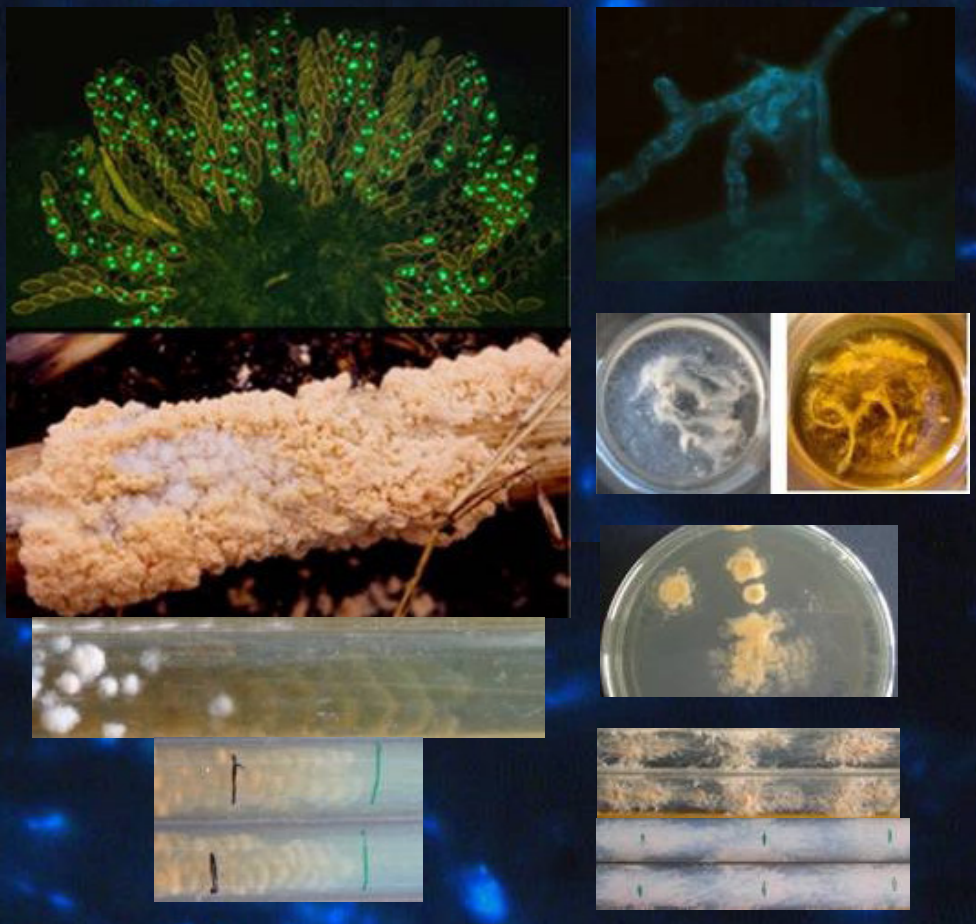
SORBOSE
AND
THE HYPHAL BRANCHING RHYTHM
IN *NEUROSPORA CRASSA*

THE INFLUENCE OF SORBOSE ON GROWTH, MORPHOLOGY, PERIOD AND TEMPERATURE COMPENSATION



MASTER THESIS 2008

HEIDI BRATTHAMMER

University of Stavanger

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Abstract

The glucose analogue sorbose has an interesting effect on the growth of the fungus *Neurospora crassa.* On sorbose *Neurospora crassa* wild type *(wt)* grows slower and with a marked difference in morphology. The appearance of a noncircadian hyphal branching rhythm with a lack of temperature compensation is a known effect of sorbose grown *wt Neurospora crassa*.

The aim of this study was to determine if previous results reported by Feldman on the phenomenon of the hyphal branching rhythm and its lack of temperature compensation, were reproducible. The hyphal branching rhythm is a specific rhythm which appears when *wt Neurospora crassa* goes into hypherbranching morphology. This results in seemingly timed "wave fronts" where the hyphal branching is most pronounced.

The hyphal branching rhythm is in sharp contrast to the free running, circadian and temperature compensated conidiation rhythm of *Neurospora crassa* in constant dark conditions (DD) and a constant temperature range between 18˚C and 32˚C. *Neurospora crassa `s* conidiation rhythm is the timed and rhythmic execution of spore formation which is visually observable and can be seen as a rhythmic formation of orange hives of spores in a glass tube. The conidiation rhythm disappears in constant light conditions (LL). The rhythm is governed by an internal genetic /transcriptional biological clock or oscillator where the key element is the gene *frequency* (*frq*) and its product, the protein FREQUENCY (FRQ).

In 1974 Feldman reported that the period of a *wt Neurospora crassa* strain grown on sorbose/sucrose media was poorly temperature compensated and not influenced by LL conditions. Feldman found that the period length of the hyphal branching rhythm increased from 21.1 h to 93.1 h over a range of constant temperatures from 30˚C to 20˚C. The control media without sorbose produced a steady conidiation rhythm with circadian properties.

The results from this thesis confirm Feldman's results that the hyphal branching rhythm is poorly temperature compensated and that the period length of the hyphal branching rhythm increase with decreasing constant temperature. Contributing to the hyphal branching rhythm are morphological changes, such as tight colonial growth, extensive hyphal branching, few aerial hyphae and sporadic conidiation. The hyphae of *Neurospora crassa* grown on sorbose

are shorter, thicker and more deformed than hyphae from wt *Neurospora crassa* growing on media without sorbose. This is thought to be the results of alteration in cell wall composition, mainly the depletion of β -1,3 -glucan in the cell wall.

The question whether the hyphal branching rhythm is a biological rhythm controlled by a circadian rhythm like that which is controlling the conidiation rhythm has been examined by using the knock out mutant *frq* ^{10} in DD conditions at 29˚C. On minimal media without sorbose *frq* ^{10} grew fast, and with normal mycelial spreading, but without rhythmic conidial bands. Grown on sorbose, *frq* ^{10} showed the hyphal branching rhythm as the *wt Neurospora crassa*. It therefore appears that the hyphal branching rhythm is independent of the *frq* gene and its products.

Acknowledgements

I would like to thank Professor Peter Ruoff for his guidance, support and knowledge during the work on this thesis. The enthusiasm and interest for the thesis have been of much help. I would also like to thank the lab staff especially Grete Falkeid for help and guidance in the laboratory, Ingunn W. Jolma for her assistance and help in the lab, and for the loan of her biological dictionary. I am also thankful to the staff and administration of the Faculty of Mathematical and Natural Science at the University of Stavanger for understanding and practical help in relation to my studies.

Finally I want to thank my mother Turid Bratthammer, my brother Stein-Ove Bratthammer, and the rest of my family, friends and colleagues for their patience, support, encouragement and motivation in the duration of my studies. Thank you for believing that I could do it!

"Correction does much,
but encouragement does more.
Encouragement after censure is as the sun after a shower."
Johann Wolfgang von Goethe 1749- 1832

Whether we wake or we sleep,
Whether we carol or weep,
The Sun with his Planets in chime,
Marketh the going of Time.
Edward Fitzgerald 1809 – 1883

CONTENTS

1. Introduction

The survival and fitness of an organism is dependent on its ability to adapt to the seasonal and daily changing environment that is caused by the rotation of this planet around the sun [1, 2]. Availability of light, temperature, nutrients and water are crucial for the organism to live and thrive. As well as adaptation to positive elements that helps the organism to live and grow. Adaptation to damaging elements is also important. There are many ways in which evolution have equipped organisms with the machinery to adapt to its changing environment. Biological clocks are an example of this [1, 3, 4].

Biological clocks are internal timekeepers that allow an organism to adapt to external cues as well as keeping its own time. This allows for economic use of resources like nutrients and water, and can also prevent damage to new spores as in the case of *Neurospora crassa* which sporulate in the early morning to prevent damage of new spores by heat and sunlight.

The importance of a well functioning biological clock is evident in medical and other research. Individuals that have altered or defect clock mechanisms are often less viable than individuals with well functional clocks. Human beings that suffer from psychosis, manic depressive disorder or other psychiatric disorders are often found to have a defect biological clock in regards to sleep. In general, sleep disorders and the uncomfortable effects of jet lag are examples of the importance of being able to adapt to changing environmental conditions such as light and darkness. Research have indicated that people that rise late and go to bed late as a normal rhythm are less affected by jet lag when they travel so that they lengthen their day.[1] In the North of Norway there are months where there are only a few hours of sunlight every day. This may lead to severe depression and problems with sleeping. This may be caused by effects by the absence of light on the circadian clock.

In this study, *Neurospora crassa wt* strain *328-4A* was used in race tube experiments and fluorescence microscopy to study the effects of sorbose on the hyphal branching rhythm, growth and morphology of *wt Neurospora crassa*. The main focus was to investigate the claim by Feldman that the sorbose induced hyphal branching rhythm in *wt* is not circadian and not temperature compensated [5], and to record the difference between the circadian free

[1] http://www.helsenett.no/reisemedwap/reisemedp11.shtml

running conidiation rhythm and the hyphal branching rhythm of *Neurospora crassa wt* in constant dark conditions.

An *frq* knock out mutant (*frq*^{10} *7490A*) was used to test the dependence of the sorbose induced hyphal branching rhythm on the *frq* gene and its protein FRQ. The knock out mutant also produced the hyphal branching rhythm in sorbose containing medium in DD conditions and constant temperature 30˚C whereas the *frq*^{10} control grew normally but without conidial banding in Vogel minimal control media under the same light and temperature conditions.

Sorbose causes colonial growth and morphological changes in *wt Neurospora crassa*. A distinct rhythm of hypherbranching hyphae is also induced by sorbose. The rhythm called the hyphal branching rhythm is proved to be non-circadian and not temperature compensated by the work of Feldman in 1974 [5]. This is confirmed in this thesis. Results in this thesis also points to the possibility that the hyphal branching rhythm is independent of a functional FRQ protein because the rhythm persists in a *frq* knock out mutant.

The possible causes of the sorbose induced hyphal branching rhythm, its loss of temperature compensation, the possible independence of the hyphal branching rhythm on the FRQ oscillator and the classification of the hyphal branching rhythm as a true biological rhythm where investigated by literature research and not by scientific experiments. Calculations relating to temperature compensation have not been conducted in this study.

In this thesis, printed literature is sited in brackets [ ] and are listed in the bibliography section. Literature online, like web pages are sited in superscript and are listed as foot notes on the bottom of each page.

1.1 Model organisms

Model organisms are as the name implies models. A model organism has a genetic and biological system that is easily studied and also closely related to other more complex organisms. This mean that studies of biological and genetic mechanisms that are difficult to study in a complex organism as i.e. humans may be better understood by studying a relatively closely related model organism like mouse or fungus. Fig. 1.1-1 gives examples of some of the most widely used model organisms.

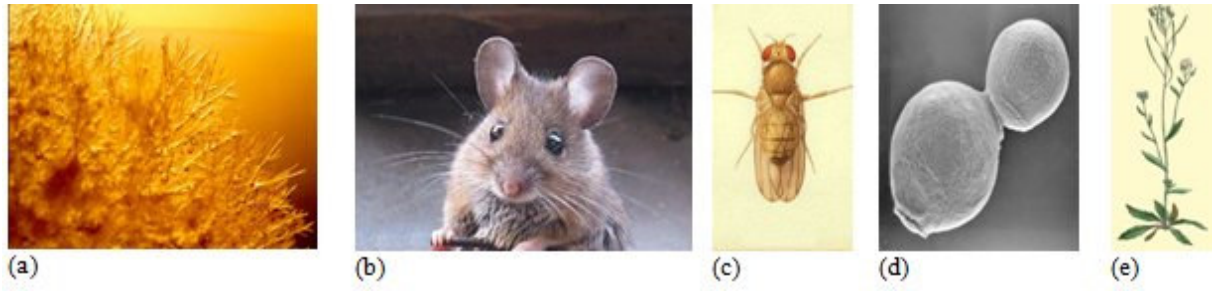


Fig 1.1-1: The most widely used model organisms. (a) *Neurospora crassa*, filamentous fungus. (b) *Mus musculus*, common house mouse. (c) *Drosophilia*, fruit fly. (d) *Saccharomyces cerevisiae*, yeast. (e) *Arabidopsis thaliana,* thale cress.

In the field of chronobiology, the field of study that deals with biological clock systems, the first organisms used in biological clock research was eukaryotes such as *Drosophilia* (fruit fly) which have been used since the 1930s and have been an important contribution to the knowledge obtained about biological clocks. The cyanobacteria *Synechococcus* was the first prokaryote used as a model in circadian system research.

It is not only in the field of chronobiology that model organisms are of great importance. Model organisms can be used to map metabolic pathways and possible roles of genes in these pathways. In medical research *Mus musculus* (the common house mouse) is often used to study effects of drugs and genetic responses. Fungi are also extensively used in the research of antifungal drugs. The plant *Arabidopsis thaliana* is used as a model organism for molecular reactions and biological systems in plants.

1.1.1 *Neurospora crassa*, a model organism

Neurospora crassa is a novel model organism. *Neurospora crassa* is a eukaryote and is relatively closely related to humans. Research on *Neurospora crassa* and other fungi may reveal helpful knowledge of human biological and genetic mechanisms. *Neurospora crassa* was used earlier in study of intermediary metabolism. Pittendrigh studied the rhythmic growth of *Neurospora crassa* on the amino acid proline. This lead to the belief that there was a possibility that the fungus contained a biological clock. Pittendrigh discovered that the rhythm were temperature compensated and therefore a true circadian rhythm [6].

E.L. Tatum and Jerry F. Feldman have used *Neurospora crassa* as a model organism since the 50s and 60s. Tatum has studied sorbose transport, accumulation and metabolism in general in *Neurospora crassa* as well as the circadian system of conidiation [7-10]. Tatum won half of the Nobel price in Physiology or Medicine in 1958 for his work on genetic research with *Neurospora crassa* [2]. Feldman have studied the genetic machinery of circadian rhythms and the hyphal branching rhythm in *Neurospora crassa* [11, 12].

Neurospora crassa is non pathological, which means that it is not dangerous and do not cause illness, a trait that is advantageous when using it in microbiological studies. Other advantages are that *Neurospora crassa* is easily grown in the laboratory grows rapidly, and is easily manipulated genetically because of its haploid life cycle.

[2] http://nobelprize.org/nobel_prizes/medicine/laureates/1958/tatum-bio.html

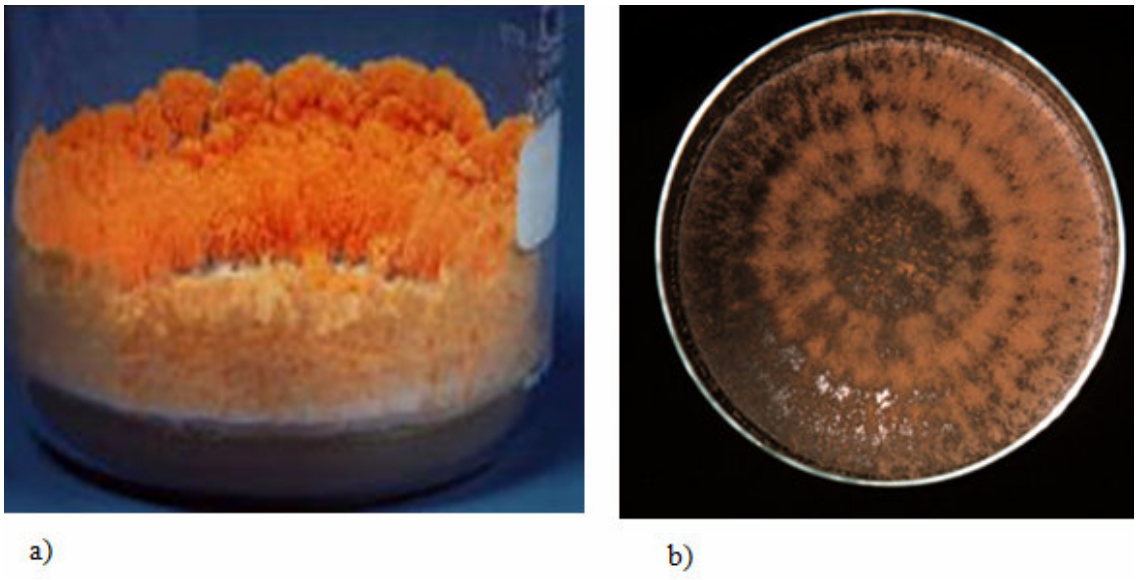


Fig 1.1-2: *Neurospora crassa* grown in the laboratory a) Grown on solid media in a beaker [3] b) on a petri dish on solid agar media [4].

Neurospora crassa is often grown in long glass tubes called race tubes. This is an easy way of assaying the conidiation rhythm and growth rate of the fungus (Fig 1.1-3).

Strain	Growth Rate (cm/day)	Period (hrs)
1858	3.6	22.1
2225	7.8	ND
2225 tfm10	2.7	22.4

Fig 1.1-3: Various *Neurospora crassa* mutant strains in race tubes containing 1 x Vogel medium N, 0.1% glucose, 0.17% arginine, and 1.5% agar in DD conditions in 30 ˚C [5].

[3] http://www.mmb.usyd.edu.au/mackay/selfdir2.php?goToLink=researchinterests.imag
[4] http://www.ux.uis.no/~ruoff/Neurospora_Rhythm.html
[5] http://www.fgsc.net/fgn53/beasley/fgn53beasley.htm

Neurospora crassa is a bread mold. Its orange/red spores, or conidia are clearly visible on infected bread products. *Neurospora crassa* is very adaptable and survives in many environments it can live and grow on many different nutrients that other organisms are unable to use.

Neurospora crassa can live and grow on dead organic material such as its own dead remains and also burnt material after forest fires.

Fig 1.1-4: *Neurospora crassa* growing on a tree after a forest fire [6].

The entire *Neurospora crassa* genome has been sequenced and consists of 43mb of DNA on 7 chromosomes. A base pair content of 54 % guanine /cytosine (G/C) content makes it very stable for primer construction[7]. *Neurospora* has more than twice as many genes as *S. cerevisiae*. Over 50% of the expressed *Neurospora* genes lack identifiable homologues in any organism, and only about 33% have homologues in *S. cerevisiae*. The genome of *Arabidopsis thaliana* contains approximately 120mb of DNA encoding ≈ 25000 genes. Average gene length is ≈ 2000bp. The human genome is estimated to be 2.91 billion bp.

[6] http://www.flickr.com/photos/lycogala/167259390/
[7] http://www.broad.mit.edu/annotation/genome/neurospora/neurospora.html

1.2 The biology of Neurospora crassa

Neurospora crassa consist of three main cell types: Hyphae, conidia and ascospores. Hyphae are vegetative cells meaning they are not reproductive cells. Conidia are the asexual reproductive cells or spores and ascospores are the sexual spores of the organism.

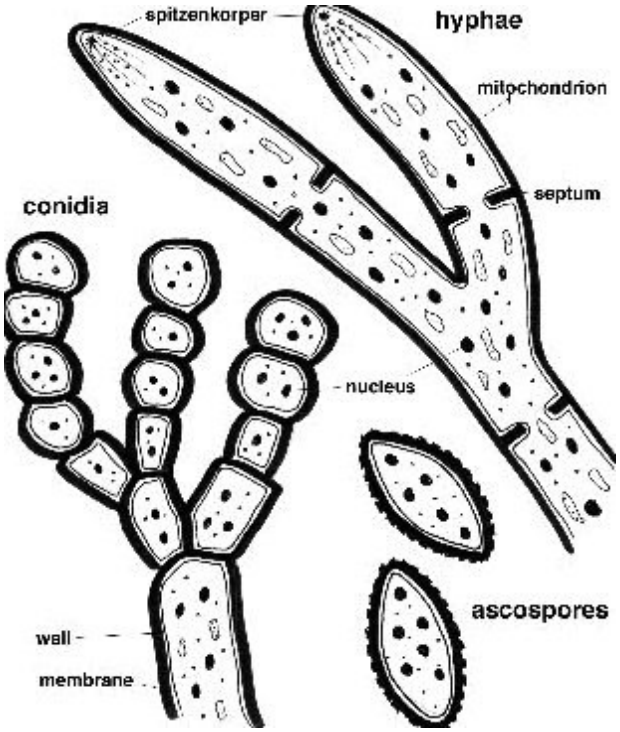


Fig 1.2-1: The three main cell types of *Neurospora crassa*[8].

Biologically *Neurospora crassa* belongs to a family called *Ascomycetes*. The name comes from the term **ascus** from ascospores, the membranous sacs where sexual spores are produced, and **mycetes (mycota)** from the network of fungal cells called mycelium. *Ascomycetes* are haploid and produce two kinds of spores; each is produced depending on which life cycle is induced.

Ascospores are produced following sexual reproduction and require the presence of and contact between two different mating types of *Neurospora crassa.* The mating type is determined by the mating type region ***Mat A*** and ***Mat-a*** in specialized zones of the *Neurospora crassa* genome.

[8] http://www.fgsc.net/Neurospora/sectionB3.htm

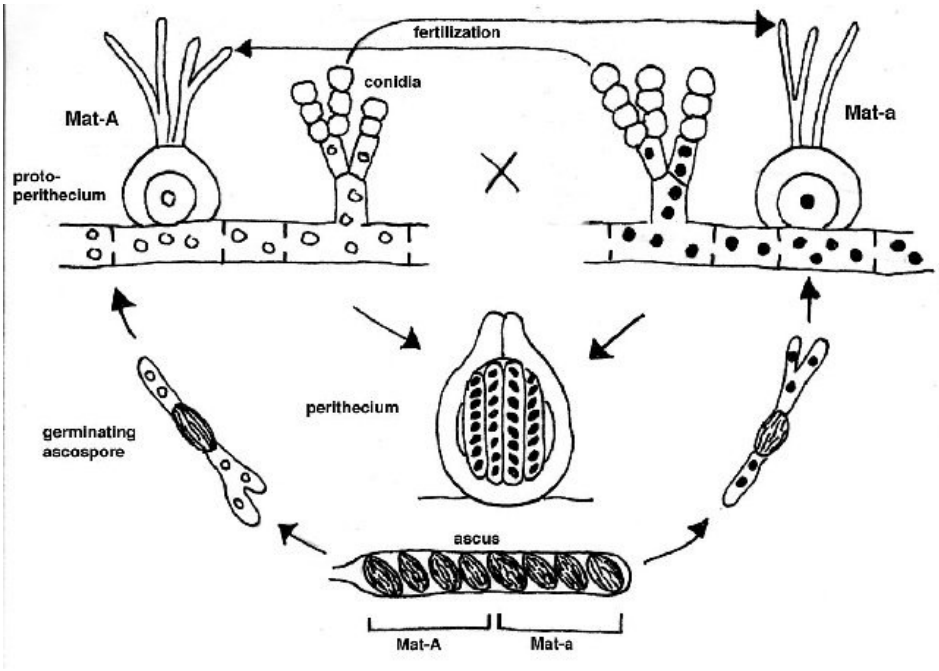


Fig 1.2-2: The mating of two mating types and sexual production of ascospores [9].

Neurospora crassa can produce two types of asexual spores, macroconidia and microconidia. Normally *wt Neurospora crassa* –strains in a laboratory produce macroconidia in abundance. Macroconidia are orange spores with two or more nuclei. Conidia are produced by budding from the tips of specialized aerial hyphae [6, 13, 14]. Microconidia are have one single nucleus and are produced as single spores from vegetative hyphae. Microconidia are produced in late stationary –phase agar cultures and because of poor viability; they are not very useful in study of developmental processes [6, 13, 14]

There are three distinct phases of the asexual life cycle [14]

1. Conidial germination
2. Vegetative growth
3. Conidiation

[9] http://www.fgsc.net/Neurospora/sectionB2.htm

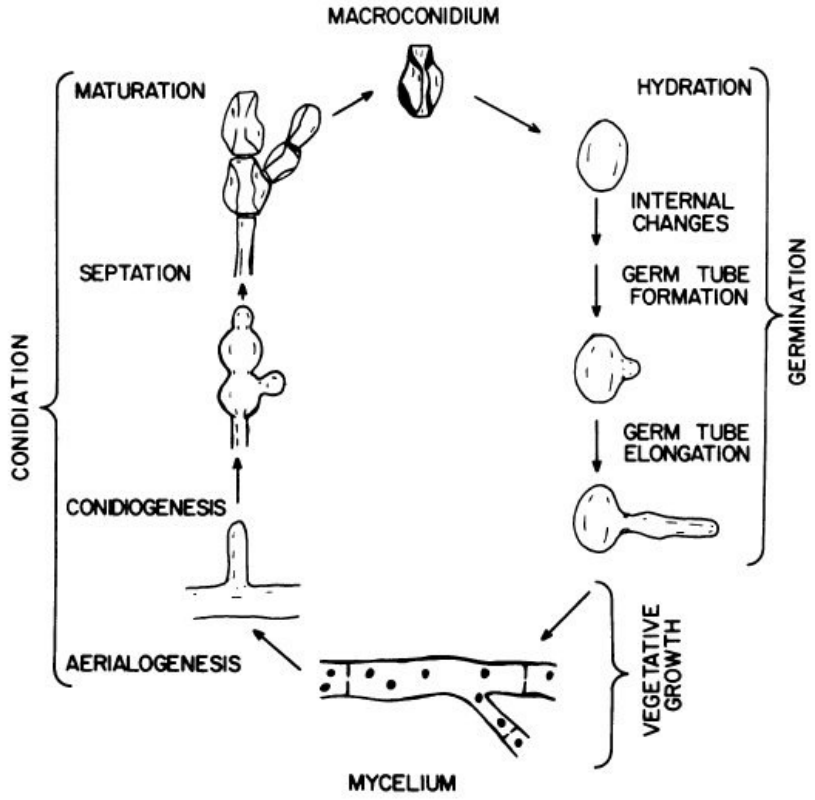


Fig 1.2-3: The asexual life cycle of *Neurospora crassa* from germination to mature conidia [14].

Conidial germination

Conidial germination is the waking up of dormant spores and results in young hyphae (germ tubes) growing out of the spores and the start of vegetative growth of mycelium. Inside the spores (conidia) are reservoirs of energy and nutrient sources that will be metabolized and used as soon as germination goes forth. To germinate, the spores require water and the loss of the hydrophobic layer and solubility of exocellular enzymes. The uptake of water is termed hydration and does not seem to require metabolic energy. The internal metabolic processes that occur for the emergence of the new hyphae from the germ tubes require energy in the form of exogenous carbon and some inorganic salts [4, 14].

