1995). Therefore, other initial media with presence of substrates that can induce regulatory genes should be used (Aro, et al. 2005)

In general, my results must be interpreted with caution because of the possibly unstable nature of proteins in liquid and the limitations of the method. The methodology which was used in this study was a basic protocol for protein determination, based on the absorbance of UV light at the wavelength of 280 nm. This absorbance is reflecting the residues of the aromatic amino acids tryptophan and tyrosine and by cysteine (Simonian, 2002), which might not dominate the extracellular fraction of *R. undulata*. Further improvement in the methodology of protein examination can be suggested. For instance, according to Kalb and Bernlohrn (1977), the wavelength of 230/260 nm can be used in order to decrease the dependency of changes in amino acid compounds of measured proteins.

Evaluation of the PM method

The study showed that PM method has capacity in characterizing the enzymatic potential of *R. undulata* strain. However, there are some weaknesses in the methodology of PM techniques regarding pH and temperature settings. As mentioned before, the enzyme induction is a complex process that demands specific environmental conditions. The pH range can influence the activity of induced enzymes, and temperature has a direct effect on mobility of enzymes in the media (Bommarius et al. 2008). Thus, the most adequate method of PM assay would be to sort all the nutrients using the relevant genes as a criterium. For example, the cellulose and polycellulases activity of the fungus in studies of Norkrans (1963) was detected to be higher in pH between 3.5-3.0. Thus, this is adequate condition for expression of these specific enzymes and for utilization of compounds. However, the acidity of the media in Biolog plates was around 5.5 pH, which may limit activity of some catabolitic enzymes. Furthermore, in cellulotic system of filamentous fungi *Trichoderma reesei* and *T. harzianum* the use of high temperatures accelerates rates of enzymatic reactions, lower bacterial contamination, while high temperature and inadequate pH conditions

will lead to enzymatic inactivation, thus low or absence of catatbolic processes (Colussi et al. 2012). The study of Colussi et al. (2012) shows that the exoglucanase activity of *Trichoderma (Th) Cel7A* increase in the range from pH 3 to 5, and decreases again from pH 5 to 7. While the activity of this enzyme was 76% below pH 3, at pH 7 the activity was zero. In addition, there were no changes in *Th Cel7A* within a range of 2-8 pH and under temperature of 25 ^{o}C (Colussi et al. 2012).

Conclusions

To summarize, the study indicated that the PM method provides novel possibilities to explore the functional enzymatic capacity of *R. undulata*. The results of this pilot test indicate e.g. that while the fungus seems to prefer nitrogen and carboxylic acids, it has limited capacity to consume sulfur and phosphorus. The implications of these results for practical forest protection are not straightforward, but for instance use of phosphite treatments as low-environmental effect plant protection agent, which may stimulate plant resistance and be of low value for the fungus, could be explored further. The tested conditions were not favorable for sexual or asexual sporulation of *R. undulata*. However, the observed cell aggregation on OMA media which could be an apothecia primordium should be studied further. In order to optimize culturing condition for the development of cell aggregates, the available set of preferred nutrients substrates from PM assay can be used. Additional experiment within protein assay could potentially point on the level of *R.undulata* pathogenicity, and its ability to degrade plant tissue. Possible suggestions towards improvements of the PM techniques would mainly concern pH and temperature settings.

In order to avoid seedling losses, burning in mature coniferous stands could be considered as a management option. Furthermore, this is more beneficial from the conservation point of view (Granström, 2001) and also might reduce the burden on the company since the FSC standard requires less burned hectares (by factor of 3) if areas with standing forest are burned (FSC, 2010).

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Appendices

Appendix A: Recipes.

All prepared media were sterilized in an autoclave at 121°C for 15min.

Malt Extract Agar (MEA)

	[g/l]
Malt extract	30
Agar	18
Aqua dest.	1000

Oatmean Agar (OMA)

	[g/l]
Oatmeal agar	75
Aqua dest.	1000

Malt Extract Broth (MEB)

	[g/l]
Malt extract	30
Aqua dest.	1000

Oatmeal Broth (OMB)

	[g/l]
Oatmeal flakes	30
Aqua dest.	1000

Appendix B: Substrate composition on the Phenotype Microarrays. Four types of plates PM1, PM3B, PM4A with 95 carbon sources (A2-H12) and a negative control (A1) for each plate were used (source: www.biolog.com).