Vegetative growth

Once the new hyphae (germ tubes) have left the spore, they begin the phase of vegetative growth. The new growing hyphae form a network that grows on the surface of the media and

extend by apical growth and occasionally branch [14]. Apical growth and branching will be handled in later chapters in this thesis.

The process of conidiation begins with the differentiation of vegetative hyphae into aerial hyphae that grows into the air away from the media. The aerial hyphae branch and macroconidia are formed by budding from the tips of the aerial hyphae. After the first conidium is formed, new buds are formed after it. This results in long chains of buds or connected conidia. Finally the buds are separated by the forming of complete cell-walls between them and they become separate cells that eventually mature and become spores that contain material for a new cycle. These cells or spores are loose and light and can be dispersed by movement or a light wind in nature. When the spores are again introduces to a suitable environment, the conidia germinate again and a new life cycle begins [6, 14].

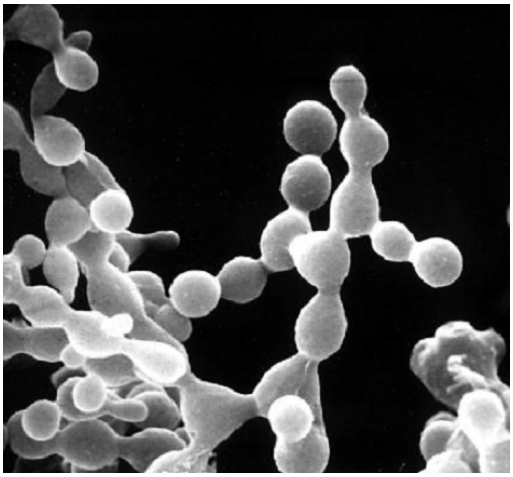


Fig 1.2-4: Electron microscope representation of *Neurospora crassa* macroconidia. Image by Bodil Aase.

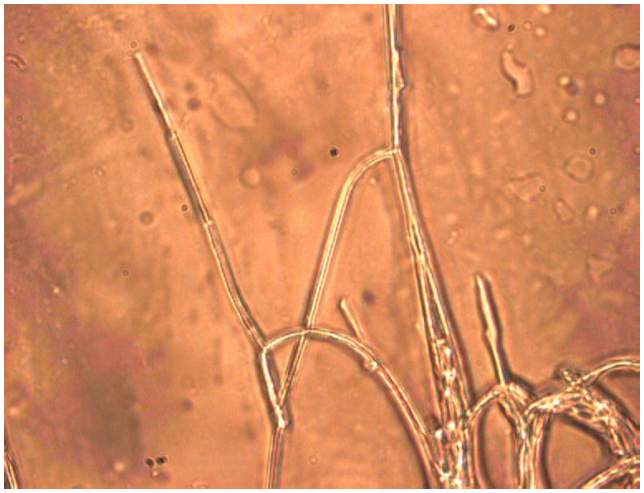


Fig 1.2-5: Light microscope representation on *Neurospora crassa* hyphae. Image by the author.

1.2.1 Hyphal growth and morphology

Hyphae of *Neurospora crassa* grow at the tip and in a straight line. This is called apical or polarized growth. Apical growth allows the fungus to grow into fresh zones of substrate[10].

[10] http://www.biology.ed.ac.uk/research/groups/jdeacon/microbes/apical.htm

The synthesis of new cell wall is only preformed at the growing tip. The hyphae grow and elongate for a while and then eventually branch out to form mycelium. A hypha is surrounded by a rigid cell wall consisting mainly of chitin and β -1,3 -glucan. Apart from multiple nuclei inside the cell, organelles like mitochondria, Golgi apparatus or dictosyme, endoplasmic reticulum appear in zones behind the tip growth zone [4, 13].

As the hypha grows, incomplete cell walls form pores to separate the new portion of the hypha from the old part. These pores, called septa allow cytoplasm, vesicles and other material to pass trough the pores to the growing tip. If the hypha bursts, the septa become plugged to prevent further damage to the remaining hypha [4, 6, 15]. The septa and cell wall of *Neurospora crassa* can be visualized by using the fluorescent dye calcofluor, a substance which bind to the chitin in cell walls and septa, and fluorescence blue under a fluorescent light. If the dye used is DAPI, a substance that fluorescence blue when it binds to DNA, the nuclei inside the hypha is seen as blue dots. In this thesis experiments with calcofluor dye was only conducted on *wt Neurospora crassa* in Vogel control media without sorbose (results not shown except in Fig 1.2-6. In this thesis only DAPI dye method was used to compare *wt Neurospora crassa* grown with or without sorbose.

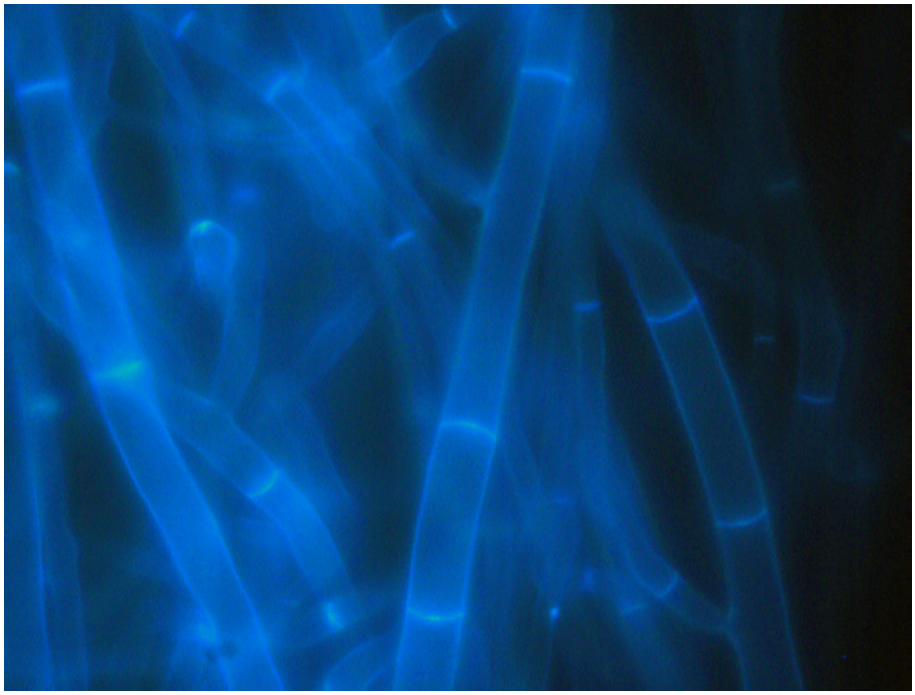


Fig 1.2-6: ***Neurospora crassa*** **hyphae dyed with the fluorescent dye calcofluor. The blue stripes across the hyphae are the septa. Image by the author.**

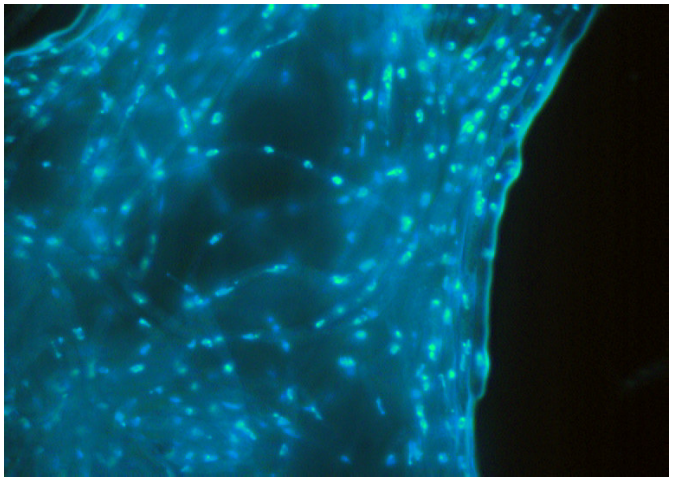


Fig 1.2-7: Microscopic fluorescence photograph of ***Neurospora crassa*** **cultured in liquid media. Mycelium containing hyphae dyed with DAPI.. The blue dots are the nuclei. Image by the author.**

It has proved difficult to use both dyes at once. This may be due to the fact that they both fluorescence blue and mask each other and/or that the dyes may react with each other.

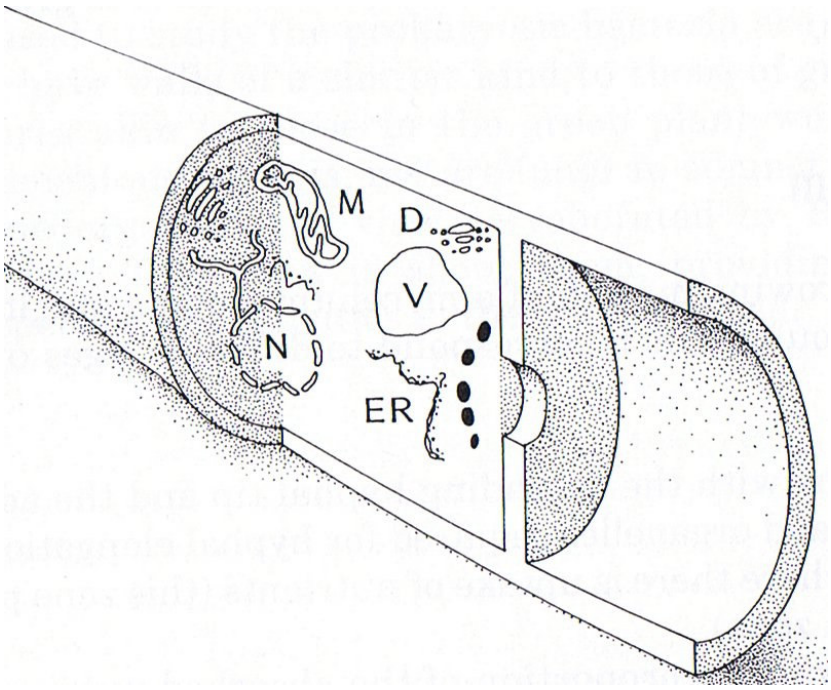


Fig 1.2-8: **Cross section of a *Neurospora crassa* hypha showing the different organelles cell are endoplasmic reticulum, dictosyme or Golgi apparatus, vacuole, mitochondrion and Wornin bodies which are filled with proteins that can block the septa pore in case of rupture** [4]**.**

As the hypha grow and ages, specialized zones appear [4]. These zones are named after the primary function of each zone. The zones are as follows

1. The growth zone
2. The absorption zone
3. The storage zone
4. The senescence zone (the oldest zone)

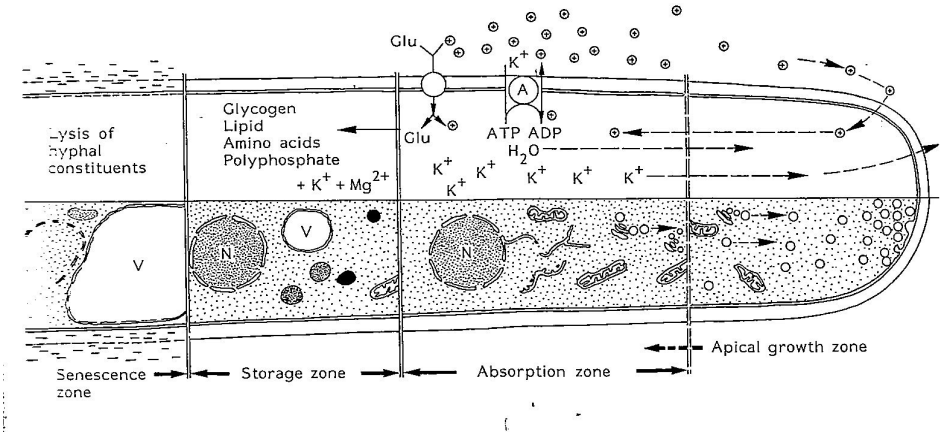


Fig 1.2-9: Representation of the growth zones of the *Neurospora crassa* hyphae [4].

The growth zone

The growth zone is where the machinery and mechanisms for growth appear. An important part of apical growth is the synthesis of new cell wall. This will be discussed later in this thesis. There are very few organelles, and no nuclei near the growing tip. Embedded in actin microfilaments, is the Spitzenkôrper, a vesicle supply center that supply the materials for cell wall synthesis, enzymes, nutrients and energy required for hyphal elongation [6]. Proteins that are involved in the mechanism and maintenance of the polar growth are; a regulatory subunit of cAMP dependent protein kinase, encoded by the *cot-1* gene [16-18], the catalytic subunit of calcineurin encoded by *cna-1* [19, 20] and protein phosphatase 2A encoded by *pph-1* [21].

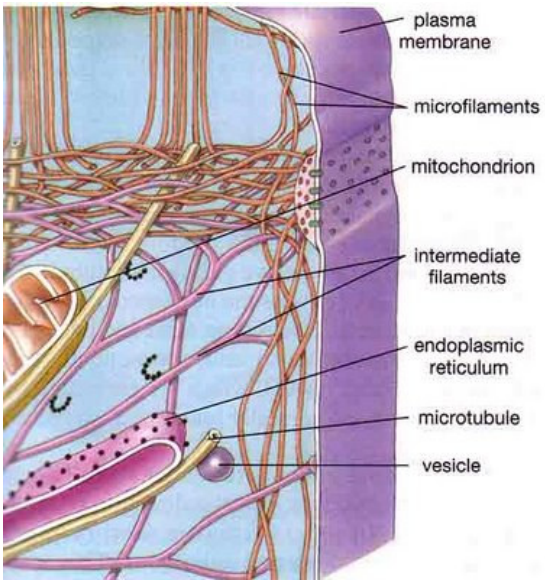


Fig 1.2-10: The organization of the cytoskeleton [11].

Hyphal elongation, the lengthening of the hyphae is thought to be regulated by an internal tip high generation of a Ca^{2+} gradient that is part of a Ca^{2+} -cAMP signaling pathway. The Ca^{2+} gradient is maintained and regulated partly by Ca^{2+} permeable channels, Ca^{2+} pumps, and Ca^{2+} exchangers, P-type ATPases and the uptake of Ca^{2+} into vacuoles [6, 19].The Ca^{2+} gradient itself is thought not to be essential for growth, but is important for apical domination. Apical domination means that elongation is dominating. The protein calcineurin is responsible for regulation of the steep a Ca^{2+} gradient and depletion of calcineurin results in growth arrest and is considered vital to hyphal elongation and growth [20]. cAMP induces the activation of cAMP dependent protein kinase subunits which is involved in the organization of the actin cytoskeleton involved in polar growth. Mutations in cAMP dependent protein kinase alleles results in colonial morphology, and cAMP deficiency is known to result in excessive branching [6].

In the growth zone specialized enzymes are released to the outside via the growing tip. These enzymes are capable of making available substances and substrates that usually are not readily available to the growing fungus [4]. The preferred carbon source for *Neurospora crassa* is the monosaccharide glucose. In the laboratory, the disaccharide sucrose is most often used as the carbon source. Sucrose is also an important ingredient in bread, which *Neurospora crassa* can grow on. Sucrose is made up by units of glucose and fructose. In order for *Neurospora crassa*

[11] http://scienceblogs.com/clock/2006/11/cell_structure.php

to get to the glucose in the sucrose, the sucrose must be broken into its monosaccharide parts. This is done by the enzyme invertase [6]. After the sucrose in broken into fructose and glucose the monosaccharides are transported into the interior of the cell in the absorption zone [6].

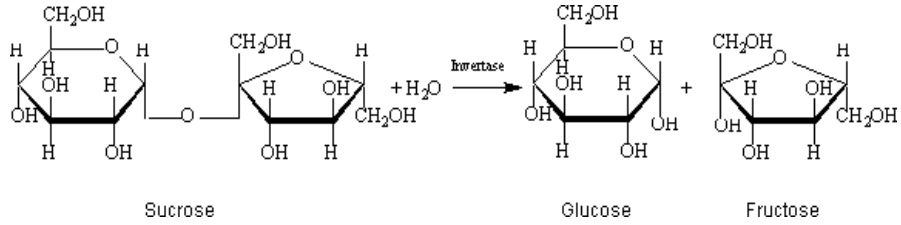


Fig 1.2-11: The enzyme invertase hydrolyses sucrose to glucose and fructose[12]

The absorption zone

The absorption zone is located behind the growth zone. Uptake of nutrients and water is the primary function of this zone. The absorption of the nutrients from the external medium is achieved mostly by the activity of a proton extrusion pump that is anchored in the plasma membrane. This pump is an ATPase which uses the energy molecule ATP supplied by mitochondrial ATP synthase [4, 6, 13].

[12] http://www.lsbu.ac.uk/biology/enzyme/practical1.html

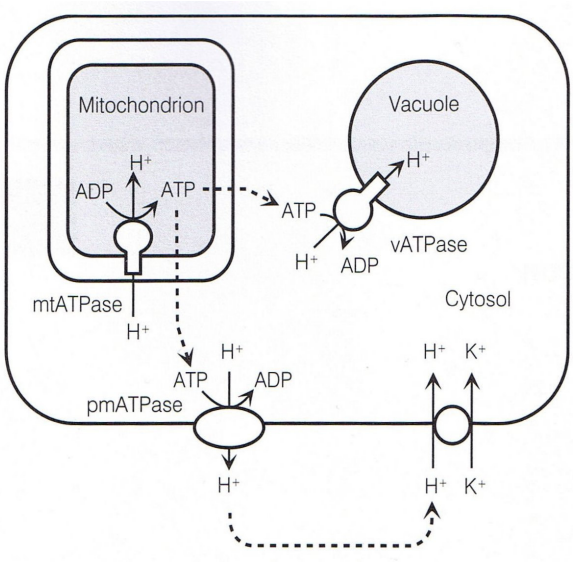


Fig 1.2-12: The proton extrusion pump in the plasma membrane and its coupling to the mitochondrion ATPase [6]**.**

The function of the plasma membrane ATPase is to create a proton motive force. ATPase pumps out protons that come in from the external medium via the growing tip. This again results in absorption of positive ions like K^+. K^+ accumulates and diffuses to the tip. The action of the ATPase results in a net negative charge inside the cells of 200- 300 mV, and a pH difference of 3.0 units between the inside and the outside of the cell. The free energy of the electrochemical potential crated by the difference in the charge outside and inside the cell, drives the movements of solutes across the plasma membrane [4, 6, 13]. The specific type of transport of H^+ to the external medium and import of K^+ is by a symport. A symport transports different molecules in the same direction either out or in across the plasma membrane. Glucose is transported into the cell at the same time as H^+ [4]. Fig 1.2.1-8 show some forms of transporters.

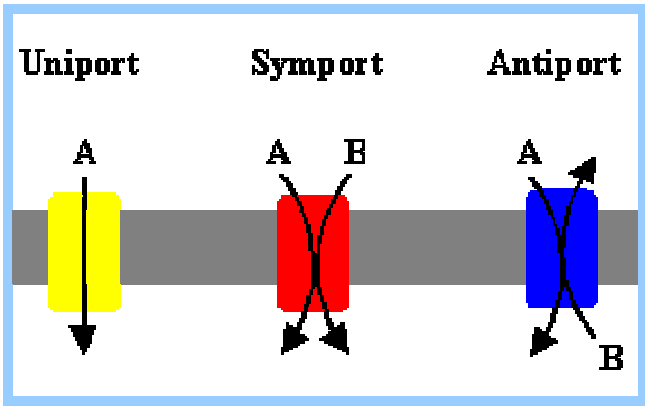


Fig 1.2-13: The three main types of carriers /transporters across the membrane. Uniport, Symport and antiport [13]**.**

Potassium contributes to the osmotic potential of the hypha and is involved in maintaining the volume of the hypha. The osmotic potential is important in the uptake of water [4].

The storage zone

In the storage zone, substances that are in excess and not required for growth are stored in an insoluble form in the vacuoles. This storage is reserved for when conditions are less favorable, and accesses to external sources are scarce. Carbon is stored as glycogen or lipid. Nitrogen is stored as amino acids or as proteins. Phosphorus is stored as polyphosphate that binds to potassium, magnesium or amino acids. The stored substances are incorporated into spores that will release and use the reserves upon germination. The storage and release of reserve substances can be a way of responding to osmotic changes [4, 6, 22].

The senescence zone

This zone is the oldest zone of the growing hyphae. Special destruction enzymes that are involved in self destruction of the mycelium are stored in compartments in vacuoles and are released when metabolism breaks down or a hypha is damaged. The appearance of greenish or black pigments in the mycelium is the first visible sign of the mycelium aging [4].

[13] http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/images/uniport.gif

1.2.2 Hyphal branching

As the hyphae grow, they branch and new tips are being made. This is to ensure the accesses to new nutrients [23]. Normally, the more nutrients in the medium, the less branching occurs. If the nutrients in the medium are scarce, the branching will increase. In *Neurospora crassa* there have been identified over 100 loci that encode products that can affect tip growth and branching. Mutations in these genes often lead to a phenocopy of increased branching and slow growth. Decreased branching is seldom observed as mutant phenocopy [23].

Fig 1.2-14: Hyphae of *Neurospora crassa* branching [14].

The actual mechanism behind branch initiation is not clear. Several theories have been submitted. One theory is that the accumulation of vesicles destined to tip growth causes the point of branching [13, 23, 24]. Other theories have stated that branching is triggered by a signal deep inside the colony. The idea is that the signal to branch is dependent on the previous branch points and that new branching is induced from information at the previous branch points [25].

[14] http://www.ux.uis.no/~ruoff/videos.html

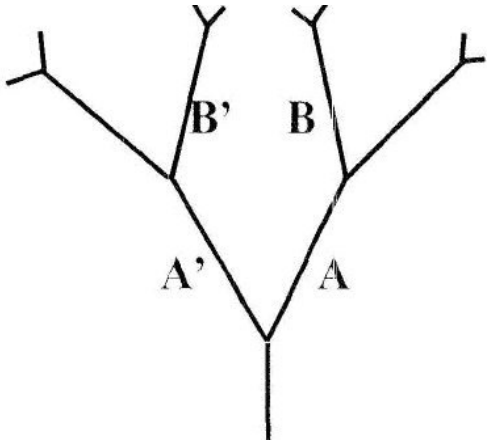


Fig 1.2-15: The distribution of branch points in *Neurospora crassa* hyphae. A branch interval is defined as the distance between tandem two branch points [25]**.**

Vesicles triggers branching when flow of material supply is greater than that of consumption [25]. This mass flow is thought to be driven, probably by turgor pressure [26]. A weakening of the cell wall may also trigger branching [23, 24]. Watters et al [23] showed that the distribution of branch intervals is independent of tip extension rate as controlled by temperature. A trait with hypherbranching mutants is that they also grow slow or stops growing. This can be a reason for accumulation of vesicles that cause branching. When the growth is slow the supply of vesicles with cell wall material may extend the rate at which the materials are incorporated at the growing tip. This leads to some signal to branch in order to spread the accumulated vesicles.

1.2.3 The *Neurospora* crassa cell wall

The fungal cell wall is an integral and important element in fungal biology. The cell wall is rigid and protects the fungal cell from the environment and the infection of pathogens, and at the same time allowing enzymes, proteins and substances to pass through to deliver nutrients and water. The importance of the cell wall is apparent and is a novel target for antifungal drugs. For the fungus, loss or depletion of cell wall can be lethal. The destruction of cell wall with antibiotics derived from fungi has been used to treat bacterial infection for many years[15].

In *Neurospora crassa* cell wall is synthesized at the tip of the growing hyphae. The materials needed for the cell wall synthesis are transported to the growing site in vesicles that are pumped along the hyphae by the actin skeleton and turgor pressure [6]. For new cell wall elements to be inserted hydrolytic enzymes that brake bonds are also needed, these enzymes are in vesicles within the cell lipid membrane and are transported to the tip and fuse with the plasma membrane and come in direct contact with the expanding wall and dissolves it so that new cell wall material can be inserted [13].

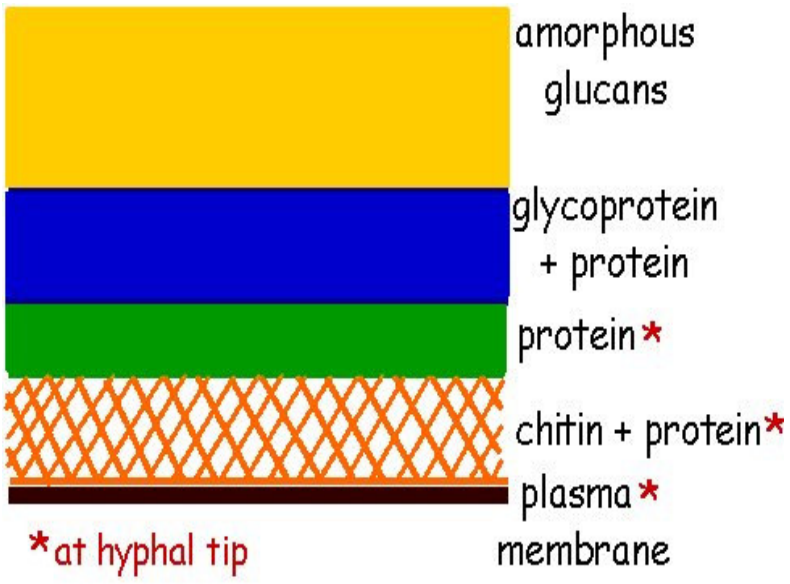


Fig 1.2-16: A representation of the composition of the fungal cell wall [16].

[15] http://bugs.bio.usyd.edu.au/Mycology/UsesOf_Fungi/industrialProduction/fungalDrugs.shtml
[16] http://www.fungionline.org.uk/3hyphae/2fungal_walls.html

The cell wall is composed of a network of polymer sugars and glucans. The distribution of the *Neurospora crassa* cell wall components is 7-10 % chitin, 25 %, β -1,3 -glucan, 35 % other glucans and 10 % proteins. Glucans are polymers of β-D-Glucose. They contain up to 250 000 glucose units. β -1,3 -glucan in *Neurospora* is thought to be linked to β -1,6 -glucan and cross-linked to chitin [13].

β -1,3 -glucan and β -1,6 -glucan are synthesized after UDP – glucose (UDP-glc) is synthesized from glucose 6-phosphate catalyzed by phosphoglucomutase (PG MUTASE) and uridylyl transferase via glucose-1 phosphate as a side reaction in the first step of glycolysis.

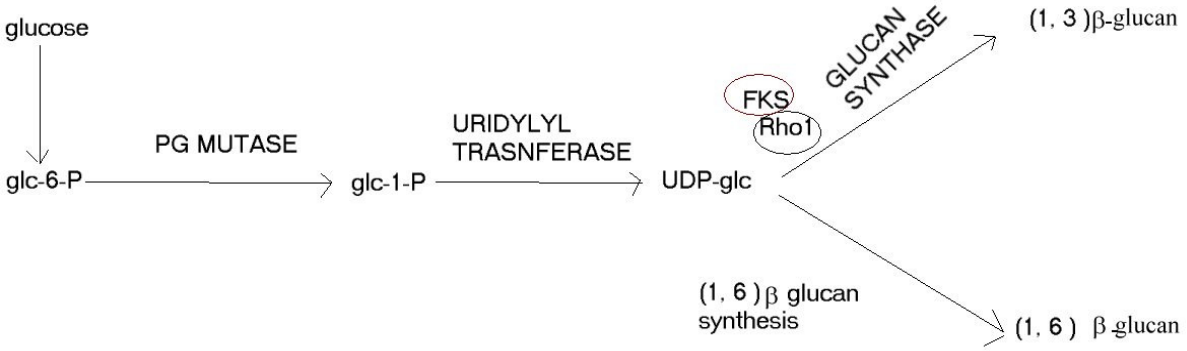


Fig 1.2-17: The synthesis of β -1,3 -glucan and β -1,6 -glucan from glucose-6- phosphate [13]**.**

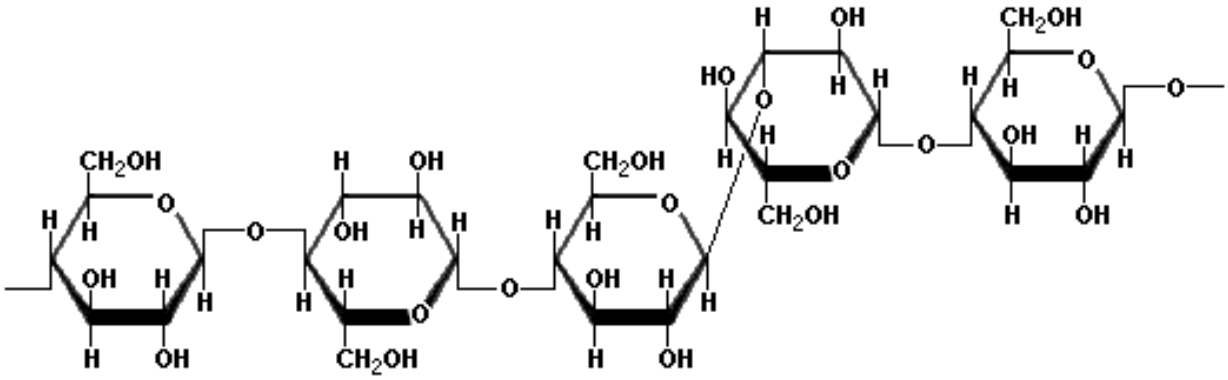


Fig 1.2-18: β -1,3 -glucan [17] **.**

β -1,3 -glucan synthase is a enzyme complex which consists of two proteins, the β -1,3 -glucan synthase catalytic sub unit encoded by the gene *FKS*, and a regulatory sub unit Rho1 encoded by the *rho-1* gene [13]. The suggested reaction sequence is that the enzyme binds an activator, forming activator -enzyme complex. The complex binds UDP-glc which is

[17] http://www.scientificpsychic.com/fitness/carbohydrates2.html

hydrolyzed, releasing UDP, while the glucose residue remains associated with the enzyme. The enzyme-activator-glucose complex binds another UDP-glc. This is the step where it is likely that linear competitive inhibitors act. Initial polymerization occurs when a disaccharide is made still connected to UDP and the enzyme. UDP is then released and enzyme-activator-disaccharide binds another UDP-Glc and Glc is covalently added. Uncompetitive inhibitors are thought to be affecting enzyme activity as further polymerization occurs by addition of glucose (from UDP-glc) to the growing glucan chain [27].

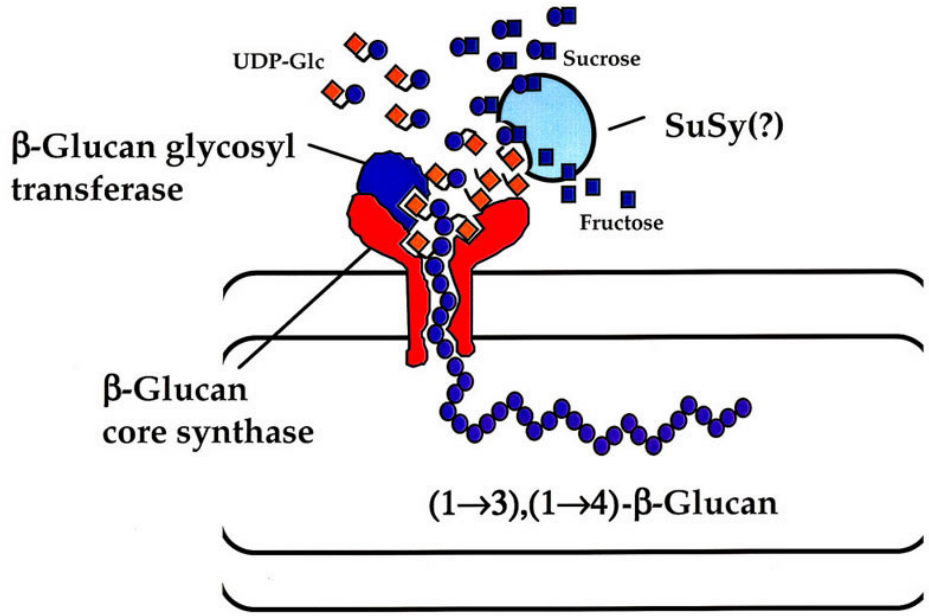


Fig 1.2-19: glucan synthase[18]**.**

β -1,3 -glucan synthase have been shown to be inhibited by sorbose [7] but the exact mechanism is not known. The result of the inhibition is depletion of β -1,3 -glucan and a local weakening of the cell wall. This again results in altered morphology of the fungus. The substrate for chitin synthase is UDP-N-acetylglucosamine (UDP –glcNAc). This is synthesized from fructose-6-phosphate via the enzymes ketol- isomerase, acetyl transferase, PAG mutase and pyrophosporylase. Chitin is thus synthesized from precursors of the second step in the glucolytic pathway [13].

[18] http://www.btny.purdue.edu/Research/Profiles/

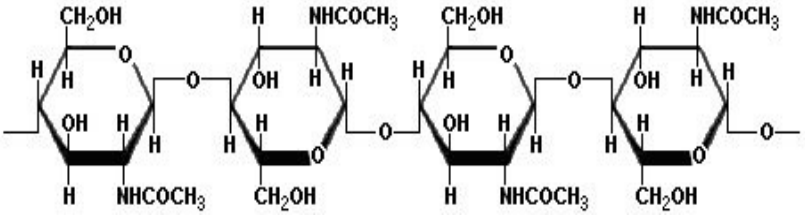


Fig 1.2-20: Chitin 11.

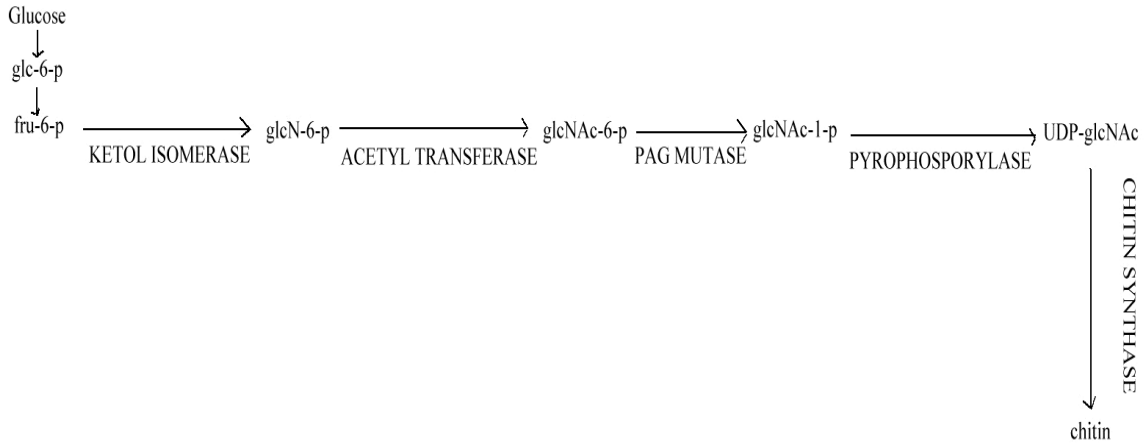


Fig 1.2-21: The synthesis of chitin from fructose-6-phosphate [13]**.**

1.3 Carbon metabolism in *Neurospora crassa*

Neurospora crassa is a heterotrophic organism. This means that it is dependent on getting its nutrients from external sources like organic materials from dead organisms or supplied sugar. Carbon is necessary for *Neurospora crassa* as source of both energy and growth. The way the carbon source is obtained, transported and used in the *Neurospora crassa* is similar to many other eukaryotes. *Neurospora crassa* activates more resources to metabolism when grown on a poor carbon source. Growing in minimal media is stressful for the fungus [28].

To metabolize carbon, *Neurospora crassa* use the Ebden –Meyerhof pathway (EM pathway) of glycolysis coupled with the Pentose phosphate pathway (PPH pathway) [6, 13]. The EM pathway starts with the phosphorylation of glucose or fructose by kinases. The end product of the metabolism is pyruvate. Pyruvate can be transported to the mitochondrion where conversion to acetyl-CoA, that enters the citric acid pathway or to alcohol trough Fermentation [6, 13, 29]. When external sources of carbon are scarce, noncarbohydrates like lipids can be used to produce glucose. This is done though gluconeogenesis. gluconeogenesis is reversal of the steps of glycolysis [6, 13].

Glycolysis is the stepwise degradation of glucose and other simple sugars. The details of the pathway were studied in the first half of the 20th century by Otto Warburg, G. Ebden and O. Meyerhof [6]. The steps of gluconeogenesis are all but two carried out by the same enzymes of glycolysis. This is possible because most of the steps are reversible. The two irreversible steps are the reactions catalyzed by pyruvate kinase (PYK) and 6-phosphofructokinase (PFK), (Fig 1.3-1). To get around these irreversible steps phosphoenolpyruvate carboxykinase (PEPCK) is used to convert oxaloacetate to phosphoenolpyruvate at expense of ATP and fructose bisphosphatase (FBP) hydrolyses fructose 1,6-diphosphate to form fructose 6 phosphate [6, 13]. Oxaloacetate is provided from the citric acid cycle [6]. The EM pathway consumes ATP at three stages and NAD is reduced to NADH + H^+. The PPH pathway consumes ATP at one stage and reduces NADP to NADPH+ H^+ in the first and second step. The details of the EM-Pathway and the PPH pathway can be seen in Fig 1.3.-1 and abbreviations of the involved enzymes are listed in Table 1.3-1

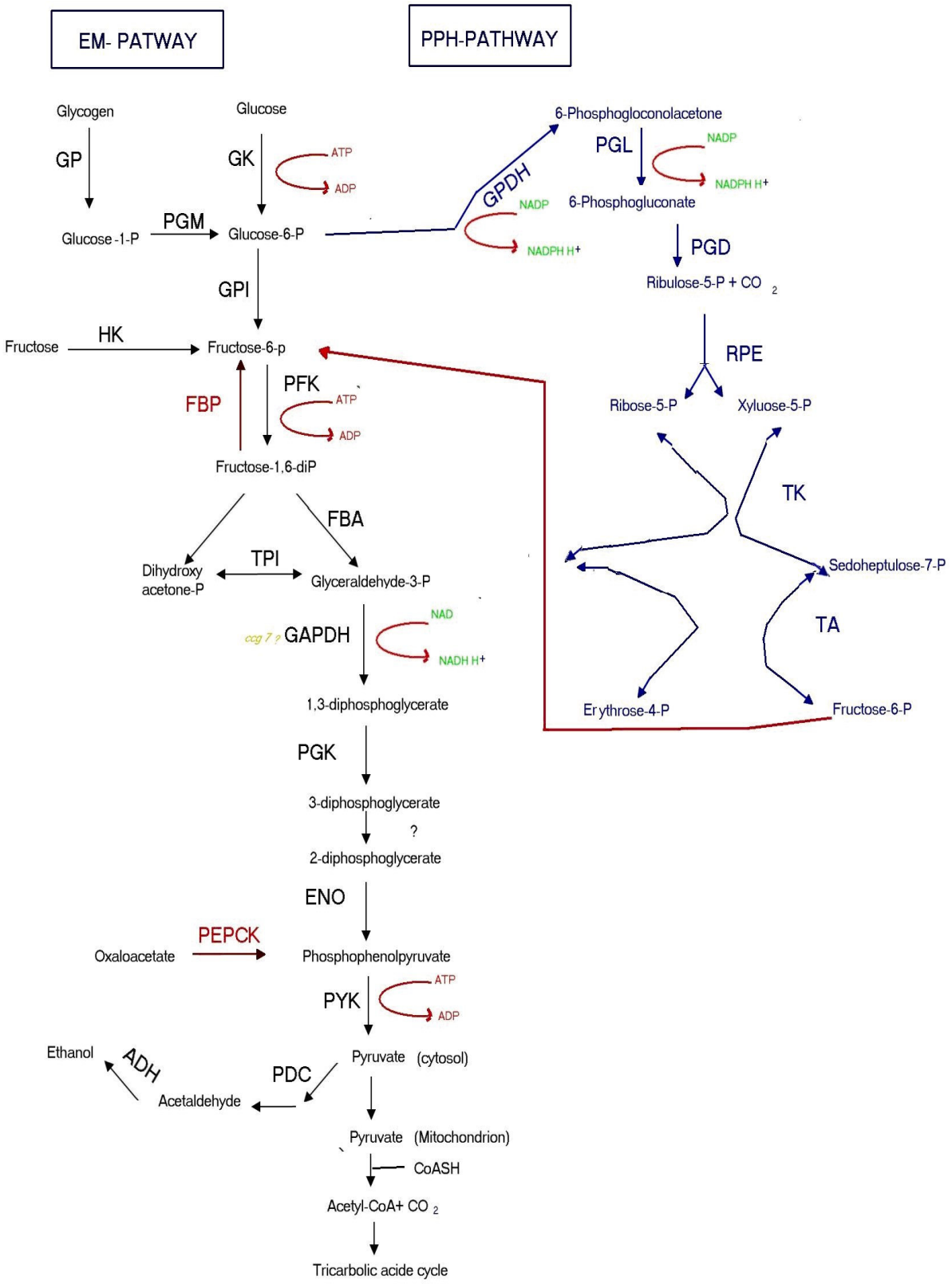


Fig 1.3-1: The EM- (black) and PPH (blue) pathways of glycolysis, irreversible steps circumvented in gluconeogenesis is in brown. The enzymes are abbreviated and are above the reaction arrows. The enzymes are listed in Table 1.3-1.

Table 1.3-1: Enzymes in carbon metabolism.

Abbreviation	Enzyme	Pathway
GP	Glycogen phosphorylase	hexose phosphorylation
HK	Hexokinase	Hexose phosphorylation
GK	Glucokinase	Hexose phosphorylation
PGM	Phosphoglucomutase	EM glycolysis
GPI	Glucose-6-phosphate isomerase	EM glycolysis + gluconeogenesis
PFK	6-phosphofructokinase	EM glycolysis + gluconeogenesis
FBA	Fructose-biphosphate aldolase	EM glycolysis + gluconeogenesis
TPI	Triose-phosphate isomerase	EM glycolysis + gluconeogenesis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	EM glycolysis + gluconeogenesis
PGK	Phosphoglycerate kinase	EM glycolysis + gluconeogenesis
ENO	Enolase	EM glycolysis + gluconeogenesis
PYK	Pyruvate kinase	EM glycolysis + gluconeogenesis
GPDH	Glucose-6-phospate 1 dehydrogenase	PPH glycolysis
PGL	6-phosphogluconol aconase	PPH glycolysis
PGD	6-phosphogluconic dehydrogenase	PPH glycolysis

RPE	Ribulose-phosphate 3-epimerase	PPH glycolysis
TK	Transketolase	PPH glycolysis
TA	Transadolase	PPH glycolysis
PDC	Pyruvate decarboxylase	Fermentation
ADH	Alcohol dehydrogenase	Fermentation
FDP	Fructose-bisphosphatase	Gluconeogenesis
PEPCK	Phosphoenolpyruvate carboxykinase	Gluconeogenesis

1.3.1 Circadian control of carbon metabolism

In relation to the topic of this thesis, the question about a coupling between carbon metabolism and the circadian clock in *Neurospora crassa* is of interest. The clock controlled gene 7 (*ccg-7*) have been reported to be linked to EM-glycolysis by the enzyme Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [30, 31]. The enzyme seems to be regulated on a daily basis, with mRNA accumulating in the late night and CCG 7 (GAPDH) activity peaking several hours later. Jay. Dunlap and Shinohara concluded that *ccg-7* was not induced by environmental stress such as glucose or nitrogen starvation [31].

1.3.2 Carbon catabolite repression

Neurospora crassa has a regiment of different ways to survive. The organism efficiently possesses systems in which, depending on the available carbon source, will induce either repression or derepression of systems that can transport and use the most available carbon source [6]. Upon the sensing of glucose, a signal will start repression of the transport and use of less usable carbon sources. This repression can include repression of transcription, destabilization of enzymes or mRNA. cAMP may also contribute by stimulating glycolysis. Glucose starvation leads to derepression and the start of systems to facilitate the use and transport of less usable sugars [6, 32, 33].

1.3.3 Sugar transport in *Neurospora crassa*.

To be able to use the sugars provided by the growth media, the sugars have to be transported into the organism across the plasma membrane. This can be done in several ways. Glucose is the sugar that is most easily used carbon and energy source. It can be used directly in the metabolism, and its presence or absence is also a signal that may induce different processes of transport and further use. This is facilitated by a glucose sensing function at the cell membrane that excludes transport systems for alternative carbon sources when glucose is sensed in the growth media [29, 34, 35]. Two glucose transport systems are known in *Neurospora crassa* [6, 29, 33-35].

System I

The low affinity system has a K_m of 8- 25mM and can transport glucose in a diffusion like manner. The system needs little energy and continued uptake of glucose is dependent on the phosphorylation of glucose. This system can transport glucose and 3-O-methylglucose which is not phosphorylated [6, 36].

System II

The high affinity system has a K_m of 10-40 µM and appears when the organism is starved for extracellular glucose or other repressing metabolites. The system allows transport over the membrane of usable carbon sources at low concentration and is facilitated by the glucose -H+ symport and a substantial change in the depolarization of the membrane. The sugars transported by system II is glucose, galactose, sorbose, fructose, xylose, mannose, tallose, 2-deoxyglucose and 3-O-methylglucose [6, 34, 35, 37].In addition there are two systems for transport of single sugars. The fructose uptake system, K_m = 0.4mM which is inhibitable by sorbose, and the galactose transport system km 400mM [6, 37].

1.4 Chronobiology

Chronobiology can be described as the scientific study of biological timekeeping.
Since life began on earth the organisms living there had to experience changes in their environment. The earth orbits the sun and at the same time it rotates around itself. This and the fact that the moon orbits the earth are responsible for the creation of cycles that synchronize endogenous biological clocks [1, 3, 38].

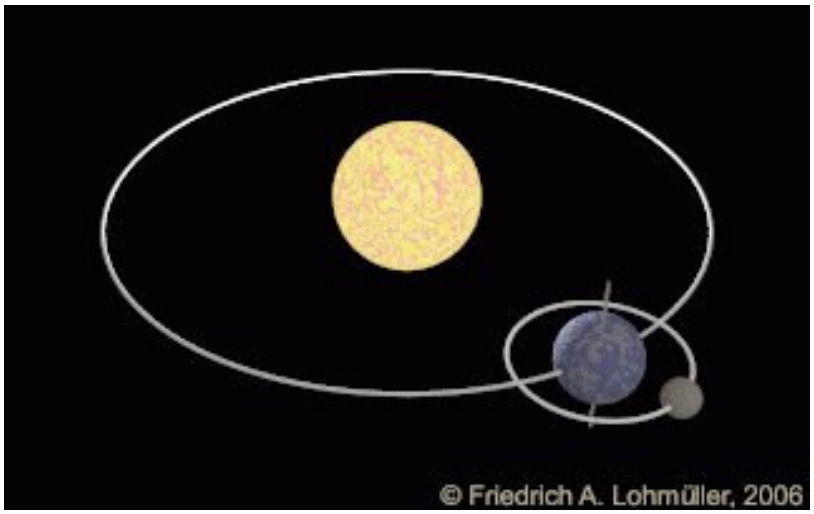


Fig 1.4-1: The interaction between the sun, earth and moon that creates environmental cycles like seasons[19].

Fig 1.4-2: Representation of the 4 seasons.

[19] http://www.f-lohmueller.de/pov_tut/animate/planet_00ani.gif

An organism will have great advantages in survival if it is able to adapt to these environmental changes quickly and correctly. This adaptation has been achieved though the process of evolution. Organisms have developed molecular and biological systems, often biological oscillators or clocks that switch on developmental and physiological processes in accordance with the cyclical changes caused by the planets movements. These switches are triggered by cues from environmental changes such as sunrise or sundown, day and night, tides, moon phases and seasons[2-4, 39].

Fig 1.4-3: The moon phase cycle [20].

The clues are named “Zeitgeibers” (time givers) and function as input signals to a biological oscillator that drives the biological rhythm that gives the output of the rhythm. Temperature and the sun (light) are significant zeitgeibers which entrains the rhythm. Entrainment of a biological clock system is the alignment of its own period and phase to the period and phase of an external rhythm. Because events like day and nigh, seasonal changes, moon cycles and tides are highly predictable it is possible for an organism’s biological clock to switch on clock controlled processes a little in advance to be ready for the environmental change [1, 40]. If removed from the cyclical environment and placed in constant conditions such as in constant darkness, he switches will still go on and of and the rhythm is considered as free running.

[20] http://www.sunna.info/souwar/data/media/10/lune.jpg

Research is done in many fields, on many different oscillators and on many different organisms. Sleep is an important field of study in chronobiology research in humans. Sleep deprivation is a known problem in psychiatric illnesses, and is also used as a form of torture in some countries. The disruption of the sleep cycle can render people less capable of tackling problems and can influence mental stability even in otherwise healthy people [21].

Why study rhythms of life?

The question of why so many scientists involve themselves in the study of biological rhythms is interesting. For many the field of chronobiology is simply interesting in it self. Others see the clear benefits of learning more about these rhythms since disturbances and abolishment of these rhythms have such impact in individual's life and well being. If we understand these rhythms, we may be able to fix what may be broken, or avoid substances or conditions that are harmful to these rhythms. Adaptations can be more readily accomplished, and again life quality and health benefit.

There are many biological rhythms in bodies. They govern sleep, blood pressure and hormone levels. Even capability to retain memories cycle! It has been shown that short time memory is best in the morning, whereas long time memory is best in the evening. Alertness is usually best in the afternoon.

Tolerance to pain and fear may also cycle. The phenomenon of premenstrual syndrome (PMS) is well known for most women. That time of the months where every little thing can make us cry. This is because the release of hormones in the later part of the cycle causes mood swings, migraine and abdominal cramps. Other such cycles involving tolerance to pain has also been observed. A dentist discovered that there were times a patient could tolerate a visit in the dentist chair, and others when the patient was not able to stand it. The dentist started to schedule his appointments to the times he knew the patient would be more receptive to treatment [39]. This made the appointments more comfortable for both parties. It may also have taken the sting out of the terror of a dentist appointment.

[21] http://www.talkaboutsleep.com/circadian-rhythm-disorders/circadian-rhythm-sleep-disorders/09-the-importance-of-melatonin.htm

The time that one individual is most productive may be different form another individual. This is because some people function better later in the evening than in the morning and so on. Today's society is ordered so that most people have to start work early in the morning and finish in the evening. Some have evening work and some have night work and some shift work. There is not yet much care taken to choose the right individuals to the right work time. Think of the benefit and resources, that would go into the work force if the right person was put to work at the right time according to his or hers biological rhythm.

1.5 History of biological clocks

The rotation and the planets have had effects on the life on earth since life itself began and have been noticed since early times. Daily rhythms in plants and animals where observed and recorded as early as in the time of Alexander the greats reign. Alexander's scribe Adrosthenes observed that certain trees opened at daytime and closed in nighttime [39].

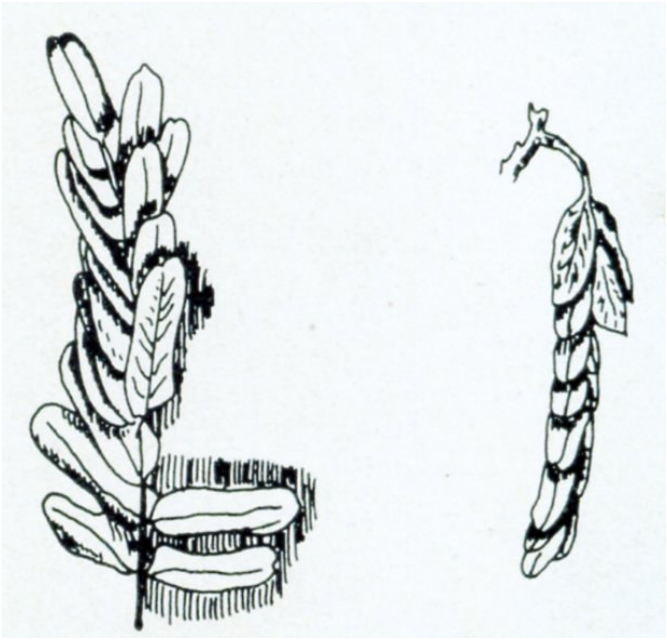


Fig 1.5-1: Discovery by Adrosthenes 400 BC. Leaves of trees open in the day and close in the night.

In 1889 Charles Darwin observed that the leaves of the mimosa plant opened and closed at specific times of a 24 h day. This had already been proven by the Frenchman De Mairan 1729. He also proved that the rhythm responsible was free running because the movements of the plants continued at the same times when the plants where placed in continuous darkness (Fig 1.5-2, 1.5-3 and Fig 1.5-4, 1.5-5) [39].