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-D,L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

PM4A MicroPlate™ Phosphorus and Sulfur Sources

A1 Negative Control	A2 Phosphate	A3 Pyrophosphate	A4 Trimeta-phosphate	A5 Tripoly-phosphate	A6 Triethyl Phosphate	A7 Hypophosphite	A8 Adenosine- 2'-monophosphate	A9 Adenosine- 3'-monophosphate	A10 Adenosine- 5'-monophosphate	A11 Adenosine- 2',3'-cyclic monophosphate	A12 Adenosine- 3',5'-cyclic monophosphate
B1 Thiophosphate	B2 Dithiophosphate	B3 D,L-α-Glycerol Phosphate	B4 β-Glycerol Phosphate	B5 Carbamyl Phosphate	B6 D-2-Phospho-Glyceric Acid	B7 D-3-Phospho-Glyceric Acid	B8 Guanosine- 2'-monophosphate	B9 Guanosine- 3'-monophosphate	B10 Guanosine- 5'-monophosphate	B11 Guanosine- 2',3'-cyclic monophosphate	B12 Guanosine- 3',5'-cyclic monophosphate
C1 Phosphoenol Pyruvate	C2 Phospho-Glycolic Acid	C3 D-Glucose-1-Phosphate	C4 D-Glucose-6-Phosphate	C5 2-Deoxy-D-Glucose 6-Phosphate	C6 D-Glucosamine-6-Phosphate	C7 6-Phospho-Gluconic Acid	C8 Cytidine- 2'-monophosphate	C9 Cytidine- 3'-monophosphate	C10 Cytidine- 5'-monophosphate	C11 Cytidine- 2',3'-cyclic monophosphate	C12 Cytidine- 3',5'-cyclic monophosphate
D1 D-Mannose-1-Phosphate	D2 D-Mannose-6-Phosphate	D3 Cysteamine-S-Phosphate	D4 Phospho-L-Arginine	D5 O-Phospho-D-Serine	D6 O-Phospho-L-Serine	D7 O-Phospho-L-Threonine	D8 Uridine- 2'-monophosphate	D9 Uridine- 3'-monophosphate	D10 Uridine- 5'-monophosphate	D11 Uridine- 2',3'-cyclic monophosphate	D12 Uridine- 3',5'-cyclic monophosphate
E1 O-Phospho-D-Tyrosine	E2 O-Phospho-L-Tyrosine	E3 Phosphocreatine	E4 Phosphoryl Choline	E5 O-Phosphoryl-Ethanolamine	E6 Phosphono Acetic Acid	E7 2-Aminoethyl Phosphonic Acid	E8 Methylene Diphosphonic Acid	E9 Thymidine- 3'-monophosphate	E10 Thymidine- 5'-monophosphate	E11 Inositol Hexaphosphate	E12 Thymidine 3',5'- cyclic monophosphate
F1 Negative Control	F2 Sulfate	F3 Thiosulfate	F4 Tetrathionate	F5 Thiophosphate	F6 Dithiophosphate	F7 L-Cysteine	F8 D-Cysteine	F9 L-Cysteinyl-Glycine	F10 L-Cysteic Acid	F11 Cysteamine	F12 L-Cysteine Sulfinic Acid
G1 N-Acetyl-L-Cysteine	G2 S-Methyl-L-Cysteine	G3 Cystathionine	G4 Lanthionine	G5 Glutathione	G6 D,L-Ethionine	G7 L-Methionine	G8 D-Methionine	G9 Glycyl-L-Methionine	G10 N-Acetyl-D,L-Methionine	G11 L- Methionine Sulfoxide	G12 L-Methionine Sulfone
H1 L-Djenkolic Acid	H2 Thiourea	H3 1-Thio-β-D-Glucose	H4 D,L-Lipoamide	H5 Taurocholic Acid	H6 Taurine	H7 Hypotaurine	H8 p-Amino Benzene Sulfonic Acid	H9 Butane Sulfonic Acid	H10 2-Hydroxyethane Sulfonic Acid	H11 Methane Sulfonic Acid	H12 Tetramethylene Sulfone

Appendix C: The utilization rate of each individual nutrient and chemical compound in Phenotype Microarray plates (PM1, PM3B, PM4A).