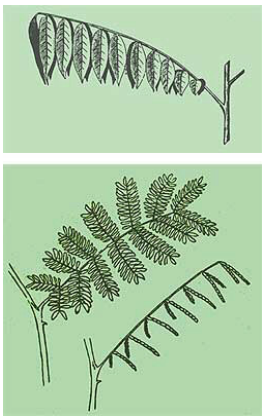


Fig 1.5-2: Mimosa leaves, top closed during the night, bottom open during the day [22] **.**

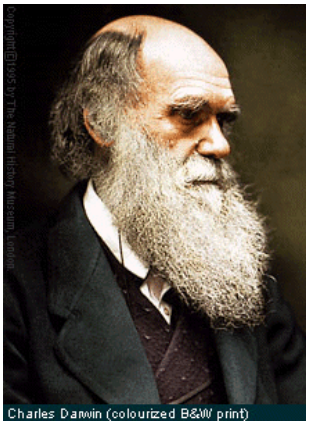


Fig 1.5-3: Charles Darwin (1808-1882) [23] **.**

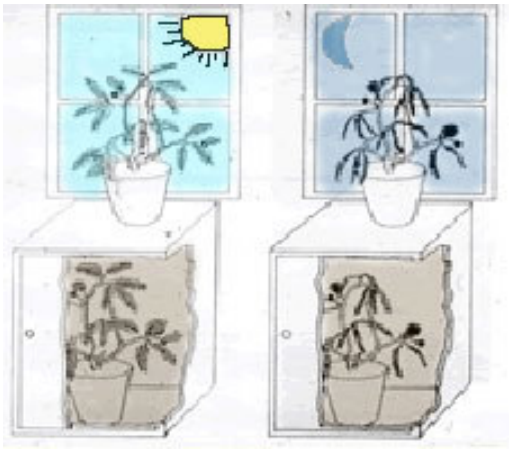


Fig 1.5-4: The free running rhythm of leaf movements in mimosa plants[24] **.**

Fig 1.5-5: De Mairan (1678-1771) [25] **.**

In 1751Carl von Linnè created a garden from the observation that different kinds of flowers opened and closed their petals at a regular time different times during the day. Fig 1.5-6 shows the Linnè flower clock where these flowers are represented.

[22] http://www.glimmerveen.nl/LE/biological_clock.html
[23] http://javalab.cs.uni-bonn.de/research/darwin/
[24] http://thebrain.mcgill.ca/flash/d/d_11/d_11_p/d_11_p_hor/d_11_p_hor.html
[25] http://www.sil.si.edu/digitalcollections/hst/scientific-identity/CF/by_discipline_display_results.cfm?Research_Discipline_1=Physics

Fig 1.5-6: Carl von Linnès flower clock [26]**.**

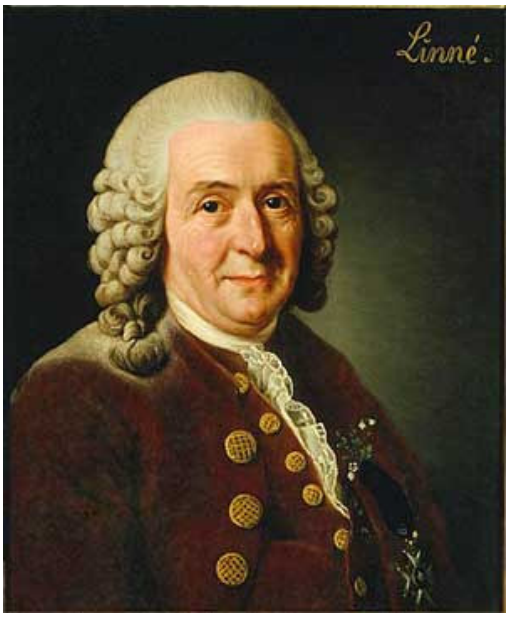


Fig 1.5-7: Carl von Linnè (1707-1778) [27] **.**

Karl von Frish observed bees returning to collect nectar in flowers at specific times of the evening. They still returned when external cues like light where removed [2, 39, 41]. The response to the relative length of day and night is termed photoperiodism and was proven in the 1920s by W.W. Garner and H.H. Allard [39]. They conducted experiments and noticed that the tobacco plant would only flower if it was exposed to light a certain number of hours a day. This photoperiodism is also thought to be a cue in bird migration cycles. Starling birds use the sun as a compass when migrating. This was confirmed by the work of Gustav Kramer in the 1950s. Kramer proved that the internal clock of starling birds where reoriented in the direction with the moving sun. This clock persisted in dim light and was synchronized to the local time by the influence of the local environment [39, 41].

Colin Pittendrigh studied the fruit flies and the emergence of an adult insect from a pupal case or an insect larva from an egg. Pittendrigh found that this process was a daily rhythm. The adults emerged at the same time at dawn with intervals of ≈ 24 h. He also proved temperature compensation by proving that the larva hatched at the same time regardless of temperature or light [2, 39].

[26] http://www.kirchersociety.org/blog/?p=1237
[27] http://obits.eons.com/tribute/gallery/11110?section=carolus-linnaeus-section&category=carolus-linnaeus

1.6 Biological oscillators

The basic properties of any biological rhythm consist of 3 parts as shown in Fig 1.6-1.

The s parts of a basic biological rhythm are:

1. The input.
2. The central oscillator
3. The output

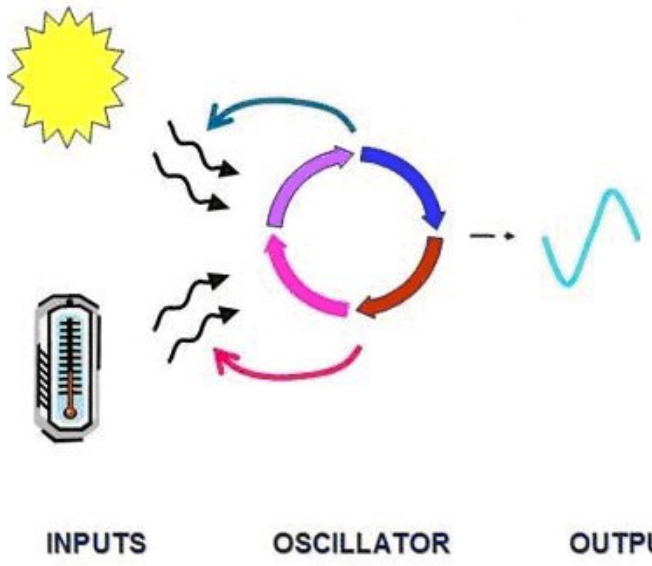


Fig 1.6-1: The structure of a basic biological clock oscillator.

Input

The input detects the zeitgeibers. Zeitgeibers are among others light, temperature, etc. Light is by far the strongest type of input.

The central oscillator

The central oscillator generates the rhythm. At the core of most biological oscillators are transcriptional/translational negative feedback loops. Negative feedback loops are driven by positive and negative elements. The positive elements induce transcription of a clock gene and the negative elements prevent this transcription. The protein product of this transcription

is the clock protein and it becomes a negative element when the concentration of the clock protein has reached a certain level. The negative element then stops the transcription until the time is right to start transcription again. The details of such an oscillator and a feedback loop are described in the chapter on the FRQ-oscillator.

The output

The output translates the rhythm from the oscillator into overt rhythms. This is done by transcription and translation of clock work genes that signals to start specific rhythmic processes.

Biological rhythms are classified according to the period length of the rhythm. The naming of these classes is based on Latin and refers to the period length of the rhythm. The classification is as follows:

- Ultradian
- Circadian
- Infradian

Ultradian Rhythms

Refer to rhythms with period lengths much shorter than 24 h. They are often repeated many times during the course of a day. Examples of such rhythms are the sleep cycles, constant breathing, heart rate, and hormonal release, regulation of body temperature, nostril dilation and appetite. Fig 1.6-2 shows the sleep cycle in mammals.

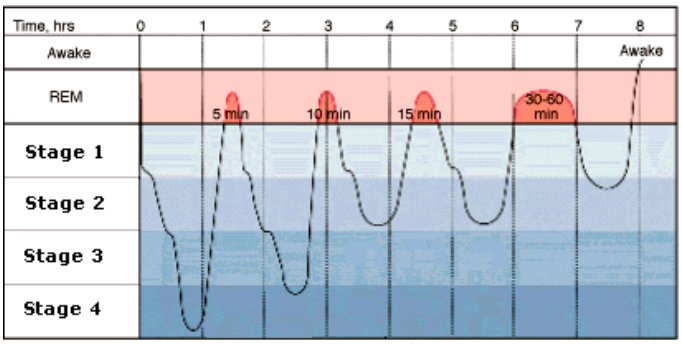


Fig 1.6-2: The sleep cycle is approximately 90 minutes [28] **.**

Circadian rhythms

Circadian rhythms have periods of ≈ 24 h. The circadian clock affects the daily rhythms of many physiological processes. It is known that concentration, alertness and memory are at its sharpest at special times of the day. Statistics have shown that most heart attacks happen early in the morning at the time when the rice of the blood pressure is sharpest [42]. Fig 1.6-3 shows different circadian cycles and physiological processes that are heightened and lowered during the day.

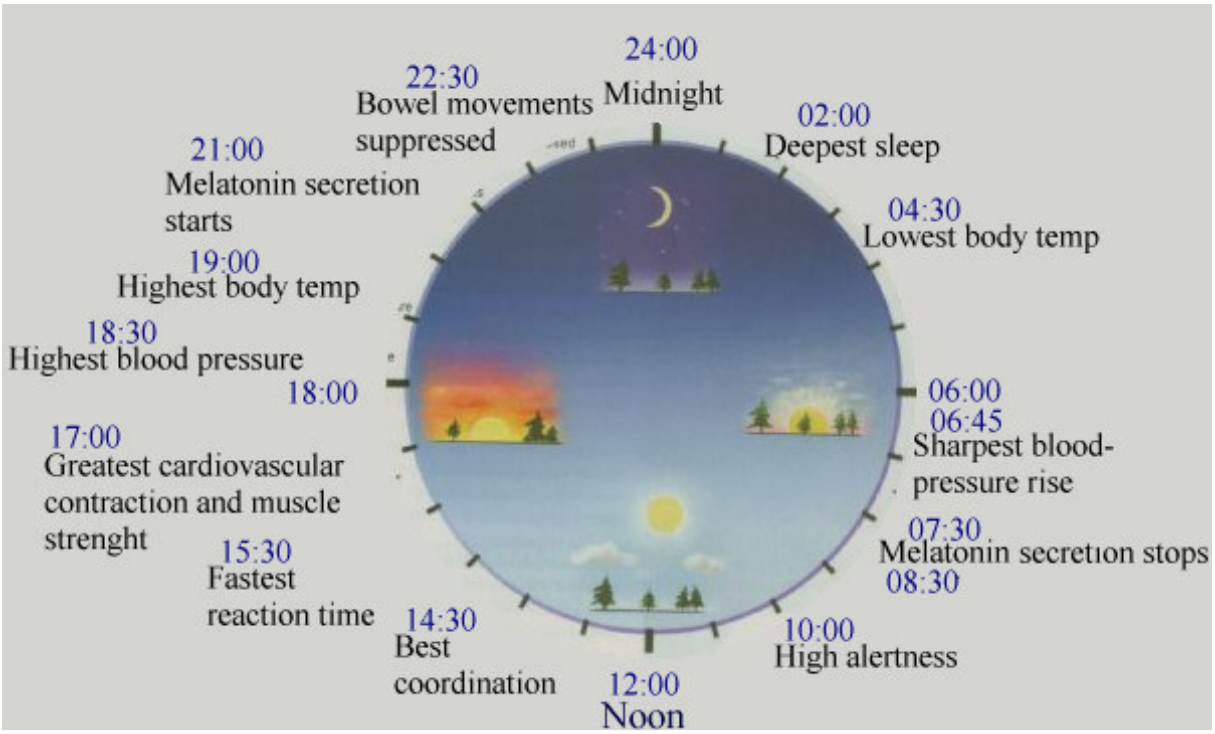


Fig 1.6-3: The daily cycle of biological rhythms [29] **.**

[28] http://www.hypnosis-and-health.com/sleep.html

The central circadian clock in humans is located in the suprachiasmatic nucleus (SCN) which is that only part of the mechanism where time is kept. The (SCN) lies within the hypothalamus in the brain. Information about day length is sensed by receptors in the retina is lead to the SCN through the pathway called the retinohypothalamic tract. The SCN interprets the information and passes the information on to the pinal gland behind the hypothalamus. The pinal gland secrets the hormone melatonin in response to the message from the retina about the length of the day [30]. Melatonin is a hormone that helps the body wind down and get ready for sleep.

Disturbances in melatonin levels are related to depression, shorter life span and cancer [31]. Melatonin release is inhibited by daylight. Therefore the melatonin level rises in the night. The circadian release of melatonin is a free running rhythm, because the release of melatonin will cycle even when light cues are absent. Destruction of the SCN will make circadian rhythms disappear completely. The pathway of the central circadian clock is shown in Fig 1.6-4 [3].

[29] http://www.glimmerveen.nl/LE/biological_clock.html
[30] http://science.jrank.org/pages/880/Biological-Rhythms.html
[31] http://www.talkaboutsleep.com/circadian-rhythm-disorders/circadian-rhythm-sleep-disorders/09-the-importance-of-melatonin.htm

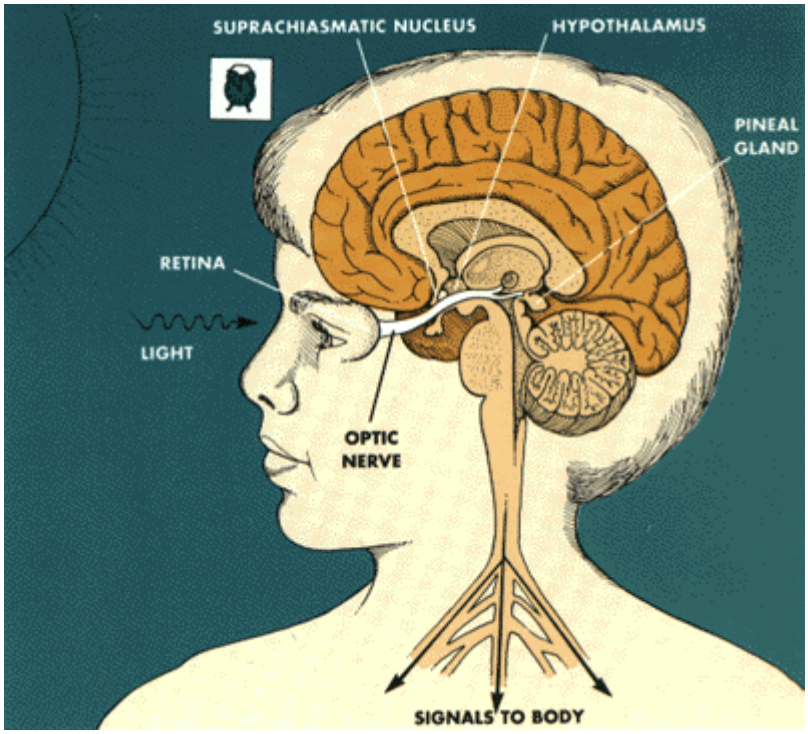


Fig 1.6-4: The central circadian clock in humans [32] **.**

Infradian rhythms

Rhythms with period lengths greater than 24 hours are infradian. Infradian rhythms can again be divided into subcategories depending on the relative period. Circannual rhythms are seasonal or yearly rhythms like the migration of birds. Monthly cycles like the menstrual cycle are circalunar (monthly).

[32] http://www.sfn.org/skins/main/images/brainbriefings/bio_clocks_illus.gif

1.6.1 Temperature compensation in biological rhythms

Temperature compensation means that the period length of a biological rhythm will remain constant over a physiological temperature range of constant temperatures. Temperature compensation is dependent on contribution on both negative and positive elements of the rhythm.

Chemical reactions will go faster the higher the temperature because the temperature lowers the activation energy of the process. A temperature coefficient Q_{10} gives the factor to which a rate constant R rises with a temperature rise of 10 degrees. The formula for Q_{10} is given in Eq 1.6-1

$$\frac{R\left(T+10^{\circ}C\right)}{R(T)}=Q_{10} \qquad \textbf{Eq : 1.6-1}$$

If two temperatures are not quite 10 units apart another equation (Eq 1.6-2) can be used to give an estimate of Q_{10}

$$Q_{10}=\left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2-T_1}\right)} \qquad \textbf{Eq. 1.6.-2}.$$

Most physiological rhythms have a Q_{10} of ≈2 because temperature rises have a large effect on the rate constant. Temperature compensated biological clocks have a Q_{10} of ≈1. Circadian rhythms are dependent on a regulated speed of reactions to keep the right time. This means that a temperature compensated rhythm must be less sensitive to changes in constant temperatures [43]

The sensitivity of temperature compensation in biological rhythms can be calculated by using the rate constants involved in the turnover of both negative and positive products in the feedback loop that makes up the actual oscillator. The effect of the positive and negative elements on the rhythms sensitivity is represented in the form of sensitivity constants also

called control coefficients (C_i) Eq : 1.6-3 . The C_i-s are calculated according to rate constants and period length of the rhythm [43, 44].

$$C_i = \frac{\partial \ln P}{\partial \ln k_i} \qquad \textbf{Eq 1.6-3}$$

When these constants are summarized, the sum should be -1. The activation energies (Ea) for each reaction in the rhythm are multiplied with the corresponding C_i. The activation energy (Ea_i) can be obtained by using the Arrhenius equation. When the product of the sum of sensitivity constants and activation energies for both negative and positive elements are balanced the rhythm in question is temperature compensated Eq : 1.6.1-4) [43, 44].

$$\sum_i \left(C_i \cdot Ea_i\right)_{neg} = \sum_j \left(C_j \cdot Ea_j\right)_{pos} \qquad \textbf{Eq 1.6-4}$$

This means that to disturb this balance and loose temperature compensation either the rate constants or the activation energy must be altered. Rate constants can be altered if the constant turnover of products in the rhythm is lower or higher than usual. This can happen if degradation is inhibited in some way. Inhabitations of degradation may occur when the protein destined for degradation are rendered more stable so that degradation takes longer, or from inhabitation of other elements of degradation such as the proteaosome which carries out the degradation process. Inhabitation of degradation often results in longer period lengths.

1.6.2 Protein degradation, an important way of regulating pathways

Internal protein turnover is of importance to many organisms. The turnover of proteins and enzymes are involved in regulation of many pathways and oscillators. In metabolic pathways as well as in biological oscillators, the regulation will consist of a feedback loop where a certain max concentration of the product will inhibit production of more of the product. The removal of a feedback protein from the feedback loop is important for a biological oscillator to function properly. The presence of the regulated protein will inhibit its own production and the prolonged presence of the product due to inefficient degradation will further result in a halt or delay in the pathway or a halt or delay in the period of the oscillator [44, 45].

To facilitate the degradation of proteins, special protein complexes called proteaosomes are used to cut the target proteins into its peptide parts. This removes the protein from the surroundings and at the same time amino acids become available for the organism. Fig 1.6-5 illustrates the function of the proteaosome.

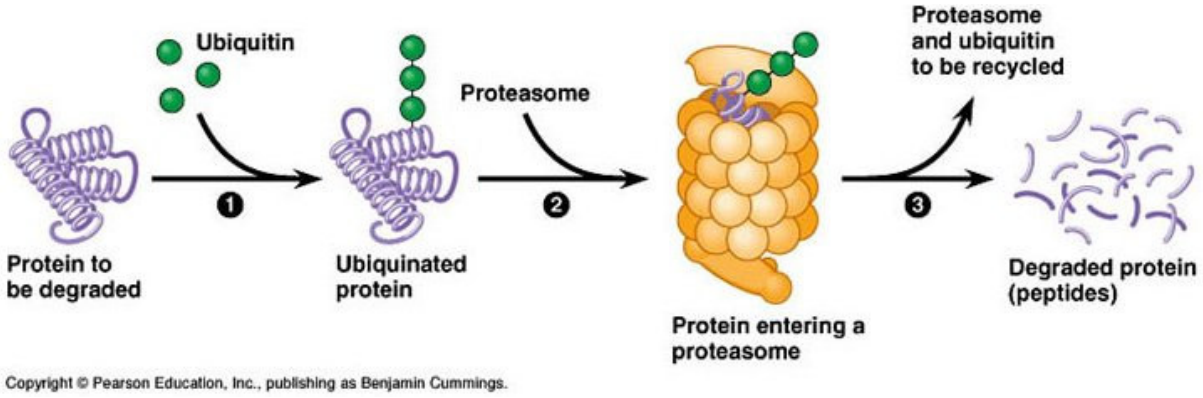


Fig 1.6-5: Illustration of the function of the proteaosome. After the unneeded protein is ubiquinated (marked for degradation) the proteaosome degrades the protein and cuts it into peptides [33].

Carbon and energy in the form of ATP is important to the degradation machinery. The proteaosome is also important in the removal of damaged proteins. Proteaosome inhibition can lead to cell death and causes a significant lengthening of the circadian period in rat embryo cells (Rat-1 cells) [46].

[33] http://fig.cox.miami.edu/~cmallery/150/cells/c19x12proteosome.jpg

1.7 Circadian Rhythms

Circadian rhythms are oscillatory processes with ca 24 h periods. To be considered a true circadian rhythm certain traits must apply to the rhythm other than the ≈24 h period length.

Circadian rhythms are:

- Free running.
- Temperature compensated.
- Entrained.
- Under genetic control.
- Adjustable.

Free running rhythm

That the rhythm is free running implies that it continues to run under constant conditions even in the absence of external environmental clues. A free running rhythm can be observed in constant dark (DD) or constant light conditions (LL).

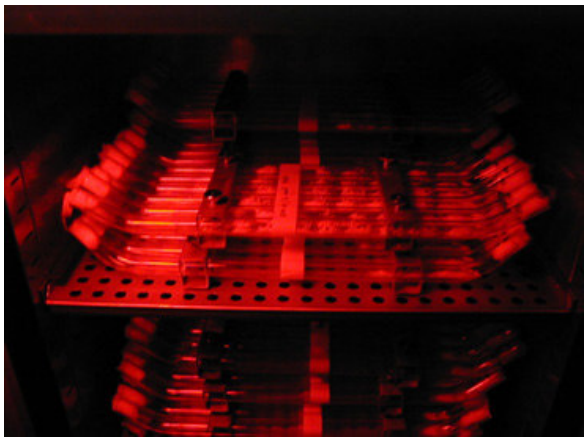


Fig 1.7-1: Race tubes with *Neurospora crassa* in (DD) constant darkness conditions. The tubes are placed in a dark room with only red light This is because the red light has no effect on the free running conidiation rhythm of *Neurospora crassa* [34].

[34] http://www.ux.uis.no/~ruoff/Neurospora_Rhythm.html

Temperature compensation

A rhythm is temperature compensated when the period length of the free running rhythm so not vary more than 2 or 3 hours when subjected to different constant temperatures. This implies that the period length must be ≈24 h for all constant temperatures in a physiological temperature range. Sudden temperature changes will induce a phase shift of the rhythm. Rhythmic temperature cycles can entrain circadian rhythms.

Entrainment

Circadian clocks can be entrained (synchronized) by light and other environmental clues. This allows for adaptation of an organism to changing day lights and seasons.

Genetic control

Mutations in clock gene alleles can alter the period length. The period can get longer or shorter. This fact makes study of clock gene mutants an important tool in chronobiology

Adjustability

Resetting of a circadian clock can be carried out by external environmental cues (zeitgeibers). When a circadian rhythm gets disturbed, it will continue to oscillate with a certain phase shift that will cause the formation of a new phase that can be advanced or delayed compared to the original rhythm. Light pulses in the middle of the night can induce such phase shifts because it tricks the organism to think that dawn is occurring earlier.

1.8 The hyphal branching rhythm in *Neurospora crassa*

Wild type Neurospora crassa grows rapidly on sucrose media. The conidiation rhythm that is expressed in DD conditions is distinctive. The rhythm is recognized as the rhythmical formation of conidial bands. Each band is formed when vegetative hyphae emerge from germinated conidia. The hyphae form mycelium that grows forward on the surface of the agar in the race tube. After a certain time the vegetative hyphae differentiate to aerial hyphae by stretching up and away from the growth medium surface. These aerial hyphae differentiate into free conidia that appear as orange hives. A new cycle starts when new hyphae grows from these conidia as spreading mycelium [6].

Each cycle is termed as a period. The period of most *wt Neurospora crassa* strains in free running conidiation rhythms are about 22 h. The rhythm is circadian and temperature compensated where the period remains practically unchanged at different constant temperatures between 20°C and 32°C in DD conditions [13]. The conidiation rhythm is known to be the result of a biological clock, where the "clock" s pacemaker is described to be transcriptional-translational negative feedback loop.

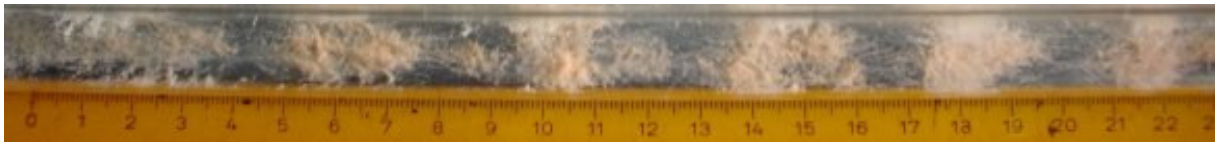


Fig 1.8-1: conidiation rhythm in *Neurospora crassa wt-328-4A*. On minimal media (Vogel [47]**) at 24.5°C in DD conditions. Time between each band (period) was ca 22.5 h, total time of growth was 4.8 days.**

Fig 1.8-2: The conidiation rhythm in *Neurospora crassa wt-328-4A*, seen from the back (under) the race tube. on minimal media (Vogel [47]**) at 24.5 °C in DD Time between each band (period) was ca 22.5 h, total time of growth was 4.8 days.**

In 1964 Alfred S. Sussman and Robert J. Lowry [48], reported the existence of a colonial mutant (*"clock")* of *Neurospora crassa*. *Clock* grew differently in the same conditions as its *wt* parent. The mutant grew in tight colonies on solid media, and bands appeared rhythmically / periodic as "wave fronts "consisting of vegetative submerged hyphae (Fig 1.8-3) [48].

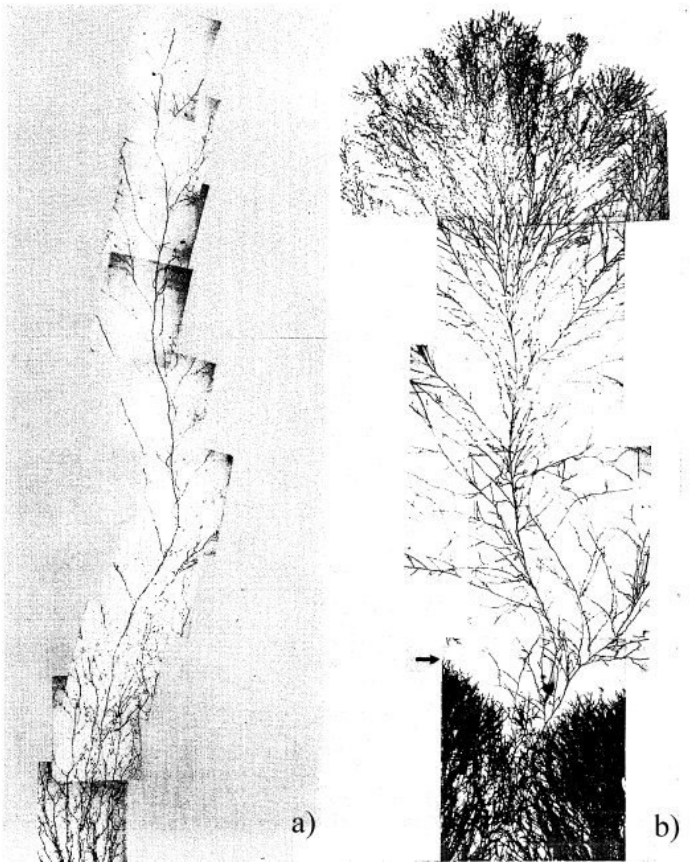


Fig 1.8-3: Branching pattern of *Neurospora crassa* hyphae in a) *wt* strain *69-1113a* on complete medium and b) The *clock* mutant with hyphal branching rhythm [48]**. The arrow in b) shows the start of the next cycle.**

These “wave fronts” are the results of extensive hyphal branching of vegetative hyphae that become aggregated and increasingly denser and finally mark a growth band. From this band, a few single free hyphae grows and branches and eventually dense to mark a new band [48].The time between each “front“ is one period. Feldman [5] asserted that the period decreases with increased temperature, so that the period will be longer in 25˚C than in 30˚C

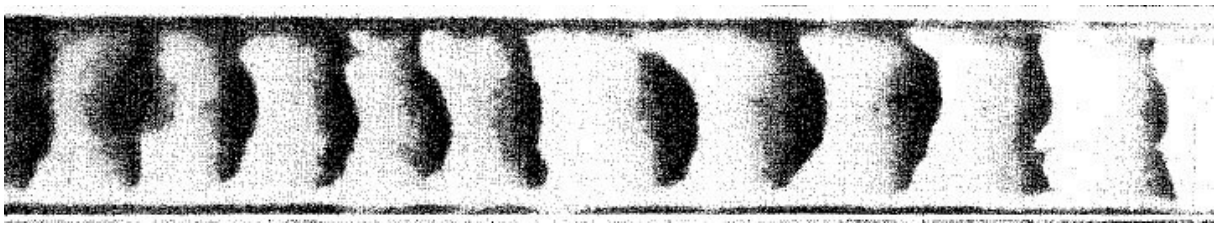


Fig 1.8-4: Representation of "clock " mutant of the *wt* strain *69-1113a* morphology when grown on complete media in a race tube [48]**.**

A phenocopy of the *clock* mutant of the *wt 69-1113a* behavior can be achieved with wild type strains when these are grown on media containing sorbose (Fig 1.8-5 and 1.8-6) [48].

Fig 1.8-5: : Hyphal branching rhythm in *Neurospora crassa wt-328-4A* induced by sorbose on minimal media (Vogel [47]**) 24.5° C in DD conditions. From the top, (over) the race tube. Time between each band (period) was ca 33.2 h, total time of growth was 37.97 days. Image by the author.**

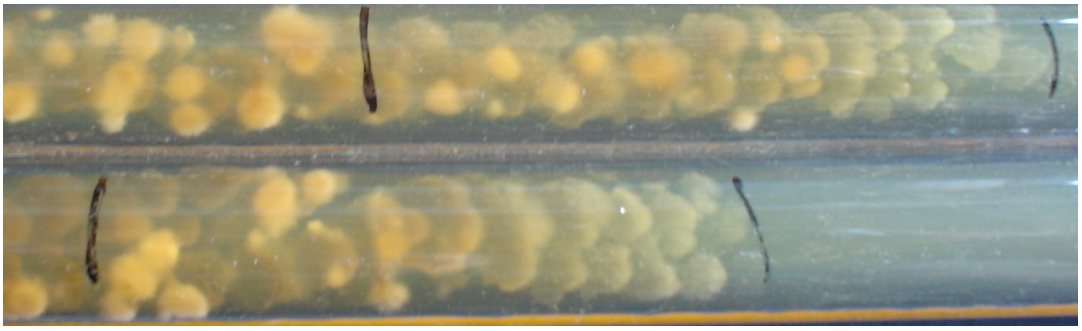


Fig 1.8-6: Hyphal branching rhythm in *Neurospora crassa wt-328-4A* induced by sorbose on minimal media (Vogel [47]**) 24.5° C in DD conditions. Seen from the front (over) the race tube. The days between each black line was 13.95 days. Time between each band (period) was ca 33.2 h. Image by the author.**

The reasons for the phenocopy effect of sorbose in not fully understood. What is known is that sorbose inhibits enzymes involved in cell wall biosynthesis [7]. There is also evidence

that *wt Neurospora crassa* when first grown on sorbose medium and then transferred to sucrose media grow normally again [49]. This would suggest that the original cause of the rhythm may not be directly a genetic mutation in genes involved in hyphal branching.

1.9 The conidiation rhythm in *Neurospora crassa*

Conidiation is the development and release of spores. These spores can then settle somewhere and begin a new colony. Conidiation is the result of the asexual cycle that gives rise to an observable sporulation as orange loose conidia. The circadian rhythm of conidiation in *Neurospora crassa* is believed to be a result of evolution and survival tactics. It is advantageous to any organism, to adapt its processes, sporulation and growth and to execute these when conditions are most favorable. This ensures best possible use of energy and nutrients.

The circadian rhythm is a result of the periodic repetition of the asexual cycle (Fig 1.9-1) and is controlled by a biological clock mechanism where the “clock” s pacemaker is described to be transcriptional-translational negative feedback loop. The most widely studied biological clock pacemaker in *Neurospora crassa* is the core clock, the FRQ/WC feedback loop.

The signal to start production of conidia is given in the late subjective night of the circadian cycle, and the signal is turned of again early the next subjective day and undifferentiated mycelium is produced. Forward growth is halted after some time as aerial hyphae and conidiation occurs. After a little while the forward growth of mycelium is commenced until a new signal is given and the cycle repeats itself. The conidiation rhythm is only evident in constant dark, DD conditions and dampens in LL conditions.

1.9.1 The FRQ oscillator

The core biological clock in *Neurospora crassa* is thought to be the FRQ oscillator. Among other things it controls the timed rhythm of sporulation, the morphological switch from mycelium with vegetative hyphae via aerial hyphae to mature spores (conidia), by inducing clock controlled genes (*ccgs*) and the vivid gene (*vvd*) (Fig 1.9-2) [50, 51]. The clock also controls metabolic events such as the nitrogen metabolism and carbon metabolism [31, 52].

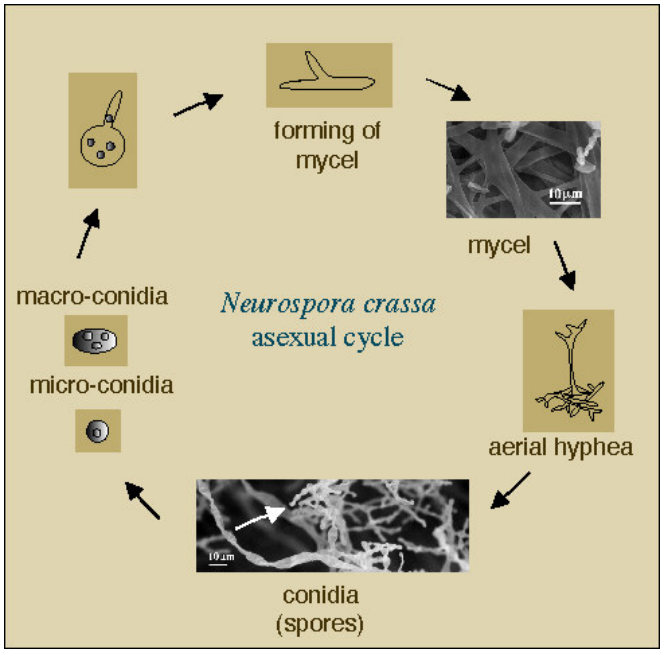


Fig 1.9-1: The asexual life cycle of *Neurospora crassa* [6]**.**

The rhythm is circadian with a period of ca 22 h (time of each cycle), and temperature compensated between temperatures of 20˚C to 32˚C. At the molecular level, the FRQ oscillator is a feedback loop that consists of negative and positive elements. The positive elements promote transcription of the *frq* gene, whereas the negative elements block its transcription. The elements of the feedback loop are the *frq* gene, its mRNA and its protein FRQ. Other key elements are transcription factors WC1 and WC2 and the protein complex WCC that results from the assembly between WC1 and WC2. The WCC complex is the positive element in the feedback loop. Its binding to the *frq* promotor starts transcription of *frq*. Dimerized FRQ is a negative element in the feedback loop as its presence in the nucleus blocks transcription of *frq* by inhibiting the WCC complex.. A more detailed description of the feedback loop follows.

At late night subjectively, levels of *frq* mRNA is low, WC1 and WC2 to the protein complex WCC. WCC binds to the *frq* promotor in two places and transcription of *frq* begins. Levels of *frq* mRNA is low at first but peak in the morning. FRQ protein appears in the cytoplasm and eventually dimerizes and interacts with the helicase FRH that is thought to stabilize FRQ and ensure its steady state [51, 53, 54]. Dimerized FRQ –FRH complex is transported into the

nucleus where its FRH mediates FRQ interaction with WCC [53]. This induces phosphorylation and subsequent splitting of the WCC. This splitting blocks *frq* transcription by inhibiting the WCCs ability to bind to the *frq* promotor. At mid day the WCC activity is at its lowest. FRQ synthesis occurs as long as there are *frq* mRNA present and FRQ levels peak at the end of the day.

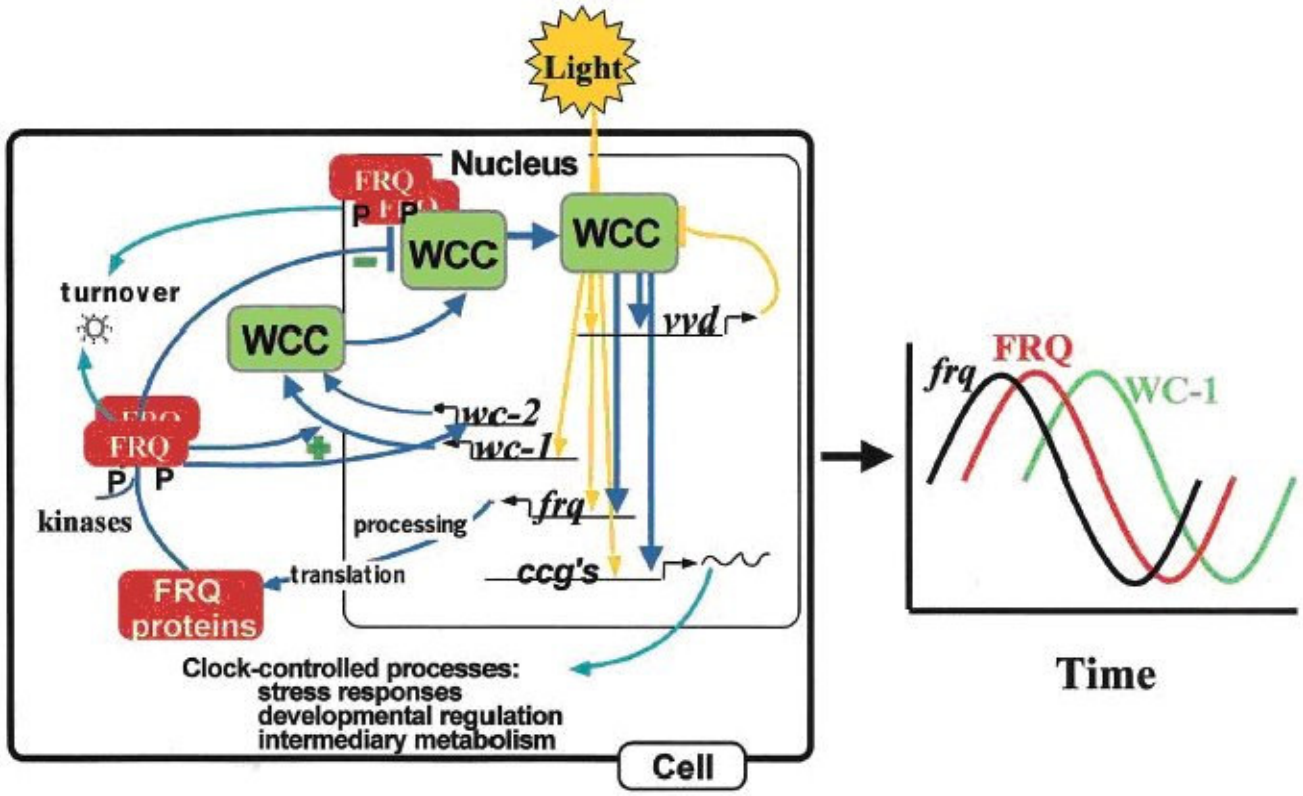


Fig 1.9-2: A model of the FRQ oscillator. The action of helicase are not shown here [13]**.**

FRQ is also believed to be involved in the promotion of WC-1 translation from an already existing *wc-1* mRNA message. The resulting low amplitude WC-1 protein rise and peaks in the night when FRQ levels are at the lowest. This result in high levels of FRQ phosphorylated inactive WCC. Eventually the turnover of FRQ is achieved by CKI, CKII (casein kinase I and II), CAMK1 and PRD4 targeting FRQ for degradation by the proteaosome by phosphorylation [51].

When FRQ disappears the FRQ mediated WC-1 synthesis and blockage of the WCC activity is lifted and the activated WCC can initiate the next cycle by binding to the *frq* promotor and resume transcription of *frq* [50].

The WC-2 remains constant due to its stability. FRQ phosphorylation and degradation is linked to both WCC reassembly and FRQ stability. FRQ stability is again linked to the property of temperature compensation. WC1 blue light receptor, maintain rhythm in the dark. Mutations in *frq* of sites may lead to change in period length, low amplitude or arrhythmic conidiation in DD conditions. Phosphorylation of these sites negatively regulates function of WC-1 and is important for function of the clock [55].

1.10 FRQ-Less oscillators (FLOs)

In later years the existence of multiple oscillators in *Neurospora crassa* has been discussed and hotly debated. It has been stated earlier that the FRQ oscillator are the core clock of the circadian system and that other oscillators independent of FRQ are slave oscillators [43, 56]. Many have argued that the FRQ oscillator is not essential for the *Neurospora crassa* circadian clock. One argument is that the FRQ oscillator may be a component of a system of connected oscillators instead of being the core circadian clock [43, 57]. This discussion is still going on and scientists are divided on the subject.

An example of a possible FRQ-Less oscillator (FLO) is the nitrogen induced nitrogen reductase activity in *Neurospora crassa* [43, 58]. The hyphal branching rhythm may also be a FLO but the underlying mechanisms of what drives the rhythm and its independence of *frq* needs to be further analyzed.

A FRQ-Less oscillator is not necessarily circadian in as to the trait of temperature compensation and it may take some time for the rhythm to appear after inoculation as in the case of the nitrogen reductase rhythm in *frq* knock out mutants [43, 58].

1.11 Sorbose

Sorbose is a monosaccharide, ketose (keto-hexose), with the molecular formula $C_6 H_{12} O_6$, and a molar mass 180.156. The IUPAC- name is (*3S, 4R, 5S*)-1, 3, 4, 5, 6-Pentahydroxyhexan-2-one and is mostly found in ring form

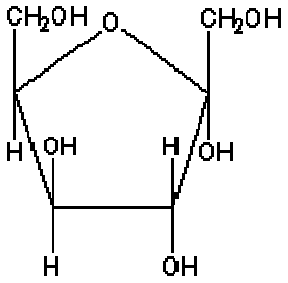


Fig 1.11-1: Sorbose (ring form).

Sorbose is most often used in the L-form, L- sorbose, which is most biologically active.

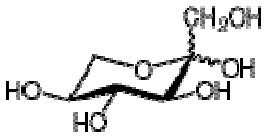


Fig 1.11-2: L –Sorbose.

L- sorbose is very similar to the important sugar glucose and is considered a glucose analogue.

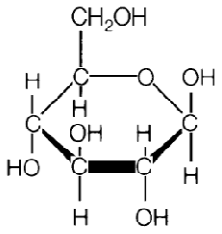


Fig 1.11-3: Glucose.

Sorbose is not abundant and is rarely used in nature but is found in the pectin of *Passiflora edulis* (passion fruit) (Fig 1.11-4), *Sorbus aucuparia* (mountain ash) (Fig 1.11-5) and from sorbitol by fermentation with *Acetobacter suboxydans*.

Fig 1.11-4: ***Passiflora edulis.***

Fig 1.11-5: *Sorbus aucuparia.*

L-sorbose is an intermediate in the commercial production of Vitamin C (ascorbic acid) [35]. Commercially sorbose is also used indirectly as a constituent of cotton, cotton fabrics, paper and paperboard that contact dry food [36]. Medically sorbose has been used by doctors to reduce the high pressure in the eyeball in glaucoma. Sorbose is known to give a positive effect on patients with diabetes[37].

[35] http://www.medilexicon.com/medicaldictionary.php?t=82940
[36] http://www.eafus.com/newindex.asp?message=detail&ad_id=1710
[37] http://www.the-tree.org.uk/BritishTrees/rowan.htm

1.11.1 The use of sorbose in fungal genetic research

Sorbose is used extensively in fungal genetic research as a tool for selection. Sorbose causes *Neurospora crassa* to grow in tight colonies on solid agar [59]. The colonial growth allows for easy selection of transformed colonies [36]. Fig 1.11-6 shows how sorbose is used for selection.

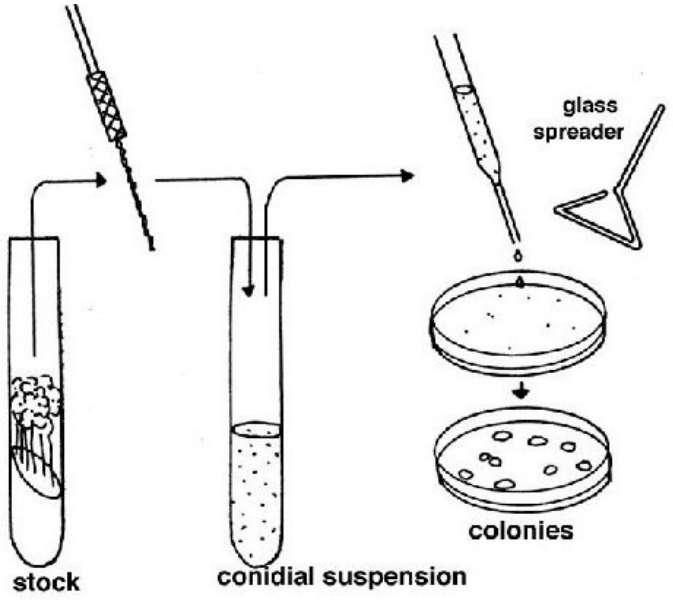


Fig 1.11-6: Plating procedure to obtain single colonial isolates [38].

Sorbose was used to produce a more robust signal of luciferase in experiments with the FRQ oscillator [60].

[38] http://www.fgsc.net/Neurospora/SECTION%20D1.htm

1.11.2 Effect of sorbose on the morphology of *Neurospora crassa*

Sorbose causes *wt Neurospora crassa* to grow slowly and in restricted colonies on agar medium. The sorbose causes *Neurospora crassa* to branch extensively. When examined with a fluorescence microscope, the hyphae of sorbose grown *Neurospora crassa* is thicker with deformed hyphal cells (Fig 1.11-7). The hyphae are also shorter than “normal” cells. The profilation from vegetative hyphae to aerial hyphae that eventually lead to conidiation are few and far between in sorbose grown *wt Neurospora crassa* [36] This may be caused by a weakening of the cell wall locally, so that the cell wall is not strong enough to support growth upwards. The failure to produce aerial hyphae my also be a lack in energy needed or damage in the circadian output that induces the switch to aerial hyphae.

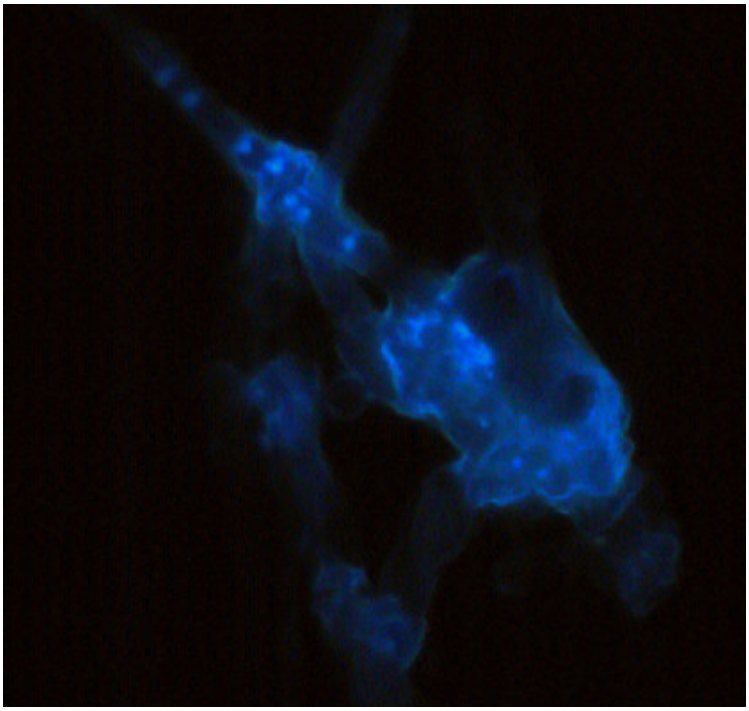


Fig 1.11-7: The effect on *wt Neurospora crassa* in sorbose media. Image by the author.

1.11.3 The effect of sorbose on the *Neurospora crassa* cell wall

The actual mechanism behind the morphological and altered growth pattern is not fully understood but it is believed that one major cause is that sorbose seems to affect the balance of cell wall components, by inhibiting enzymes involved in cell wall synthesis, and strongly reducing the amount of β-1, 3- glucan which is an integral part of the fungal cell wall [7, 9, 36, 61].

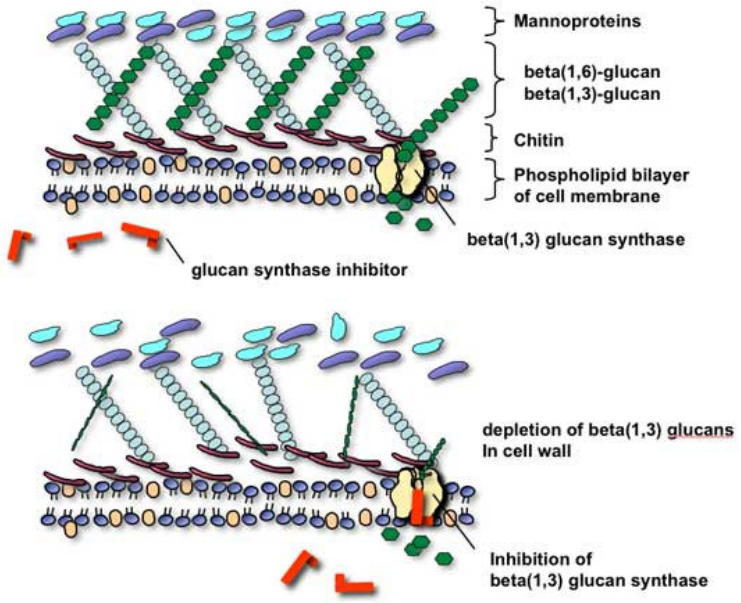


Fig 1.11-8: Components of the fungal cell wall. Depletion of β-1,3 -glucans in the cell wall, and inhibition of β-1,3 -glucan synthase [39].

Sorbose is an uncompetitive inhibitor and probably inhibits glucan synthase in the stage of continued polymerization of β-1,3 -glucan [27, 62].The inhibition seems to take place without sorbose being chemically changed [36]. This results in a weakening of the cell wall and renders it unable to withstand osmotic pressure and may influence the morphology and growth of the fungus.

[39] http://www.doctorfungus.org/thedrugs/antif_pharm.htm

1.11.4 Sorbose and carbon metabolism

The direct influence of sorbose on carbon metabolism in *Neurospora crassa* is unclear. Since sorbose is thought to be unmetabolizable, the effect it could have is unlikely to be a direct cause of the difference in molecular build. Sorbose does not inhibit phosphorylation of fructose. Sorbose has no known effects on any of the enzymes in the primary carbon metabolism [36].

Some have theorized that sorbose may cause uncoupling of oxidative phosphorylation and respiration [36]. This is because there are many similarities between sorbose and compounds that cause such uncoupling. The role of oxidative phosphorylation and respiration is to produce ATP which provides crucial energy to biological processes. Oxidative phosphorylation and respiration is the formation of ATP from the flow of electrons to oxygen (Fig 1.11-9). Partial uncoupling of this process can cause production of less utilizable energy. An increase in O_2 consumption is also seen in partial uncoupling of oxidative phosphorylation and respiration. Insufficient energy caused by this uncoupling may lead to inability drive metabolic processes that maintain integrity of the cell wall [9].

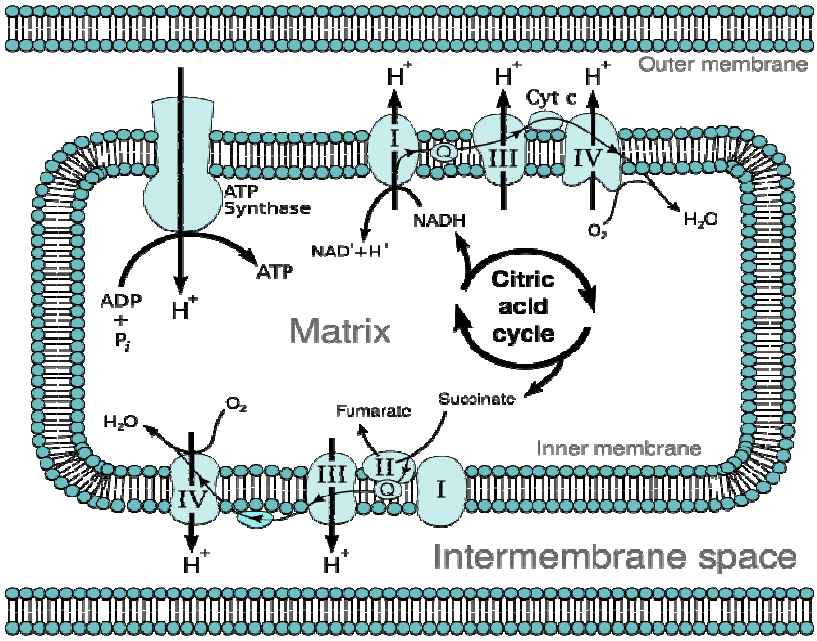


Fig 1.11-9: Oxidative phosphorylation and respiration [40].