PM1 Micro plate (Carbon sources)						
CARBOXYLIC ACIDS						
carbon source	**Well no.**	**Level of utilization**		**Carbon source**	**Well no.**	**Level of utilization**
D-Saccharic Acid	A4	0.005		Fumaric Acid	F5	0.024
Succinic Acid	A5	0.004		Propionic Acid	F7	0.007
D-Glucuronic Acid	B5	0.131		Mucic Acid	F8	0.01
D-Gluconic Acid	B6	0.008		Glycolic Acid	F9	0.012
L-Lactic Acid	B9	0.001		Glyoxylic Acid	F10	0.002
Formic Acid	B10	0.033		Tricarballylic Acid	G2	-0.004
D-Galactonic Acid-γ - Lactone	C2	-0.003		Acetoacetic Acid	G7	0.01
D,L-Malic Acid	C3	0.116		D-Malic Acid	G11	0.004
Acetic Acid	C8	0.009		L-Malic Acid	G12	0.006
D-Glucosaminic Acid	D3	-0.004		p-Hydroxy Phenyl Acetic Acid	H2	0.009
α-Keto -Glutaric Acid	D6	0.054		m-Hydroxy Phenyl Acetic Acid	H3	-0.003
α-Keto -Butyric Acid	D7	0.009		Pyruvic Acid	H8	-0.003
M-Tartaric Acid	E2	0.015		L-Galactonic Acid -γ - L actone	H9	0.003
α-Hydroxy Glutaric Acid- γ-Lactone	E6	0.004		D-Galacturonic Acid	H10	0.006
Citric Acid	F2	0.003				
CARBOHYDRATES						
L-Arabinose	A2	0.016		D-Melibiose	C11	0.002
N-Acetyl-D-Glucosamine	A3	0.009		α-Methyl –D-galactoside	D8	-0.002
D-Galactose	A6	0.021		α-D-L actose	D9	0.002

D-Trehalose	A10	0		Lactulose	D10	0.004
D-Mannose	A11	0.002		Sucrose	D11	0.003
D-Sorbitol	B2	0.011		β-Methyl-D-glucoside	E8	0
L-Fucose	B4	0.013		Maltotriose	E10	0.018
D-Xylose	B8	0.022		M-Inositol	F3	0.016
D-Mannitol	B11	0.002		D-Cellobiose	F11	0.001
D-Ribose	C4	0.021		N-Acetyl-β -D-Mannosamine	G8	0.009
L-Rhamnose	C6	0.006		Mono Methyl Succinate	G9	0.007
D-Fructose	C7	0.011		Methyl Pyruvate	G10	0.004
α-D-Glucose	C9	0.016		D-Psicos e	H5	0.016
Maltose	C10	-0.001		L-Lyxose	H6	0.019
OTHER CARBON SOURCES (Alcohols, Amines, Amides, Amino acids, Aromatic chemicals, phosphorylate chemicals and polymers)						
L-Aspartic Acid	A7	0.009		D-Fructose -6 - Phosphate	E4	0.003
L-Proline	A8	0.005		Tween 80	E5	-0.019
D-Alanine	A9	0.002		α-Hydroxy Butyric Acid	E7	0.008
Dulcitol	A12	0.002		Adonitol	E9	0.013
D-Serine	B1	0		2-Deoxy Adenosine	E11	-0.001
Glycerol	B3	0.002		Adenosine	E12	-0.001
D,L-α-Glycerol - Phosphate	B7	0.006		Glycyl -L -Aspartic Acid	F1	0.008
L-Glutamic Acid	B12	0.009		D-Threonine	F4	0.01
D-Glucose -6 -Phosphate	C1	0.006		Bromo Succinic Acid	F6	0
Tween 20	C5	-0.016		Inosine	F12	0.008
Thymidine	C12	0.008		Glycyl -L -Glutamic Acid	G1	0.001
L-Asparagine	D1	0.010		L-Serine	G3	0.009

D-Aspartic Acid	D2	0.006		L-Threonine	G4	0.003
1,2 -Propanediol	D4	-0.002		L-Alanine	G5	-0.001
Tween 40	D5	0.052		L-Alanyl -Glycine	G6	0.019
Uridine	D12	0.002		Glycyl -L -Proline	H1	-0.005
L-Glutamine	E1	-0.001		Tyramine	H4	0.002
D-Glucose -1 -Phosphate	E3	-0.005		Glucuron amide	H7	0.001
2-Aminoethanol	H12	0.008		Phenylethylamine	H11	0.021