[40] http://en.wikipedia.org/wiki/Oxidative_phosphorylation

1.11.5 Sorbose metabolism in *Neurospora crassa*

Sorbose is considered a nonmetabolizable sugar. Sorbose is not phosphorylated in *Neurospora crassa* [35, 36], a step that is essential for the glucolytic metabolism of glucose and fructose. This lack of phosphorylation may be the reason for the fact that sorbose not being used in metabolism. There have been reports however of strains of *Neurospora crassa* being able to metabolize sorbose via sorbitol, but only after considerable time of adaptation [9]. The thought is that instead of being metabolized in most *wt* strains of *Neurospora crassa*, the sorbose is accumulated and left free to inhibit enzymes like glucan synthase and possibly other reactions.

1.11.6 Sorbose resistant *Neurospora crassa* mutants

Sorbose resistant mutants can be a valuable tool in the search for the reason for the effect of sorbose on *Neurospora crassa* in genetic terms. Several sorbose resistant mutants exist. The mutants presented here are the ones most likely to be relevant for this thesis. The mutants strain *T9* has been investigated for the excretion of extra cellular enzymes and it was found that this strain was resistant to the colonizing effect of sorbose [63]. This resistance was explained by a change in the cell wall composition of the mutant. The change in cell wall (or cell membrane) rendered the strain resistant to sorbose but did also make the fungus more sensitive to high osmolarity in the medium, higher activity of extra cellular acid phosphatase. The fact that some sorbose resistant mutants like the *sor* mutants do not have altered levels of β -1,3 -glucan the cell wall and this is thought to be the reason for the resistance to sorbose in these mutants. Some *sor* mutants were able to metabolize sorbose to glucose. This may be another reason for the sorbose resistance.

2 Materials and methods

Neurospora crassa strain *wt-328-4A* from The Dunlap laboratories (Dartmouth medical school), and the mutant strain *frq*^{10} *7490*A from Fungal Genetics Stock center (FGSC), was used in this study. Wild type means that the strain has no known mutations, manipulated or natural, that would lead to any special behavior. The mutant strain *frq*^{10} is an *frq* knock out mutant and express no functional FRQ protein. The mutant strain is thus seen as arrhythmic and non temperature compensated.

2.1 Culture methods solid agar medium

2.1.1 Race tube experiments:

To ascertain if the growth rate and period length of *wt Neurospora crassa 328-4A* were differed in Vogel media with and without sorbose, and Feldman media with and without sorbose clean race tubes filled with the respective media was marked with media type, autoclaved for 12 minutes, balanced, and left a day for the agar to stiffen. The tubes were then gathered, and inoculated with *wt-328- 4A* which had been grown in Horowitz media glass tube for at least 3 days on the laboratory bench or in constant light at 25˚C. After inoculation the tubes were placed in incubators at 19˚C, 24.5 ˚C and 29˚C in DD conditions for ca 7 days for sucrose control, 7-10 days for Feldman control, and 14 to 49 days for sorbose media. The controls were marked daily for growth fronts, whereas the sorbose race tubes were marked the 1st day, the 3rd day, after 14 days and the last day. The race tubes were photographed next to a ruler for the purpose of documentation.

The *wt* controls where only tested at 24.5˚**C** because it is well documented that *wt Neurospora crassa* when grown on the Vogel control medium in DD conditions show temperature compensation with a period of ca 22 h in the temperature range of 18-32˚**C [5, 30, 43, 64, 65].**

Fig 2.1-1: Race tubes. Image by Stein-Ove Bratthammer.

To test if the hyphal branching rhythm may or may not be independent of *frq,* the knock out mutant where inoculated in race tubes containing Vogel minimal control medium or Vogel sorbose/sucrose medium. These race tubes were then placed in DD conditions at 29˚C. The controls were marked everyday, whereas the sorbose tubes were only marked the 1st, second and the last day. The race tubes were photographed next to a ruler for the purpose of documentation.

Fig 2.1-2: Horowitz tubes with *Neurospora crassa* cultures ready for inoculation on race tubes. Image by Stein-Ove Bratthammer.

2.2 Determination of the growth rate and period length

The growth rate of the conidiation rhythm was determined, by dividing the distance between the first and the last marking on the number of days between markings. The period was determined by multiplying by 24 h, the number gained by dividing the distance between the first and the last sporulation band on the product of growth rate multiplied by the number of spaces between the sporulation bands (Fig .2.2-1).

The period of the hyphal branching rhythm was determined in the same way except that instead of sporulation bands, the "wave fronts" was the period defining entity.

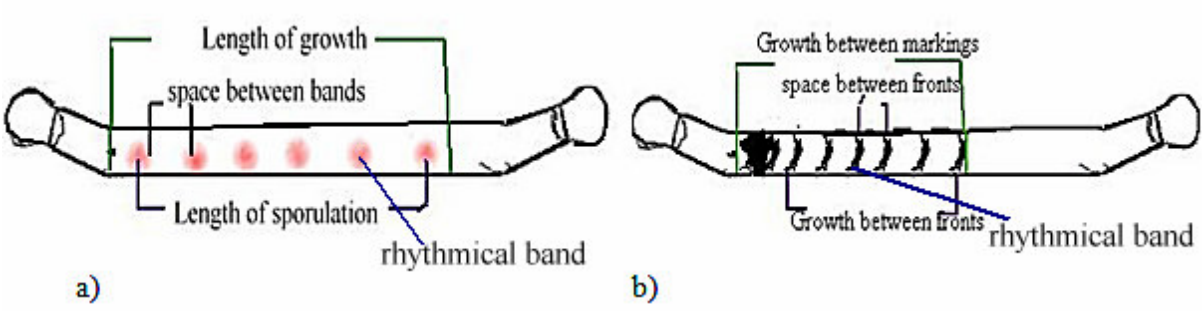


Fig 2.2-1: Visualization of measurements of growth rate on a) minimal Vogel control media. b) Sorbose media, sorbose and Feldman sorbose. Image by the author.

The *frq* ^{10} strain is usually grown in longer race tubes. This is because the sporulation occurs after a considerable induction time [66]. In this experiment the object was to investigate whether there was significant difference between the growth of *frq* ^{10} on media with and without sorbose.

Since the object was to test the theory that the hyphal branching rhythm is independent of the *frq* ^{10}allele, the growth pattern of the first week were of most importance, and there was therefore no need to grow *frq* ^{10} long enough for conidiation to occur. Fig .2.2-2: show the difference between banding in normal *bd* strain, and some mutants with altered or deleted *frq* alleles [66]. The *frq* ^{10} is a knock out mutant and shows no conidial banding in the same time span when grown in Vogel minimal control medium and sorbose/sucrose medium.

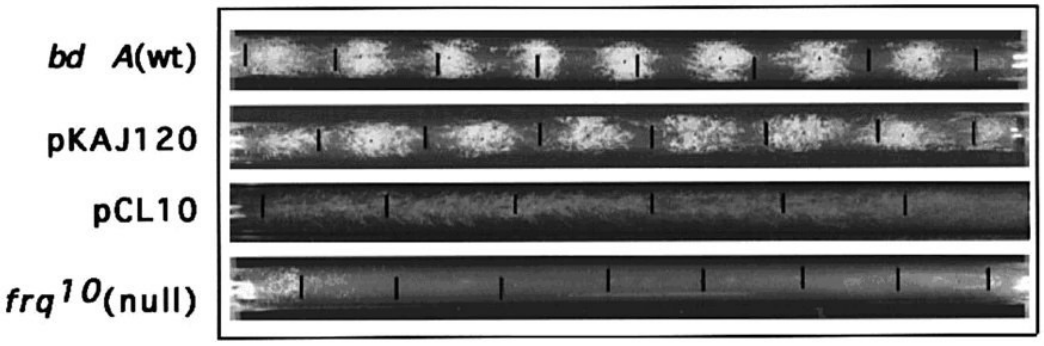


Fig 2.2-2: Different strains of *Neurospora crassa*. Period and growth pattern in DD conditions [66]**.**

Because there was no banding in *frq* ^{10} on control medium, only growth rate were determined in the *frq* ^{10} control. Growth rate and period was determined in the sorbose grown *frq* ^{10}.

A trial with Feldman sorbose media containing no sucrose was conducted at DD conditions at 24.5 ˚C and 29˚C to see if the sucrose had any effect on the growth of *wt* on sorbose media.

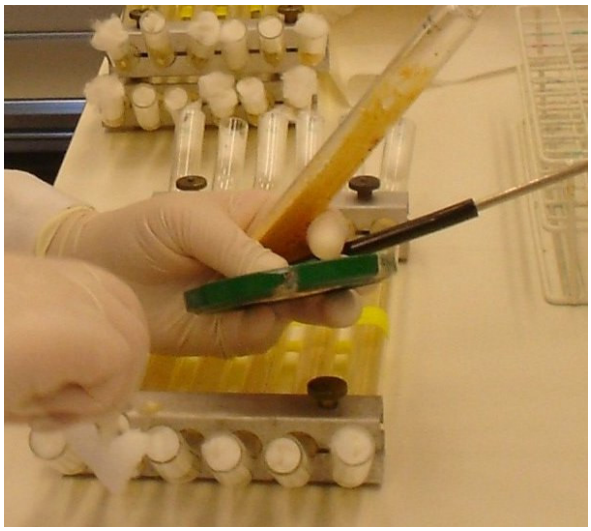


Fig 2.2-3: The author inoculating race tubes.
Image by Grete Falkeid.

2.2.1 Solid media for race tubes

Vogel control medium

for normal conidiation rhythm

(in 200 ml H_2O)

sucrose	0.8g
Vogel salts 50 X	4ml
arginine (1 mg / ml)	400µl
agar	3g

Vogel sorbose medium

hyphal branching rhythm

(in 200 ml H_2O)

sorbose	1.0g
sucrose	0.2g
yeast extract	1.0g
Vogel salts 50 X	4 ml
agar	3g

Vogel only sorbose medium

hyphal branching rhythm

(in 200 ml H_2O)

sorbose	1.0g
yeast extract	1.0g
Vogel salts 50 X	4ml
agar	3g

Feldman control medium [5]

conidiation rhythm

in 200ml H_2O

sodium acetate	2.4g
sucrose	2.0g
difcocasaminocaids	1g
casein digest	1.0g
glycerol	2.0g
yeast extract	1.0g
Vogel salts 50x	4ml
agar	3g

Feldman sorbose medium [5]

hyphal branching rhythm

in 200ml H_2O

sorbose	1.0g
sucrose	0.2g
agar	3.0g
yeast extract	1.0g
Horowitz complete salt 10 x:	20ml

Horowitz complete salt 10 x [5]

in 250ml H_2O

ammonium tartrate	12.5g
ammonium nitrate	2.5g
KH_2PO_4	2.5g
magnesium sulfate 7h2o	1.25g
sodium chloride	0.25g
calcium chloride 2.h2o	0.325g
biotin	0.25ml
trace element solution	0.25ml

2.3 Petri dish experiment

The difference of growth on solid agar in petri dishes was also investigated. Petri dishes were filled with autoclaved Feldman sorbose/sucrose media. *Neurospora crassa wt 328-4A* grown for 3 days in Horwitz tube at 27˚C and LL, was inoculated in the middle of the discs. One disc was placed in DD at 24.5˚C, and another disc at 27˚C in LL. After 6 days the dishes were examined and photographed.

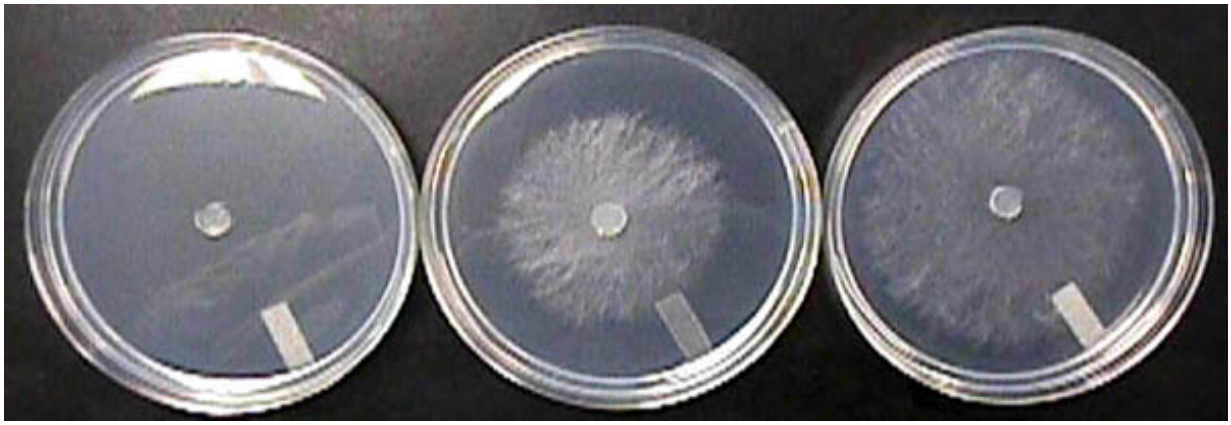


Fig 2.3-1: Inoculated *Neurospora crassa* on petri dishes with sorbose medium.

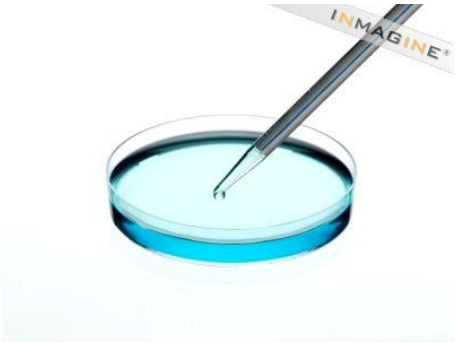


Fig 2.3-2: Inoculation of petri dish.

2.4 Liquid culture media

2.4.1 Shaking cultures

Shaking cultures were prepared for growth of *wt 328-4A* in liquid Vogel minimal media with or without sorbose. Autoclaved 100 ml Erlenmeyer flasks were filled with 25 ml of the respective autoclaved media and inoculated with *Neurospora crassa wt 328-4A*. The flasks were put on a rotary shaker at 25 ˚C in LL conditions for ca 24 h.

The cultures were then photographed and used in microscopic experiments to examine the morphology.

Fig 2.4-1: Rotary shaker with Erlenmeyer flasks.

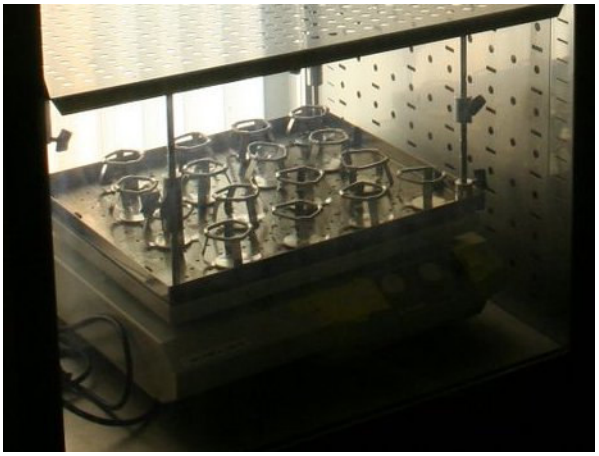


Fig 2.4-2: Rotary shaker in light cabinet. The cabinet can be set to give constant light. Image by Stein-Ove Bratthammer.

2.4.2 Shaking culture media

Vogel control media

(in 200 ml H_2O)

sorbose	1.0g
sucrose	0.2 g
yeast extract	1.0g
Vogel salts 50 X	4ml

Vogel sorbose media

(in 200 ml H_2O)

sorbose	1.0g
sucrose	0.2g
yeast extract	1.0g
Vogel salts 50 X	4ml

LL media

(in 1000 ml H_2O)

sucrose	20g
Vogel 1 X	20ml

2.5 DAPI dye method and fluorescence microscopy

Neurospora crassa shaking culture (ca 24 h) was dyed in the following way:
DAPI from freezer (1mg/ml), thawed and stirred, is diluted to 1:100, 1:10000 and 1:1000000 by filling 3 Eppendorf-tubes with 990 µl distilled H_2O and marking them with the respective dilution.

Table 2.5-1: Eppendorf tube number and the DAPI dilution factor.

Tube nr	1	2	3
Dilution	1:100	$1:10*10^3$	$1:10*10^6$

.

1. 10 µl DAPI pippetted over in tube 1. The tube is then shaken on whirler.
2. 10 µl from tube 1 is pippetted into tube 2. The tube is then shaken on Whirler
3. 10 µl from tube 2 is pippetted into tube 3. The tube is then shaken on Whirler

Preparing samples

1. Some of the *Neurospora* culture is transported to a filter paper for removal of access liquid.
2. The sample is then put on a slide and is spread so that single hypha can be distinguished.
3. The slide is marked with dilution factor and growth media.
4. 1 drop (30 µl) DAPI dilution is added to the sample on the slide.
5. A cover glass is placed over (avoid bubbles).
6. The slides are covered with aluminum foil (to avoid light intrusion).
7. After 15 -30 min the slides are ready for viewing in fluorescence microscope.

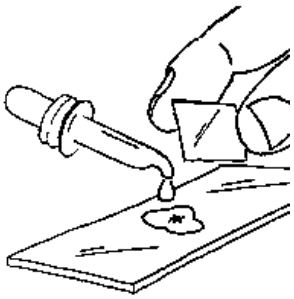


Fig 2.5-1: Preparation of samples on a microscope slide [41]

Microscopy

The samples were examined and photographed on Leica DC 100 DMLS Microscope at object size 100x/1.25 with oil. Photos of the microscopy were marked with date, dilution factor, and object size.

Fig 2.5-2: The Leica DC 100 DMLS microscope with computing software for photographing. Image by Stein-Ove Bratthammer.

41 http://www.cartage.org.lb/en/themes/Sciences/Physics/Optics/OpticalInstruments/Microscope/GlassSphere/usph_06.gif

3 Results

3.1 Results of period length compared with the Feldman study

This thesis is concerned mostly with the phenomenon of the hyphal branching rhythm as induced by the keto-hexose sorbose. The main point was to try to reproduce some of the results of work done by Feldman in the article "A direct comparison between circadian and noncircadian rhythms in *Neurospora crassa*" [5] where it is stated that the hyphal-branching rhythm induced by sorbose / sucrose media does not show circadian properties in contrast to the conidiation rhythm of the same strain

Feldman found that the hyphal-branching rhythm of *wt Neurospora crassa* when grown in DD conditions and under constant temperature, had marked difference in period, and was therefore not temperature-compensated. The period decreased greatly with increasing temperature and was well out of circadian range in the lower temperatures, in contrast with the conidiation rhythm which seems to only vary with a couple of hours and keep inside the interval defined as circadian throughout the temperature range, Growth is also non- linear and slow in sorbose induced growth as opposed to the linear rapid growth of the circadian conidiation rhythm that appears when the fungus is grown without sorbose. The results of the comparison are listed in Table 3.1-1

Table 3.1-1: Representation of results in this thesis and those form the Feldman study of period at different constant temperatures.

Results for period in hyphal branching rhythm of *wt Neurospora crassa* grown on Feldman sorbose/sucrose media in DD conditions						
Source reference Temp	**Feldman [5] 30˚C**	**This study 29˚C**	**Feldman [5] 25˚C**	**This study 24.5˚C**	**Feldman [5] 20˚C**	**This study 19˚C**
Period	**21.6±2.2 h**	**21.98±2.8 h**	**32.6±6.6 h**	**34.1±1.5 h**	**93.3±4.6 h**	**79.5±15 h**

When the data in Table 3.1-1 is plotted the resulting plot shows that the results of this study are close to that of the Feldman study. The plot is seen in Fig 3.1-1.

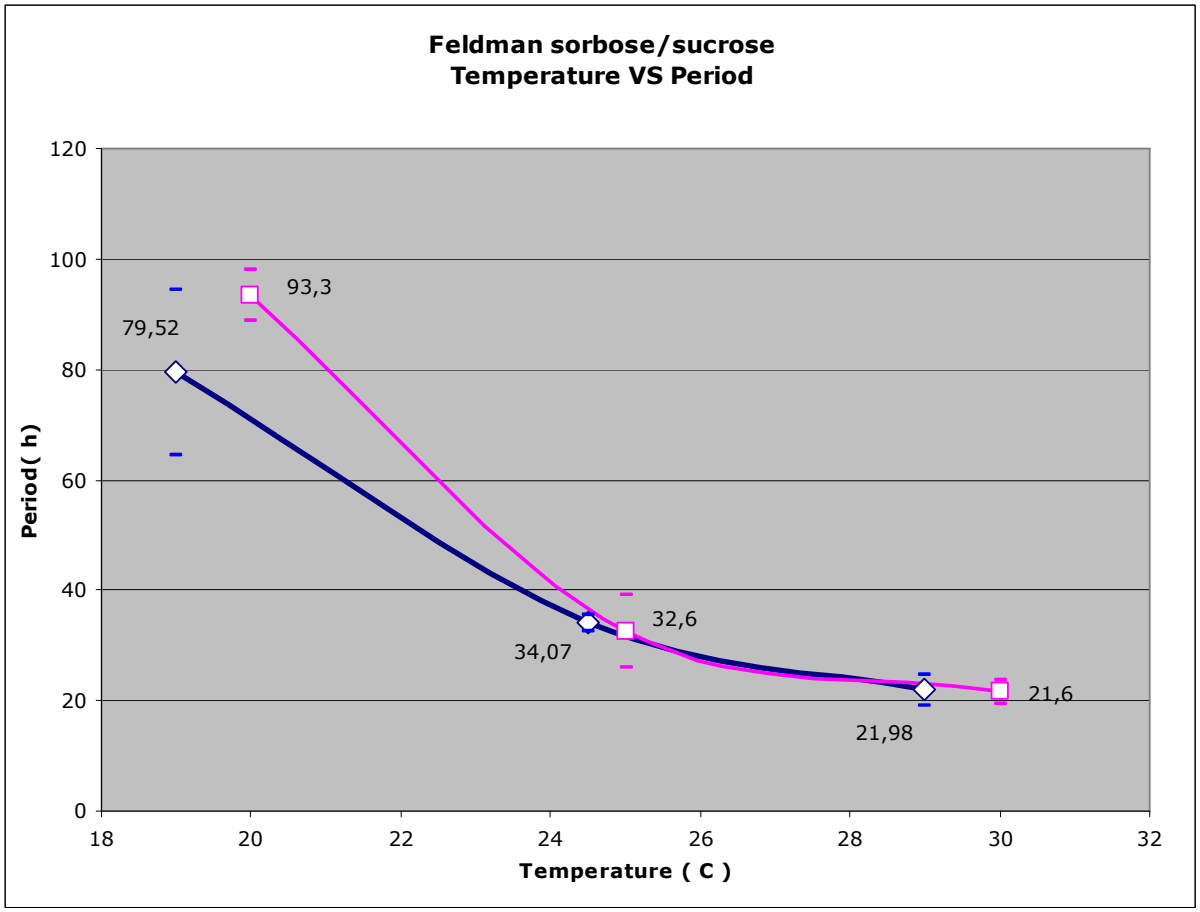


Fig 3.1-1: Graphic view of the comparison of the period in the Feldman study (pink line), and the experimental results in this thesis (blue line). The shorter segment of each color is a measure of the relevant standard deviation at each temperature.

The Feldman control had a period of 20.52 ± 2.88 h at 24.5˚C.

The influence of sucrose in the sorbose/sucrose medium was interesting. Therefore *wt Neurospora crassa* where grown on Vogel media containing only sorbose and no sucrose at two different constant temperatures in DD conditions. The experiments showed the same trend of increased period length with decreasing temperature although the periods were shorter than the ones from the experiments with sorbose/sucrose medium. In Fig 3.1-2 the "wave fronts" were clearer with seemingly loose spores of conidia at the "wave fronts" can be seen at the two constant temperatures.

The incubator cabinets used for this thesis were set to temperatures 20˚C, 25˚C and 30˚C but temperature recording devices recorded 19˚C, 24.5˚C and 29˚C. Because of this the results of this thesis are reported according to the recorded temperatures.

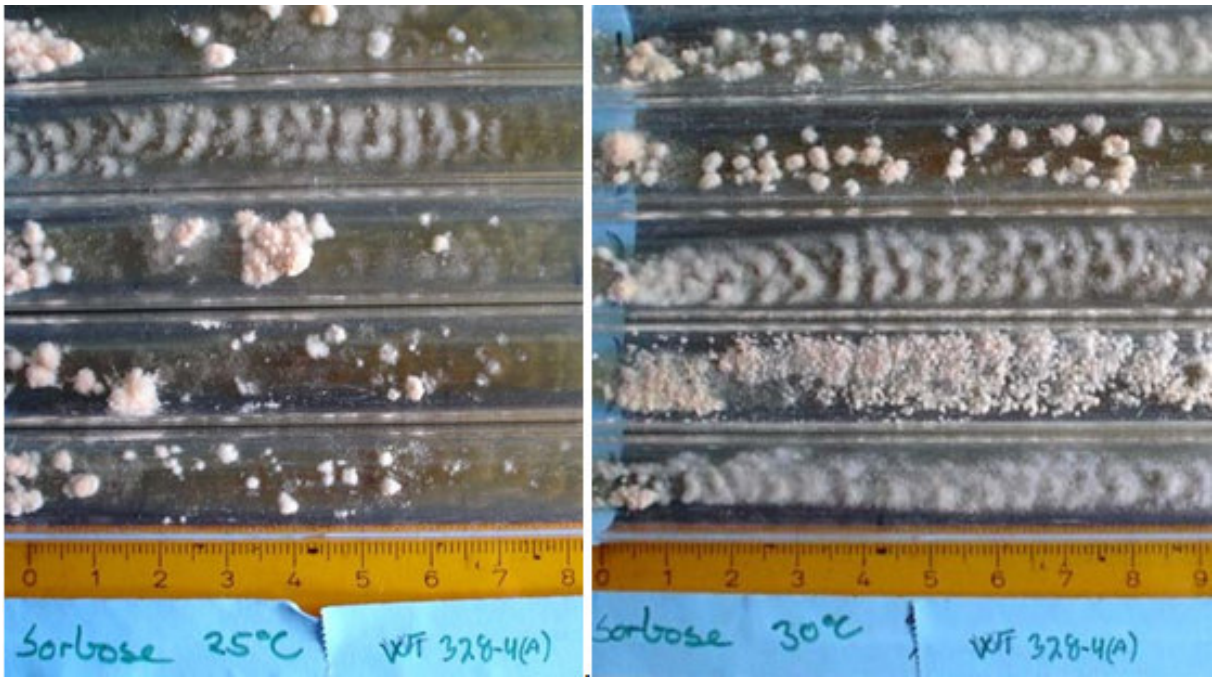


Fig 3.1-2: Photos of the hyphal branching rhythm in *wt Neurospora crassa.* The fungus is grown in sorbose media without sucrose in DD conditions in a) 24.5˚C and 29˚C for ca 22 days.