PM3B Nitrogen sources

NITROGEN SOURCES

Negative	A1					
Ammonia	A2	0.001		β-Phenylethylamine	E2	0.000
Nitrite	A3	0.009		Tyramine	E3	0.003
Nitrate	A4	0.000		Acetamide	E4	-0.005
Urea	A5	-0.003		Formamide	E5	0.003
Biuret	A6	-0.002		Glucuronamide	E6	0.571
L-Alanine	A7	-0.005		D,L-Lactamide	E7	0.036
L-Arginine	A8	0.000		D-Glucosamine	E8	0.000
L-Asparagine	A9	-0.002		D-Galactosamine	E9	-0.003
L-Aspartic Acid	A10	0.000		D-Mannosamine	E10	0.002
L-Cysteine	A11	0.019		N-Acetyl-D-Glucosamine	E11	0.000
L-Glutamic Acid	A12	-0.001		N-Acetyl-D-Galactosamine	E12	0.001
L-Glutamine	B1	-0.001		N-Acetyl-D-Manno samine	F1	-0.003
Glycine	B2	-0.002		Adenine	F2	0.000
L-Histidine	B3	-0.001		Adenosine	F3	0.001
L- Isoleucine	B4	0.007		Cytidine	F4	-0.001
L-Leucine	B5	-0.003		Cytosine	F5	-0.003
L-Lysine	B6	0.000		Guanine	F6	0.265
L-Methionine	B7	0.000		Guanosine	F7	0.005

L-Phenylalanine	B8	-0.001		Thymine	F8	-0.008
L-Proline	B9	-0.003		Thymidine	F9	-0.004
L-Serine	B10	-0.003		Uracil	F10	0.000
L-Threonine	B11	-0.001		Uridine	F11	-0.002
L-Tryptophan	B12	0.007		Inosine	F12	0.000
L-Tyrosine	C1	0.133		Xanthine	G1	0.525
L-Valine	C2	-0.002		Xanthosine	G2	0.005
D-Alanine	C3	0.002		Uric Acid	G3	0.015
D-Asparagine	C4	-0.001		Alloxan	G4	-0.002
D-Aspartic Acid	C5	-0.001		Allantoin	G5	0.001
D-Glutamic Acid	C6	-0.001		Parabanic Acid	G6	0.003
D-Lysine	C7	0.010		D,L-α-Amino-N-Butyric Acid	G7	-0.004
D-Serine	C8	-0.001		γ-Amino-N-Butyric Acid	G8	-0.001
D-Valine	C9	-0.002		ε -Amino -N-Caproic Acid	G9	-0.001
L-Citrulline	C10	-0.004		D,L-α-Amino-Caprylic Acid	G10	0.027
L-Homoserine	C11	-0.004		δ-Amino -N-Valeric Acid	G11	0.001
L-Ornithine	C12	-0.002		α-Amino -N-Valeric Acid	G12	-0.005
N-Acetyl-D,L -Glutamic Acid	D1	0.002		Ala -Asp	H1	0.002
N-Phthaloyl-L-Glutamic Acid	D2	0.000		Ala -Gln	H2	-0.004
L-Pyroglutamic Acid	D3	0.000		Ala -Glu	H3	0.001
Hydroxylamine	D4	0.005		Ala -Gly	H4	-0.003
Methylamine	D5	0.004		Ala -His	H5	0.003
N-Amylamine	D6	0.002		Ala -L eu	H6	0.000
N-Butylamine	D7	0.006		Ala -Thr	H7	0.001
Ethylamine	D8	0.001		Gly -Asn	H8	-0.004
Ethanolamine	D9	-0.005		Gly -Gln	H9	-0.001
Ethylenediamine	D10	0.215		Gly -Glu	H10	0.004

Putrescine	D11	0.001		Gly -Met	H11	0.000
Agmatine	D12	0.001		Met -Ala	H12	0.001
Histamine	E1	0.001				

PM4A (Phosphorus and Sulfur sources)