Table 3.1-2: Representation of period at two different constant temperatures with ***Neurospora crassa*** grown in medium containing only sorbose and no sucrose.

Results for period in hyphal branching rhythm of *wt Neurospora crassa* grown on Vogel only sorbose media		
Source **Temp**	**This study** **29˚C**	**This study** **24.5˚C**
Period	**18.05 ±3.39 h**	**24.5 ± 3.45 h**

Table 3.1-2 show the results of period of hyphal branching rhythm in race tubes with *wt Neurospora crassa* grown on only sorbose media. The resulting plot of period versus temperature shows the decrease of period length with decreasing temperature (Fig 3.1-3).

Period length for Vogel sorbose/sucrose medium was not assayed for other temperatures than 24.5˚C.

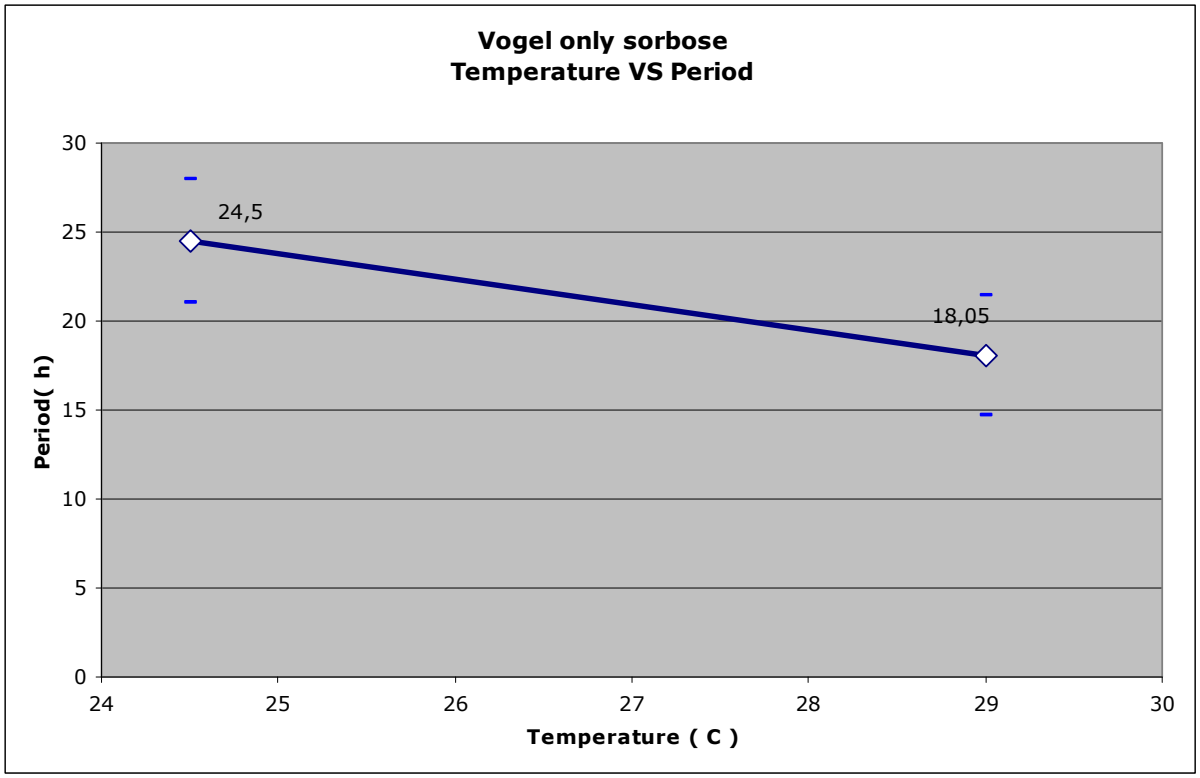


Fig 3.1-3: Graphic view of the experimental results in this thesis, (blue line). The shorter segments above and under the data points are the standard deviation at each temperature.

The results for the experiments of this thesis reflect the results Feldman found in his work which is that the period of the hyphal branching rhythm increases with decreasing constant temperature and is therefore not temperature compensated. The rhythm is not circadian in the sense that the periods are out of circadian range of 18 – 22 h at temperatures at and below 24.5˚C

3.2 The growth rate of sorbose grown *wt Neurospora crassa*

Growth of *wt Neurospora crassa* on Vogel minimal control medium was 4.54 ± 0.1 cm/day. Growth on Vogel minimal control medium gave a normal conidiation rhythm with conidiation ca every 21h. The growth of *wt Neurospora crassa* on Feldman control medium was 2.86 ± 0.45 cm/day. Feldman control medium had almost continuous extensive conidiation and this may account for the reduced growth rate. When *wt Neurospora crassa* where inoculated in sorbose medium, the growth rate where considerably lower. Growth rate in Vogel sorbose medium was higher than the growth rate in Feldman sorbose medium. Growth rate in sorbose medium at 24˚ C was 0.39 ± 0.45 cm/day. Growth rate in Feldman sorbose medium was 0.23

± 0.006 cm day at 24˚C. Growth rate of *wt Neurospora crassa* on Vogel sorbose medium without sucrose was 0.41± 0.03 cm/day.

3.3 Morphology of wt Neurospora crassa

3.3.1 Morphology of *Neurospora crassa* on solid media

When *Neurospora crassa wt – 328 –4(A)* were grown in race tubes on minimal Vogel control media, the normal growth morphology was evident. The mycelium grew with a clear growth front in a network of hyphae until aerial hyphae appeared and normal conidiation commenced. This conidiation was rhythmic and periods about 22h.

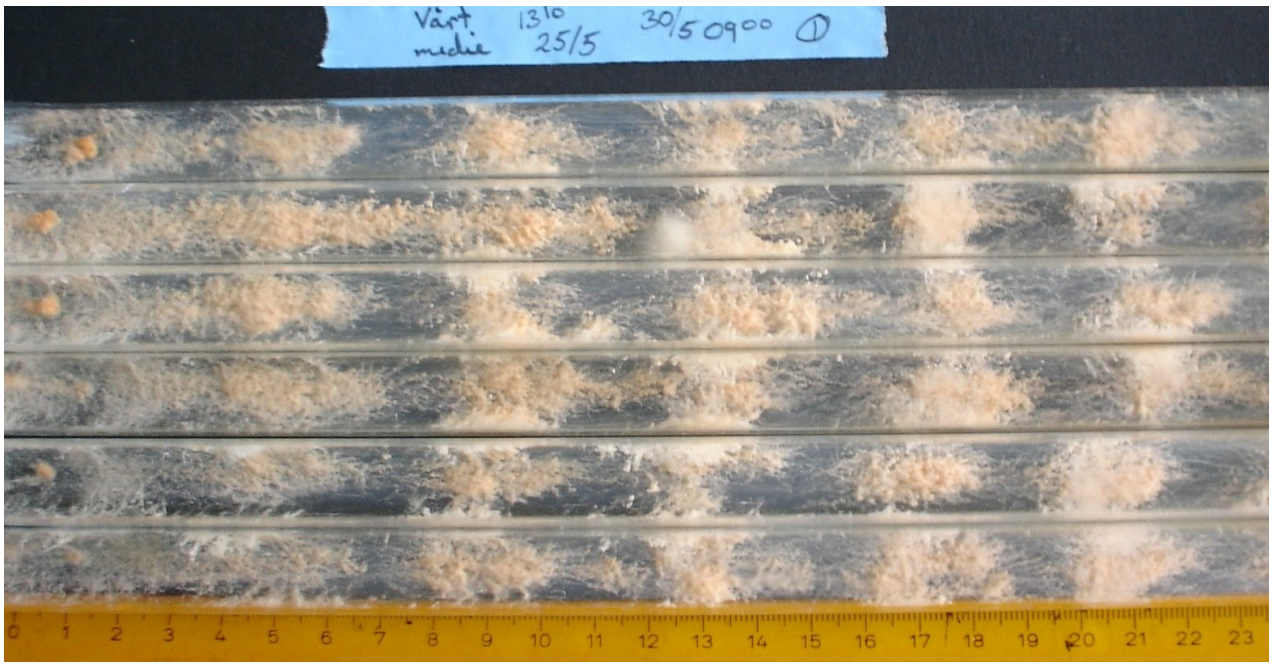


Fig 3.3-1: Normal growing *wt* on minimal Vogel control media at 25˚ C in DD conditions for 6 days. Image by the author.

The bands were clearly seen from the top of the race tubes (Fig 3.3-1) and from the bottom (Fig 3.3-2). The conidia were loose and powdery and would loosen and inoculate other areas of the race tube if the tube was moved rapidly.

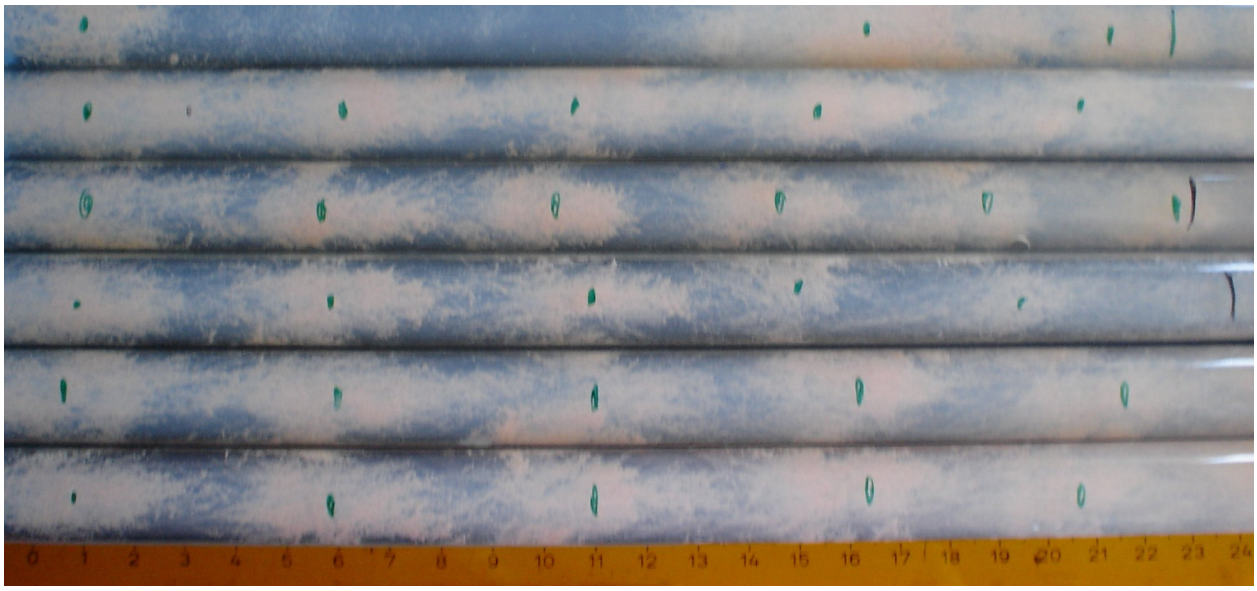


Fig 3.3-2: Normal growing *wt* on minimal Vogel control media at 25˚ C in DD conditions for 6 days. View from under the race tube. The banding is seen clearly as dots of conidia. Image by the author.

Feldman control media for conidiation grew slowly and very inherent. The myriad of extra nutrients in this media may explain the overflow of conidia. The massive conidiation may explain the slow growth, as *Neurospora crassa* is known to slow down when conidiation occur Fig 3.3-3 and 3.3-5 .

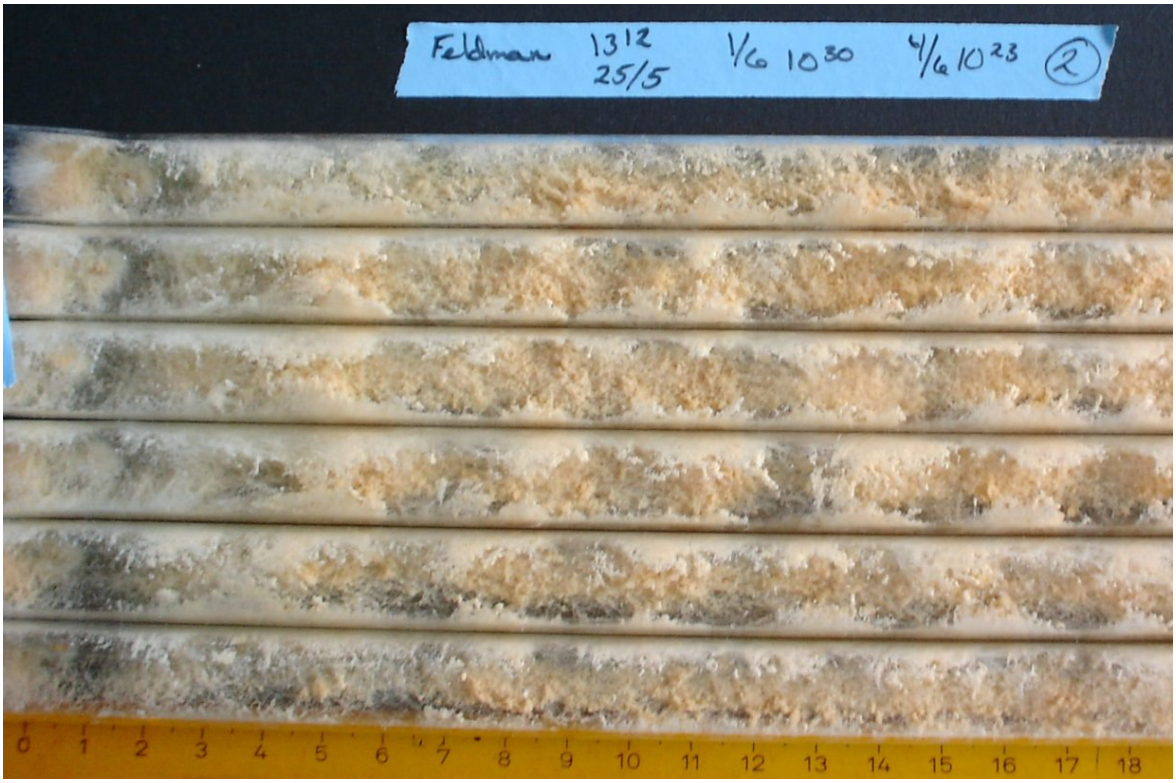


Fig 3.3-3: ***wt Neurospora crassa*** **on Feldman control media at 24.5˚ C in DD conditions for 10 days. View from above front of race tubes. Image by Grete Falkeid.**

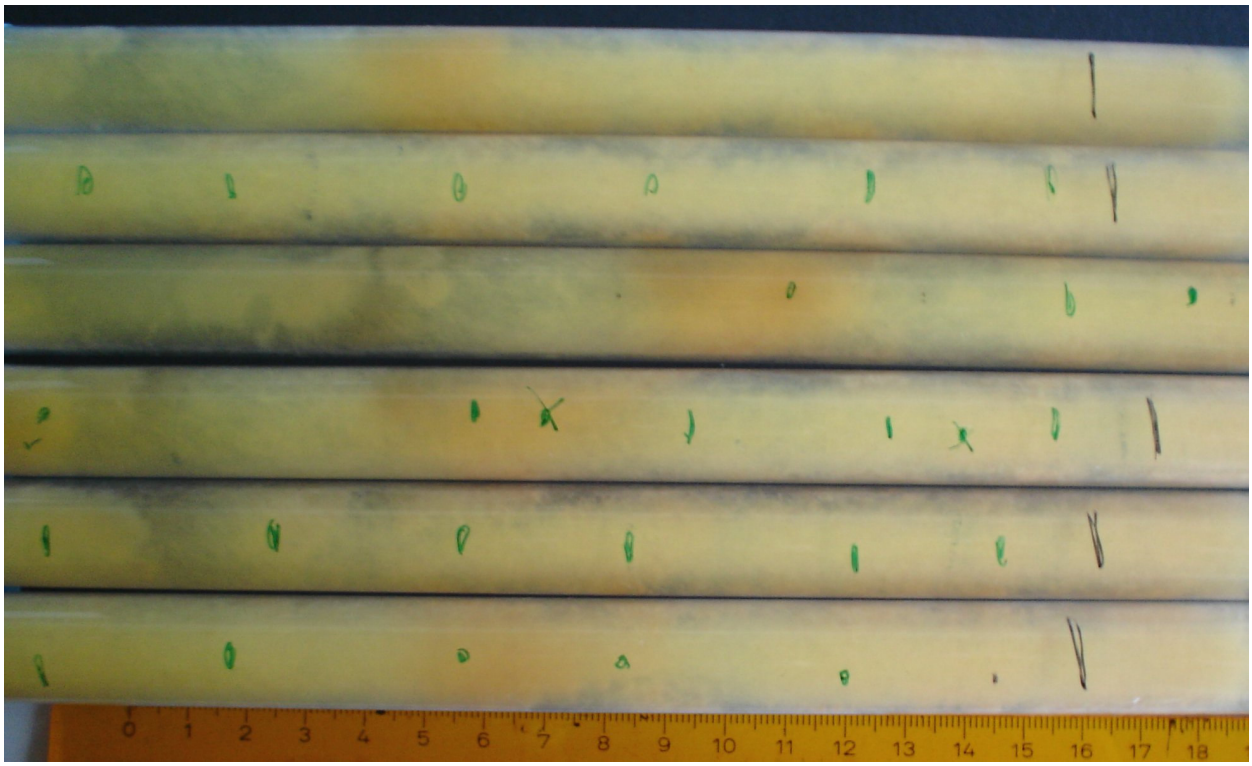


Fig 3.3-4: : ***wt Neurospora crassa*** **on Feldman control media at 24.5˚ C in DD conditions for 10 days. The bands were difficult to mark. View from under the race tubes. Image by the author.**

When grown on sorbose in race tubes the characteristic pattern of bands like “wave fronts” appeared. A view from the top of the race tubes shows these “wave fronts” and occasional tight conidiation hives on individual colonies. This conidiation came a few days after the “wave fronts”.

Fig 3.3-5: *Neurospora crassa wt* grown on sorbose media in race tube on solid media. Image by the author.

The wave “front” pattern is more easily seen from under the race tube from the bottom of the tube.

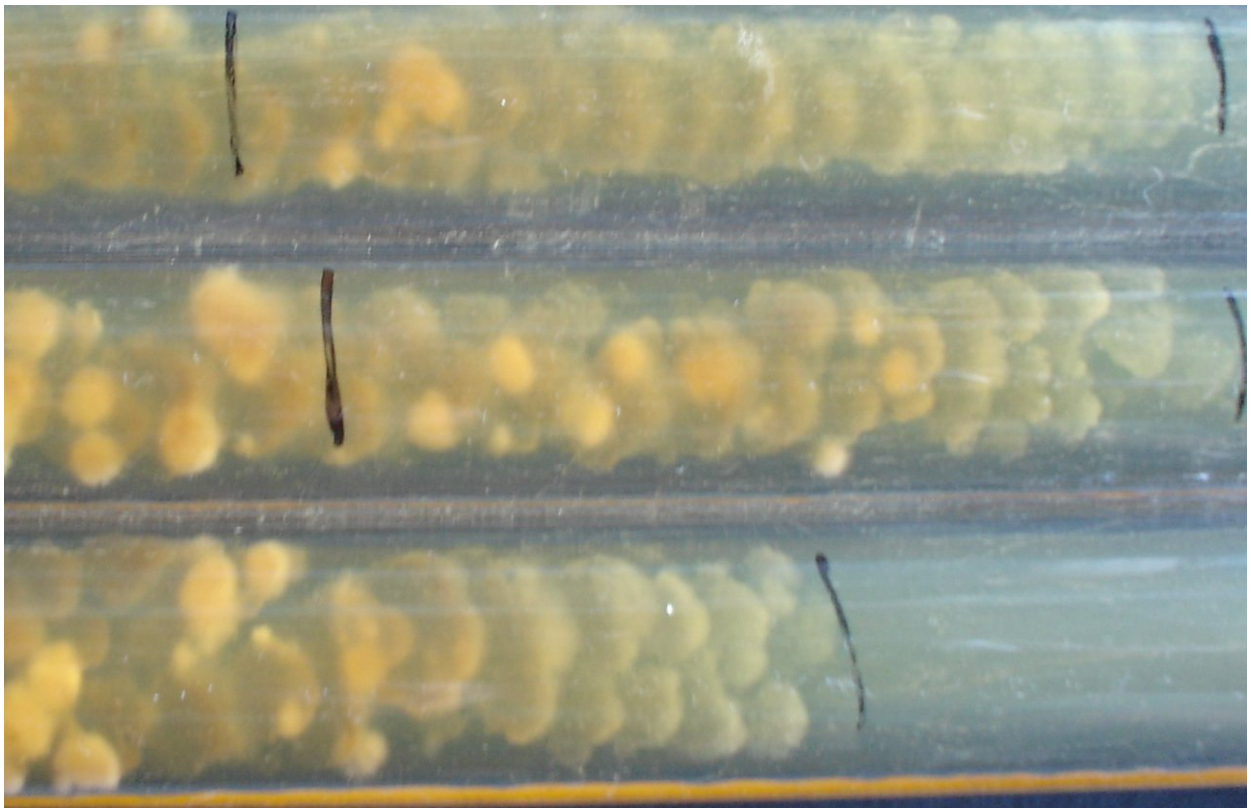


Fig 3.3-6: *wt Neurospora crassa* grown on sorbose media in race tube on solid media. View from under the race tube. The "wave fronts" can be seen clearly. Image by the author.

Wt Neurospora crassa also has a very special way of growing in race tubes. It seems to grow down into the media, for then to appear at the surface again before initiation of the next cycle

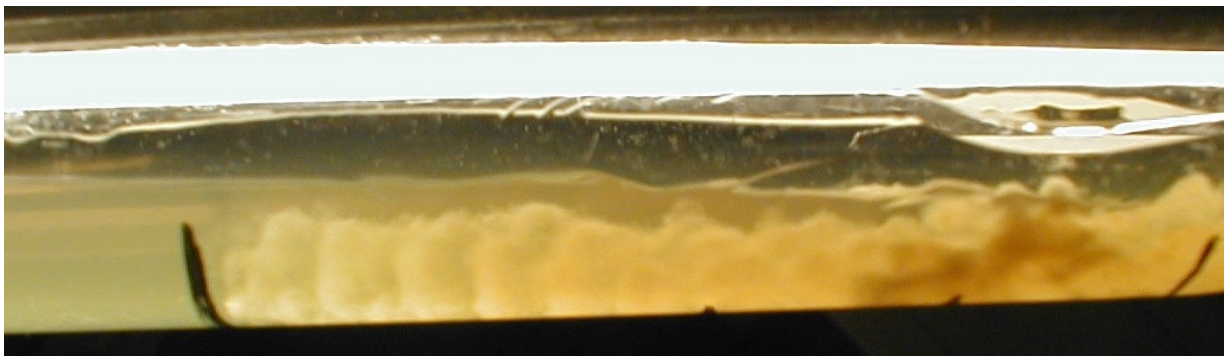
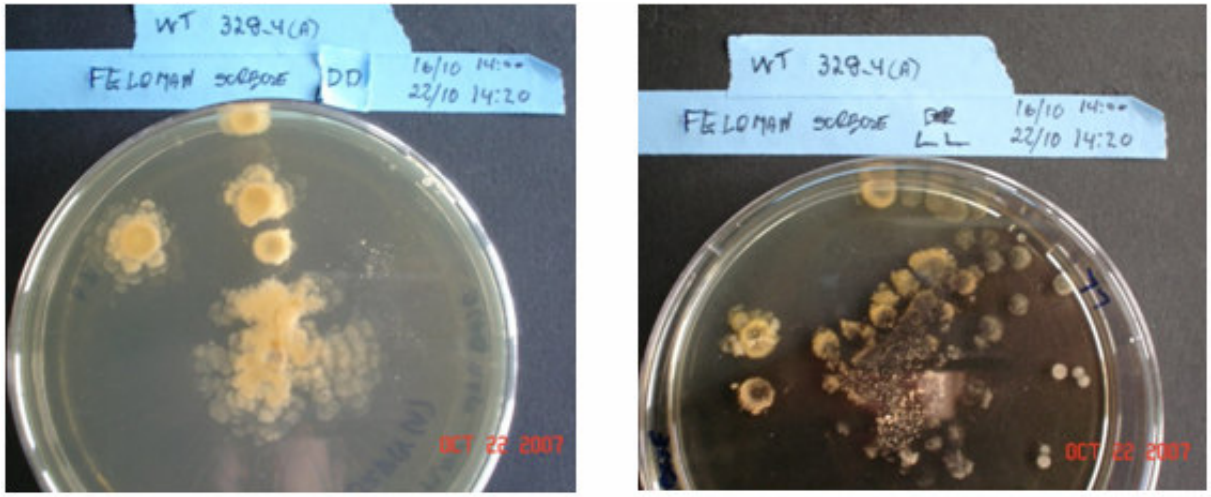


Fig 3.3-7: *wt Neurospora crassa* grown on sorbose media in race tube on solid media. The fungus grows down into the media and not on the surface as on the Vogel minimal control media without sorbose. Image by the author.

3.3.2 Morphology of *Neurospora crassa* on petri dish

When *Neurospora crassa wt* is inoculated in the middle on minimal solid agar media on a petri dish, the fungus grows outward in circular zones. When the growth media is sorbose /sucrose medium, the fungus grows slower and unfolds like a flower. In DD the growth is slow and seems to be downwards into the media. Conidiation occurs on patches on the surface and appear to be loose and normal. This may be because the conidiation occurs on islands formed of colonies that have appeared above the surface. In LL conditions it seems the fungus grow more on the surface and not in a flowery arrangement. This may be due to drought.



a) b)

Fig 3.3-8: *wt Neurospora crassa* on Feldman sorbose/sucrose media in DD conditions (a) and in LL conditions (b) in 24.5˚C for 6 days. Image by the author.