PHOSPHORUS SOURCES

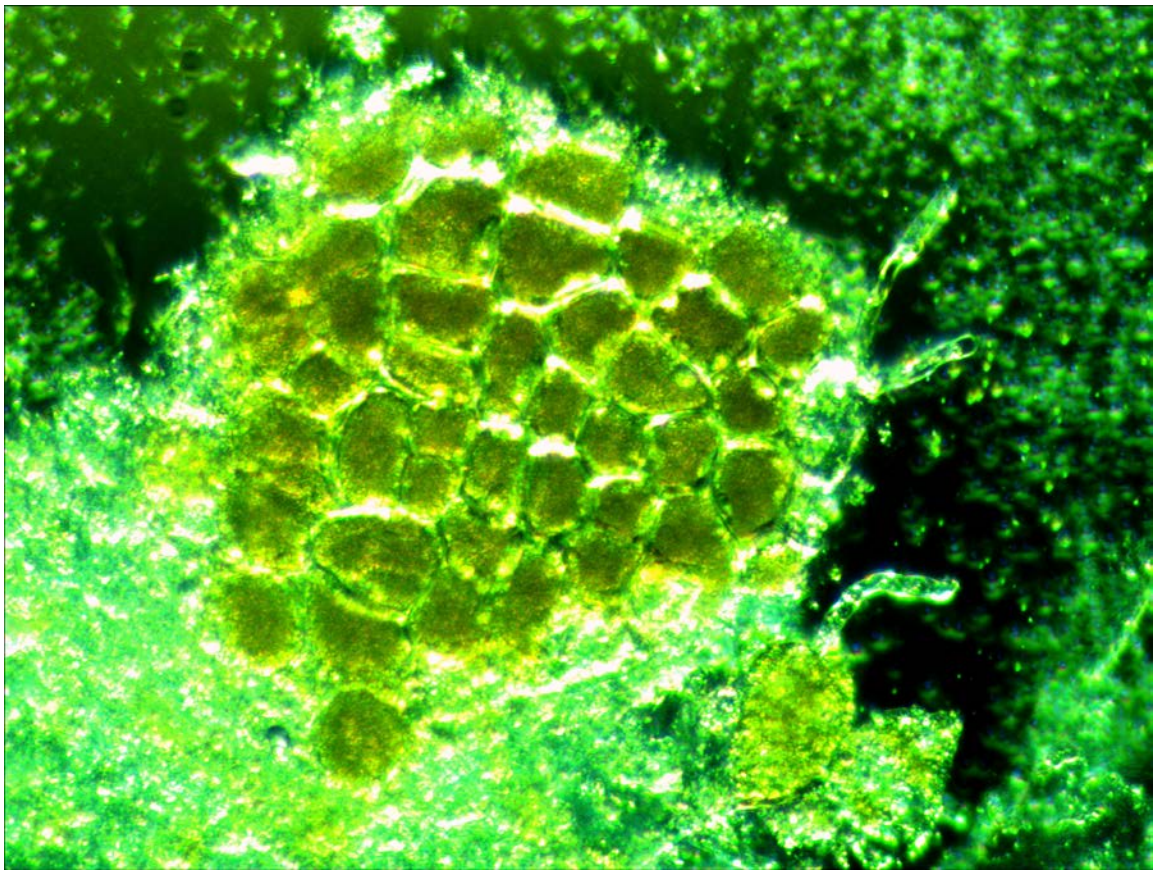
Negative	A1					
Phosphate	A2	-0.001		Cytidine - 2’ - monophosphate	C8	0.037
Pyrophosphate	A3	0.051		Cytidine - 3’ - monophosphate	C9	0.018
Trimetaphosphate	A4	-0.002		Cytidine - 5’ - monophosphate	C10	0.002
Tripolyphosphate	A5	0.010		Cytidine - 2’ ,3’ - cyclicmonophosphate	C11	0.000
Triethyl Phosphate	A6	0.025		Cytidine - 3’ ,5’ - cyclicmonophosphate	C12	-0.004
Hypophosphite	A7	-0.001		D-Mannose -1 -Phosphate	D1	0.009
Adenosine - 2’ - monophosphate	A8	-0.002		D-Mannose -6 -Phosphate	D2	0.002
Adenosine - 3’ - monophosphate	A9	-0.006		Cysteamine -S-Phosphate	D3	0.006
Adenosine - 5’ - monophosphate	A10	0.155		Phospho -L -Arginine	D4	0.000
Adenosine -2’,3’ - cyclicmonophosphate	A11	0.000		O-Phospho -DSerin e	D5	0.004
Adenosine -3’,5’ - cyclicmonophosphate	A12	0.000		O-Phospho -L -Serine	D6	0.001
Thiophosphate	B1	0.001		O-Phospho -L -Threonine	D7	0.001
Dithiophosphate	B2	0.003		Uridine - 2’ -monophosphate	D8	-0.001
D,L-α-Glycerol Phosphate	B3	0.000		Uridine - 3’ -monophosphate	D9	0.001
β-Glycerol Phosphate	B4	0.000		Uridine - 5’ -monophosphate	D10	0.007
Carbamyl Phosphate	B5	0.004		Uridine - 2’ ,3’- cyclicmonophosphate	D11	0.000