On Feldman sorbose solid media in petri dishes *wt Neurospora crassa* looked like rosettes with petals in DD conditions. In LL conditions the colonies seemed dryer and did not make these rosettes. This may be because the LL dish contained less medium than the DD dish. Sorbose grown *wt Neurospora crassa* tend to grow down into the media rather than on the surface. The dryness of the LL dish may be to lack of nutrients rather than the constant light conditions.

3.3.3 Morphology of *Neurospora crassa* in liquid cultures

When sucrose/sucrose media is autoclaved, the liquid becomes brownish in color.
This is said to have no effect on the growth and morphology of *Neurospora crassa*. The difference between *Neurospora crassa* grown in liquid shaking cultures with or without

sorbose seems to be that in the LL media (only sucrose the fungus grow more threadlike and looser than in the sorbose/ sucrose media. The sorbose grown Neurospora grow in thick strings or pellets (Fig 3.3-9).

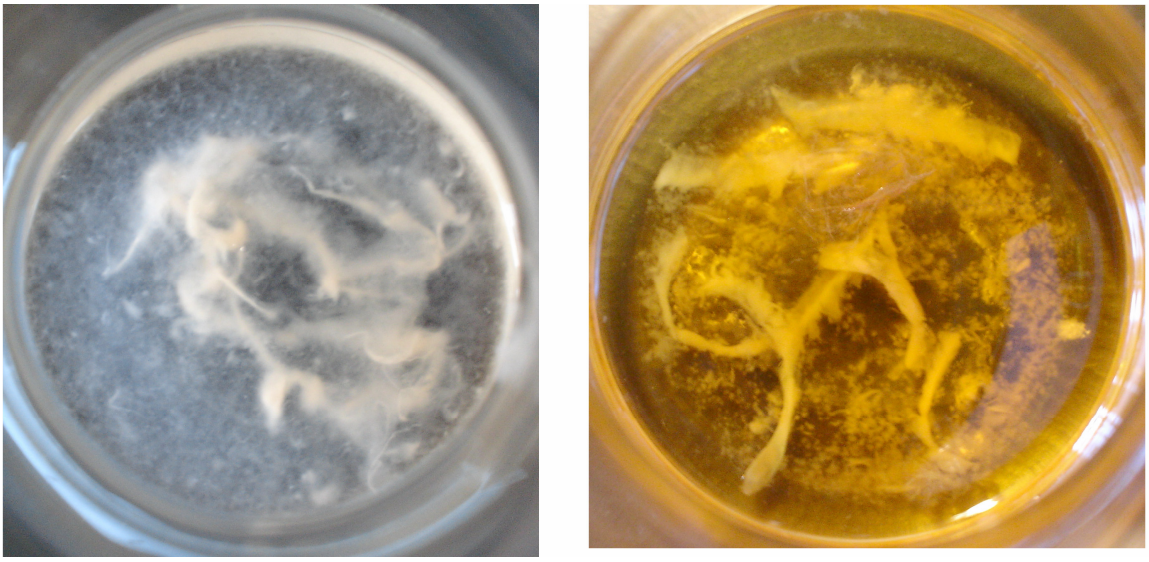


Fig 3.3-9: On the left is *Neurospora crassa* grown in shaking culture with LL media in 24 h. On the right sorbose / sucrose media grown *Neurospora crassa* 24 h in LL conditions. Image by the author.

3.4 Microscopy

DAPI dye allows the DNA in the nuclei of *Neurospora crassa* to be seen as blue colored elements under a fluorescence microscope. This again can be useful when determining morphology differences on hyphae grown in culture with or without sorbose.

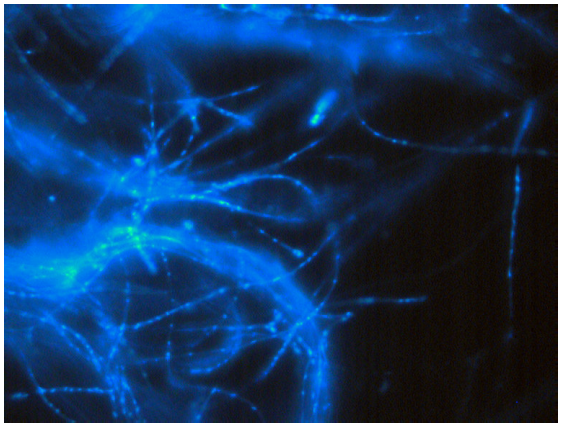


Fig 3.4-1: DAPI dyed 1:100 dilution, *Neurospora crassa wt 328-4A* shaking culture (24 h in LL media) Viewed at 40 x enlargement. Image by the author.

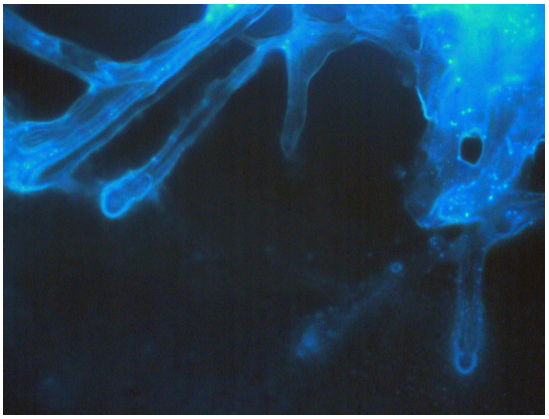


Fig 3.4-2: DAPI dyed 1:10000 dilution. *Neurospora crassa wt 328-4A* shaking culture (24 h in sorbose/sucrose media).Viewed at 100 x enlargement. Image by the author.

Neurospora crassa grown in LL medium for 24 h in LL conditions grow normally with long straight hyphae that branch. When sorbose/sucrose media is used, the hyphae become distorted and lumpy. It looks like the cell wall integrity is compromised and that there is extensive branching at the growing tip (Fig 3.4-1 to 3.4-6).

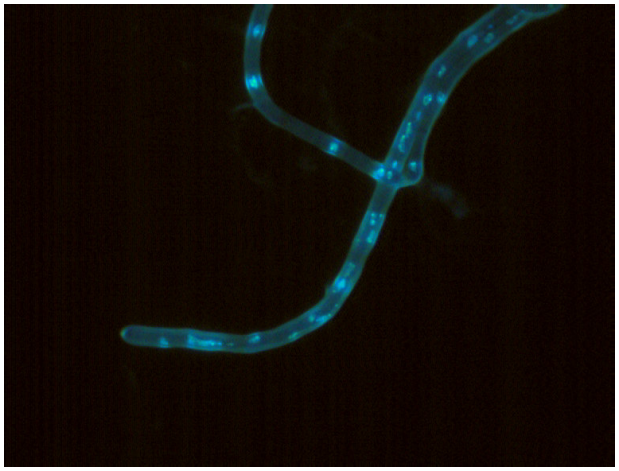


Fig 3.4-3: DAPI dyed 1:103 dilution, *Neurospora crassa wt 328-4A*. Shaking culture (24 h in LL media). Viewed at 100x enlargement. Two single hypha. Image by the author.

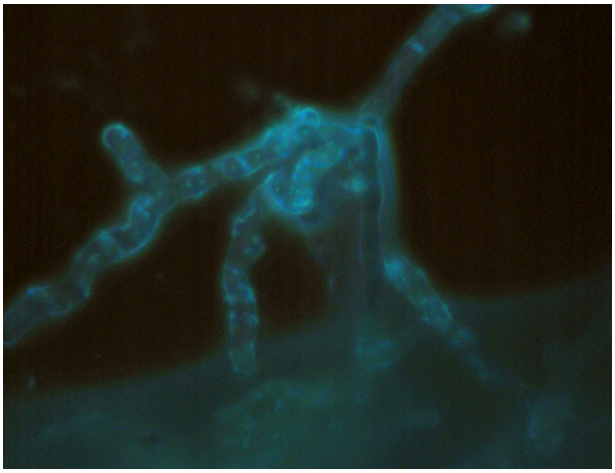


Fig 3.4-4: DAPI dyed 1:103 dilution, *Neurospora crassa wt 328-4A*. Shaking culture (24 h in sorbose/sucrose media). viewed at 100x enlargement. Hyperbranched hypha. Image by the author.

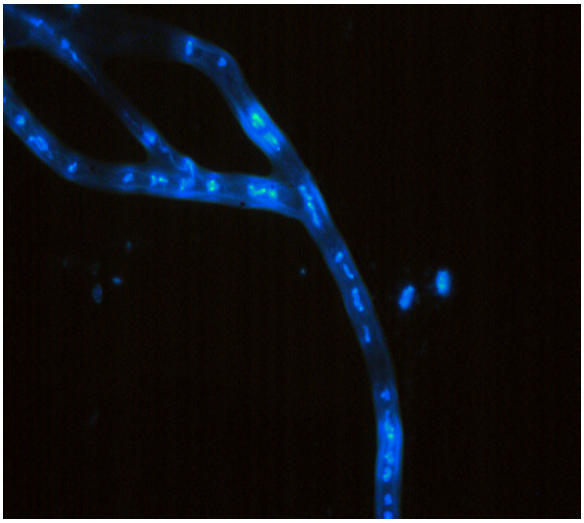


Fig 3.4-5: DAPI dyed 1:103 dilution, *Neurospora crassa wt 328-4A s*haking culture (24 h in LL media)Viewed at 100x enlargement. Branched hypha. Image by the author.

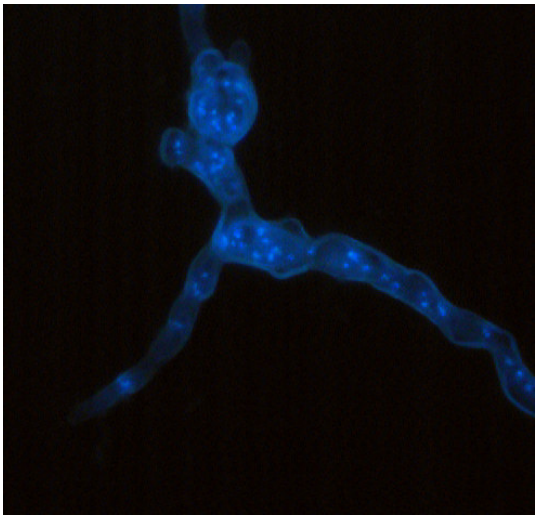


Fig 3.4-6: DAPI dyed 1:103 dilution, *Neurospora crassa wt 328-4A*. shaking culture (24 h in sorbose/sucrose media) viewed at 100x enlargement. Branched hypha. Image by the author.

3.5 The influence of *frq*^{10} on the hyphal branching rhythm

A *frq* knock out mutant *frq*^{10} was used to determine the influence of *frq* on the hyphal branching rhythm. A set of race tubes were filled with Vogel minimal control media, and one set with Vogel sorbose/sucrose media. Both sets were inoculated with *Neurospora crassa frq*^{10}, and left at 29˚C in DD conditions for 5 to 7 days.

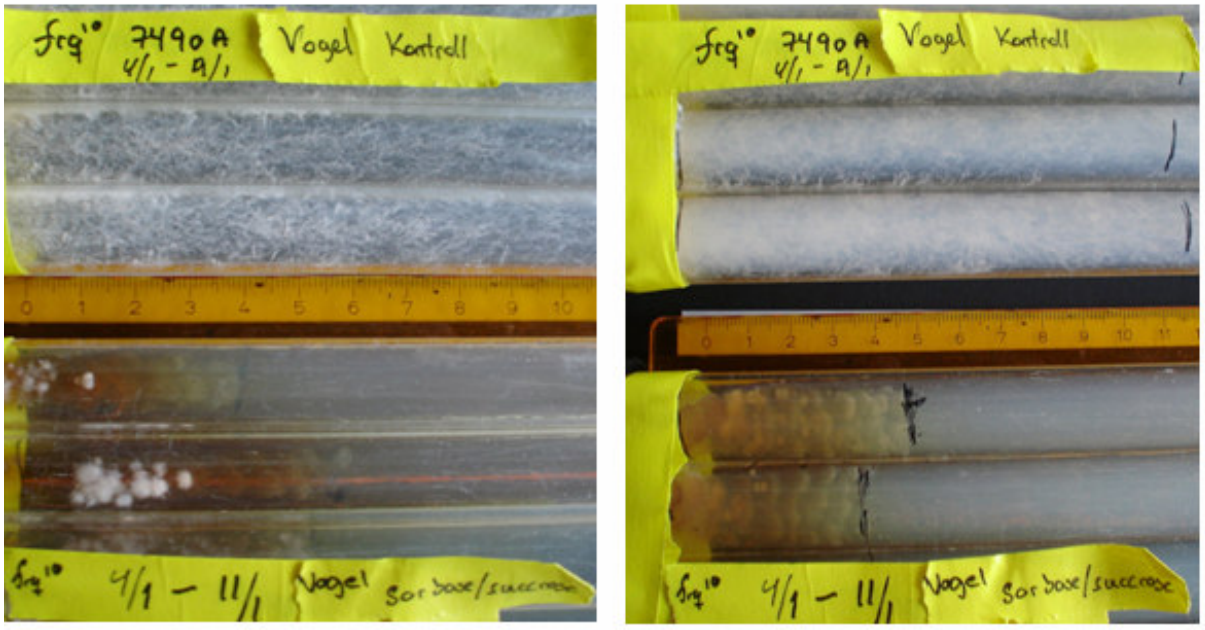


Fig 3.5-1: Comparison of race tubes inoculated with *Neurospora crassa frq*^{10} on media without (top) and with sorbose (bottom). Photo is from the top of the race tubes, the front a) and below the race tubes b). Image by the author.

Neurospora crassa frq^{10} grown in Vogel minimal control medium had normal spreading mycelium with no conidial banding. While the *Neurospora crassa frq*^{10} grown in Vogel sorbose /sucrose media showed the hyphal branching rhythm (Fig 3.5-1)

4 Discussion

One explanation for sorbose inducing extensive hyphal branching may be due to weakening of the cell wall due to β -1,3 -glucan depletion which again may lead to changed turgor pressure and increased flow of vesicles. The counterpart of turgor pressure is the synthesis and modulation of the cell wall. If these latter was disturbed, turgor pressure may lead to apical hypherbranching [20]. Sorbose resistant mutants exists that have no altered level of β -1,3 –glucan in its cell wall and that can metabolize sorbose to glucose [62]. These mutants strengthen the idea that the effect on morphology of *wt Neurospora crass* on sorbose media is most probably due to the weakening of the cell wall and /or failure to metabolize sorbose to glucose. If cell wall weakening and lack of metabolism is linked directly to the running of the hyphal branching rhythm is however unclear.

4.1.1 Carbon metabolism

The connection between sorbose induced morphology and carbon metabolism is not clear. It has been established that sorbose does not inhibit any of the enzymes in the main metabolic route of the glycolysis. The link between carbon metabolism and the sorbose induced hyphal branching rhythm have not been found in the literature used it this thesis. A gene responsible for the circadian control of a step in glycolysis is a link found explaining any circadian control of carbon metabolism. The ccg-7 gene controls the NADH + H^+ producing of conversion of glyceraldehyde-3-phosphate to 1,3- diphospoglycerate by glyceraldehyde-3-phosphate dehydrogenase in glycolysis. Studies have been preformed that showed that ccg-7 expression is not influenced by glucose starvation or metabolic stress. If sorbose causes glucose repression or starvation, this is then uninteresting in relation the ccg-7 and linking to circadian control of carbon metabolism.

4.1.2 Repression of glucose metabolism and glucose transport

It is unclear if sorbose causes glucose repression in *Neurospora crassa*. Glucose is used less efficient if sorbose in present in the growth medium [9, 36]. If this is caused by repression of the glucose metabolism or by glucose transport genes are also unclear. Changes in the excretion of invertase that converts sucrose to glucose and fructose may be another reason for less uptake of glucose in the medium. A medium containing both glucose and fructose in addition to sorbose allows *Neurospora crassa wt* to grow with almost normal spreading

morphology. It is not clear if the hyphal branching rhythm still exists or if it disappears in the same conditions.

4.1.3 Possible uncoupling of oxidative phosphorylation and respiration

Tatum and Crocken [9] reported that *wt Neurospora crassa* show an increase in O_2 consumption when grown in sorbose. The rise in O_2 consumption have been proven with other compounds that cause partial uncoupling of oxidative phosphorylation and respiration The ability of sorbose to cause partial uncoupling of oxidative phosphorylation and respiration is not extensively proven [36].

4.1.4 *frq*^{10} and the hyphal branching rhythm

This study has revealed that the hyphal branching rhythm is not dependent on *frq*, because the rhythm is present in an *frq*-knock out mutant of *Neurospora crassa*. This finding is based on visual observation of the hyphal branching rhythm grown in race tubes with sorbose in the growth media.

Luciferase experiments have proved that oscillations of *frq* occur even in nonbanding strains of *Neurospora crassa* [60]. In the luciferase experiments mentioned a sorbose assay was developed to improve signal strength of the luciferase light emissions when *frq* mRNA oscillated. Results presented in this thesis have indicated that the hyphal branching rhythm is independent of *frq* since it appears in the *frq* knock out mutant *frq*^{10} on sorbose medium but not on Vogel minimal control medium. The appearance of the hyphal branching rhythm in *frq*^{10} does not rule out the presence of the FRQ oscillator in sorbose grown *wt Neurospora crassa*, only that the hyphal branching rhythm may be independent of *frq*. The FRQ oscillator may be masked by the hyphal branching rhythm, the morphological changes overriding the output of the *frq* oscillator. The failure to produce a normal free running conidiation rhythm in DD conditions may also be due to damage, repression or inhibition of the output of the FRQ oscillator at the same time as the oscillator responsible for the hyphal branching rhythm is operating. This thesis can find no proof that sorbose is the cause of this failure.

4.2 Reproducibility of the results in the Feldman study

The results concerning the period of the hyphal branching rhythm where in accordance with the Feldman study. Although not all the data where exactly the same, the trend of period length changing with changing constant temperature where confirmed. The Feldman control media for conidiation rhythm did conididate extensively which made measurement of any conidial rhythm difficult. The extensive conidiation in Feldman control medium may explain the reduced growth rate since conidiation causes the fungus to stop growing or slow down growth during conidiation.

Period length in Vogel sorbose medium was not calculated for other temperatures than 25 C. Growth rate in Vogel sorbose medium was higher than the growth rate in Feldman sorbose medium.

4.3 Explanations for loss of temperature compensation

4.3.1 Defective protein degradation

No experiments were conducted in relation to this thesis that involved the actual stability of clock proteins or the possible inhibition by sorbose on the proteaosome facilitated degradation of any clock protein. Experiments included only the visual observation of the period in race tube experiments with or without sorbose and in three different constant temperatures. This produced a temperature/period curve that proved loss of temperature compensation. the temperature /period profile show that the period increases with decreasing constant temperature.

The temperature compensation profile of the *wt* grown on sorbose /sucrose in this study is very similar to the profile of *wt* grown. In the presence of lithium the stability of FRQ increases which again leads to a lengthening in the circadian period, and loss of temperature compensation. The inhibition of the proteaosome which facilitates the degradation of the clock protein in any biological oscillator may also produce changes in period and temperature compensation. It may also be that the organism is drained of metabolism created energy required for a fully functional degradation machinery and thereby inducing a lengthening of the period and a loss of temperature compensation.

The pattern of increasing periods with decreasing temperatures are also evident in non temperature compensated FLOs indicating that the hyphal branching may be a FLO. This is in accordance with the findings in this thesis that the hyphal branching rhythm is present in a *frq*-knock out mutant.

Whether it is increased FRQ stability or inhibition of the ubiquinone proteaosome is unclear.. Defective protein degradation machinery may also explain the period length and temperature compensation loss if another oscillator other than the FRQ/WC oscillator is involved which seems to be the case with the results in this study from the *frq*-knock out mutant experiments where the hyphal branching rhythm was present when the strain used would not produce a functional FRQ protein.

Other reasons for loss of temperature compensation

Changing the activation energy is also a way of disturbing temperature compensation. There is no documentation on sorbose as an agent to changing the activation energy of the reactions involved in the hyphal branching rhythm.

4.4 Reliability of experimental results

Period length from race tube experiments was more difficult to record with the sorbose induced hyphal branching rhythm in *wt Neurospora crassa* and *frq*^{10} than the conidiation rhythm for the same strains in Vogel control media. The conidiation rhythm is normally very easy to assay whereas the bands show clearly as bundles of red conidia every 22 h and a mark is set in the middle of each bundle. The hyphal branching rhythm, the outskirts of the colonies are considered the bands. Race tube experiments with sorbose media where conducted over long periods often over several weeks. When the race tubes where finally assayed, some of the earlier bands disappeared probably because of autolysis or senescence. The growth rate, banding type and senescence of the hyphal branching rhythm makes the reliability of the research less reliable than the results from the conidiation rhythm. The differences between the period lengths in different constant temperatures of the sorbose induced hyphal branching rhythm where however significant enough to state that the sorbose induced hyphal branching rhythm in *Neurospora crassa wt* and frq^{10} follow the pattern of reduced period length with increasing temperature and the loss of temperature compensation.

4.5 Future work

This thesis has been focused on the influence of sorbose on the hyphal branching rhythm in *Neurospora crassa*.

The practical work in this thesis included visual observations of conidiation rhythm and the hyphal branching rhythm. No enzyme assays or determining of mRNA was preformed to ascertain the levels of FRQ protein or *frq* mRNA in response to exposure to sorbose in the media. To determine if there is FRQ oscillation in *wt Neurospora crassa* when grown on sorbose media, more work should be done in the form of mRNA and protein measurements. A micro assay of the genes expressed in connection with the sorbose induced hyphal branching rhythm would be of advantage to determine if there are clock controlled genes expressed. Further testing with fully codon optimized luciferase would give indication of *frq* activity in relation to the hyphal branching rhythm.

Race tube experiments with *Neurospora crassa frq*^{10} should be preformed. In the experimental phase of this thesis the *frq*^{10} experiment where only done for one constant

temperature. frq^{10} experiments conducted in more than one constant temperature would give a picture of temperature compensation.

5 Conclusion

The focus on this thesis has been on the effects of sorbose on *wt Neurospora crassa*. The main concern has been the attempt to reproduce results from a study by Jerry. F Feldman in 1974 where he reported a poorly temperature compensated hyphal branching rhythm that appeared when *wt Neurospora crassa* were grown in a sorbose/sucrose containing medium. Results on period length and temperature compensation from the experiments in this thesis where compared to the results in the Feldman study. The results in this thesis have confirmed Feldman s findings on the lack of temperature compensation of the sorbose induced hyphal branching rhythm. The possible cause or causes of the hyphal branching rhythm has been explored by researching literature and not by practical experiments or calculations.

Race tube experiments with the *Neurospora crassa* knock out mutant strain frq^{10} was conducted in the practical part pf this thesis to test is the sorbose induced hyphal branching rhythm is dependent on the *frq* gene and its products in DD conditions at 29˚C. The hyphal branching rhythm appeared in frq^{10} grown on sorbose /sucrose media but not when $frq^{10}$was grown on Vogel minimal control medium. The period of the sorbose induced hyphal branching rhythm was lower in frq^{10} than in that of the *wt* at 29˚C and DD conditions. In Vogel minimal control medium frq^{10} showed no banding rhythm in the duration of the experiment, neither hyphal branching rhythm nor conidial banding, but showed normal spreading mycelial growth. The appearance of the hyphal branching rhythm in frq^{10} can imply that the hyphal branching is a FRQ-Less oscillator independent of *frq*.

The changed morphology and hypherbranching of *wt Neurospora crassa* grown in sorbose media was in accordance with the supplied literature. An interesting feature was the observation that the fungus grew into the growth medium when grown on sorbose. This observation is unexplained in this thesis and available literature.

The conclusion is that the hyphal branching rhythm is, as Feldman found, is a poorly temperature compensated rhythm possibly independent of the clock gene *frq* and its products. If the sorbose induced hyphal branching rhythm is a true biological rhythm, the question is whether it is a FRQ-Less-oscillator, another kind of biological oscillator or a pattern that appears to be a rhythm but which is not controlled by a genetic clock. Further work should be done to get more information about what drives the rhythm.

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Glossary

Absorption zone:
The region behind the hyphal tip where the bulk of the nutrients is absorbed.

Agar:
Jelly (gel) produced by dissolving (usually 1-2% w/v) in hot water (or an appropriate solution of nutrients, when it is terms nutrient agar) an extract of the cell walls of certain red algae. Few fungi can break down the carbohydrate forming the gel matrix (a complex of galactans (polymers of galactose)), some of which are sulphated), so nutrient agar provides an admirable solid surface for mycelial growth. The low percetage of solid in agar means that it does not have much effect on water availability.

Apical growth zone:
The growing (extending hyphal tip)and the adjacent regions of the hypha that provide the metabolites and enzymes necessary for growth.

Autolysis:
Self-digestion of senescent hyphae.

Circadian:
“About one day”.

Conidium (plural **conidia**):
Exogenously produced spore, which is found exclusively in and is typical of fungi.

Dictyosome:
Organelle found in plant cells and fungi functionally equivalent to the Golgi apparatus of animal cells. Origin: gr. Soma = body a net-like structure in the cytoplasm of animal cells (especially in those cells that produce secretions).

frequency (frq):
A gene which expresses the FRQ-protein, where FRQ due to auto regulation (negative feedback loop) is assumed to be part of the circadian pacemaker in *Neurospora.*

Hypha (adjective **hyphal**; plural **hyphae**):
The individual unit of the filamentous growth form.

Mycelium (plural **Mycelia**):
Network of hyphae.

Senescence:
Ageing of hyphae often accompanied by autolysis.

Septum (plural **septa**):
Cross-division of hyphae with a central pore (not to be confused with a cross-wall where no pore is present).

Storage:
Accumulation of nutrients within one part of a cell/hypha, often in the form of polymeric material.

Vegetative state:
Mycelium in which there are no reproductive structures.

WC-1:
White Collar 1. A zinc finger protein that as transcription factor in various genes and as a blue light receptor in *Neurospora.* Associated with WC-2.

WCC:
White Collar Complex. The WCC is a heterodimer between the proteins White Collar -1 (WC-1) and White Collar-2 (WC-2).

Yeast:
A fungus which forms distict cells; reproduction by budding or division.

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