D-2-Phosph-Glyceric Acid	B6	0.001		Uridine - 3’ ,5’ - cyclicmonophosphate	D12	-0.002
D-3-Phospho-Glyceric Acid	B7	0.001		O-Phospho -Dtyrosine	E1	0.010
Guanosine-2’-monophosphate	B8	0.001		O-Phospho -L -Tyrosine	E2	0.004
Guanosine - 3’ - monophosphate	B9	-0.001		Phosphocreatine	E3	0.000
Guanosine - 5’ - monophosphate	B10	0.002		Phosphoryl Choline	E4	0.002
Guanosine -2’,3’ - cyclicmonophosphate	B11	0.001		O-Phosphoryl - Ethanolamine	E5	0.003
Guanosine -3’,5’ - cyclicmonophosphate	B12	0.006		Phosphono Acetic Acid	E6	0.001
Phosphoeno l Pyruvate	C1	0.002		2-Aminoethyl Phosphonic Acid	E7	-0.002
Phospho -Glycolic Acid	C2	-0.003		Methylene Diphosphonic Acid	E8	0.048
D-Glucose -1 -Phosphate	C3	0.007		Thymidine-3’-monophosphate	E9	0.000
D-Glucose -6 -Phosphate	C4	0.001		Thymidine-5’-monophosphate	E10	0.003
2-Deoxy-D-Glucose 6 - Phosphate	C5	0.002		Inositol Hexaphosphate	E11	0.002
SULFUR SOURCES						
D-Glucosamine-6-Phosphate	C6	0.004		Thymidine-3’,5’ - cyclicmonophosphate	E12	-0.002
6-Phospho -Gluconic Acid	C7	0.001		D-Methionine	G8	0.000
Negative	F1			Glycyl -L -Methionine	G9	-0.001
Sulfate	F2	0,007		N-Acetyl-D,L -Methionine	G10	0.000
Thiosulfate	F3	0.000		L- Methionine Sulfoxide	G11	0.003
Tetrathionate	F4	0.002		L-Methionine Sulfone	G12	-0.001
Thiophosphate	F5	0.002		L-Djenkolic Acid	H1	0.000
Dithiophosphate	F6	0.007		Thiourea	H2	0.009
L-Cysteine	F7	0.002		1-Thio -β -Dglucose	H3	0.000

D-Cysteine	F8	-0.002		D,L-Lipoamide	H4	0.017
L-Cysteinyl -Glycine	F9	0.001		Taurocholic Acid	H5	0.051
L-Cysteic Acid	F10	0.002		Taurine	H6	0.002
Cysteamine	F11	0.004		Hypotaurine	H7	0.001
L-Cysteine Sulfinic Acid	F12	0.003		p-Amino Benzene Sulfonic Acid	H8	0.001
N-Acetyl-Lcysteine	G1	0.006		Butane Sulfonic Acid	H9	0.002
S-Methyl -L -Cysteine	G2	0.005		2-Hydroxyethane Sulfonic Acid	H10	0.006
Cystathionine	G3	0.016		Methane Sulfonic Acid	H11	0.007
Lanthionine	G4	0.001		Tetramethylene Sulfone	H12	0.000
Glutathione	G5	0.004				
D,L-Ethionine	G6	0.002				
L-Methionine	G7	0.001				

Appendix D

Structures of *Rhizina undulata* under microscope



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