1-07

Investigating the Effect of Traditional Chinese Medicine and Mushroom Extracts on Alzheimer's Using *C. elegans* as a Model

Leader: Joshua Siau (15) (3i3)
Joel Ong (12) (3i3)
Zou Jiaxin (32) (3i3)
Hwa Chong Institution (High School)

Abstract

Alzheimer's disease is a neurodegenerative disease that causes a huge problem in our society. In Singapore, 10% of people above the age of 60 suffer from dementia, corresponding to 82,000 people in 2019. This number is projected to increase to 152,000 by 2030, according to the Institute of Mental Health, Singapore (2019). Therefore, we propose the use of Traditional Chinese Medicine (TCM) extracts such as *Monascus purpureus, Hericium erinaceus* and *Ginkgo biloba* nut extracts to help delay the onset of Alzheimer's disease. In this study, we investigated the effects of the abovementioned TCM extracts on the survival rate of transgenic *C. elegans* 4176 that express the Alzheimer's proteins at 25°C, using *C. elegans* 4176 as a model organism. The survival rate of *C. elegans* was assessed by counting the number of surviving worms 7 days after upshifting the plates to 25°C. The safety of the herbs was evaluated via a toxicity assay where herbal extract was placed onto the petri dish with wild type *C. elegans* and the number of surviving worms were counted after one week. *G. biloba* was found to have limited toxicity even at high extract concentrations of 100µg/ml, whereas for *M. purpureus*, it was toxic at high extract concentrations. The optimal concentration for *G. biloba* was 100µg/ml, and for *M. purpureus* it was 25µg/ml. Both herbal extracts also displayed antioxidant activity as shown through the DPPH test. Both herbal extracts significantly increase the survival rate of transgenic *C. elegans 4176.* Both herbs show promise to be used as a safe and effective method to delay the onset of Alzheimer's disease.

Introduction

Red yeast rice (RYR) is a traditional Chinese medicine (TCM) produced by the fermentation of rice with *Monascus purpureus*. RYR is capable of lowering blood cholesterol

levels and promoting blood circulation. During fermentation, polyketides called monacolins that can inhibit cholesterol production are produced (Musselman et al., 2011). RYR also contains many other cholesterol-lowering agents, antioxidants, and anti-inflammatory agents, which have been proven to ameliorate Aβ40 infusion-induced memory. A prior study found that RYR extracts were able to reverse memory loss as well as prevent Aβ40 infusion and damage in the hippocampus and cortex in rats treated with amyloid β (Aβ) when orally administrated (Zhu et al., 2019).

Ginkgo biloba is another TCM. Both leaves and nuts of the tree have been in use for the past several centuries in traditional Chinese medicine practice. Ginkgo leaf extract is prescribed as a memory remedy in many parts of the world and is currently the most commonly used herbal remedy for Alzheimer's disease. Ginkgo leaf has antioxidant and antiapoptotic properties as well as potential inhibiting effects against amyloid-β aggregation (Luo et al., 2002). Ginkgo nuts have also been shown to have antioxidant properties. However, a 2009 clinical trial showed the use of *G. biloba* leaf extract at 120 mg twice daily did not affect the rate of cognitive decline in older adults (72–96 years of age) (Snitz et al., 2009). Despite being widely used, the effect of *Ginkgo biloba* in the prevention and treatment of Alzheimer's remains controversial and further research is needed.

In research involving Alzheimer's disease, the organism *Caenorhabditis elegans* is a useful model as C. *elegans* have neurons, skin, gut, muscles, and other tissues that are very similar in form, function, and genetics to those of humans. They can be genetically modified to produce the neurotoxic proteins TAU and Amyloid- β proteins, which causes Alzheimer's disease in humans (Griffin, Caldwell & Caldwell, 2017). Also, 38% of the genes in *C. elegans* have a human ortholog and it also has a relatively short lifespan of 2-3 weeks. The organism C. *elegans* is transparent, making it easy to be observed using simple microscopy. The strain that we will be using is CL4176, which is a transgenic strain of *C. elegans* that produces low levels of beta amyloid peptide and expressing human $A\beta_{1-42}$ in muscle cells under a temperature-inducible system (Link et al., 2003).

1.1 Objective

The objective of the experiment was to investigate the effectiveness of *G. biloba* nuts and *M. purpureus* against Alzheimer's disease , by studying the extent that they can reduce paralysis and lengthen the lifespan of the *C. elegans* strain CL4176 that have been genetically modified to produce amyloid-β. We also aimed to study the efficacy of different concentrations of these herbs in lengthening the lifespan of these *C. elegans*.

1.2 Hypothesis

We hypothesised that *G. biloba* nuts, and *M. purpureus* were effective against Alzheimer's disease and would reduce paralysis and lengthen lifespan of the *C. elegans*. The extract(s) with more antioxidative properties would reduce the damage caused by oxidative stress and amyloid-β to a greater extent, hence reducing paralysis and lengthening the lifespan of *C. elegans* by a greater extent. The greater the concentration of *G. biloba* nuts, and *M. purpureus* the greater the efficacy in preventing the onset of Alzheimer's disease

2. Materials and methods

2.1 TCM and worms

Fresh *G. biloba* nuts were bought from NTUC fairprice, and *M. purpureus* and *H. erinaceus* were bought from Sinchong TCM, a TCM shop in Singapore. Transgenic *C. elegans* 4176 that express human $A\beta_{1-42}$ in muscle cells at 25°C was bought from a laboratory in the US.

2.2 Toxicity Assay

G. biloba nut, *Hericium erinaceus and M. purpureus* were blended and ground into powder, mixed with deionised water and boiled for 1 hour. The various concentrations of herb extract of 25 µg/ml, 50 µg/ml,and 100 µg/ml were obtained through serial dilution. 100µl of the extract was added onto the NGM agar plates after OP50 *Escherichia coli* was added, and before the *C. elegans* 4176 were transferred onto the plate. The plates were incubated at 16°C

for one week, and the concentration of herbs where most worms survived was recorded as the optimal concentration and used in the paralysis assay.

2.3 *Caenorhabditis elegans* paralysis assay

Blocks of NGM containing the *C. elegans* 4176 were added to the NGM agar plates with OP50 *Escherichia coli*. The100µl of the extract was added onto the NGM agar plates before the worms were transferred onto the plate. The plates were incubated at 16°C for 3 days, and then upshifted to 25°C for one week. The number of dead, paralyzed, and worms with abnormal movement was recorded, and videos of the worm movement were also taken. A similar control set-up was used but instead of the extract, sterile water was used.

2.4 *Caenorhabditis elegans* movement analysis

The videos of the worms that we took were analyzed using ImageJ, a software that allows us to compare the motion of the worms to the typical motion of the worms so as to determine what was abnormal behaviour of the worms, paralyzed worms referred to worms that were wiggling but could not move themselves, and the worms that did not display any signs of movement were classified as dead.

2.5 DPPH assay of *Ginkgo biloba* nut *and Monascus purpureus* extract

0.1mM solution of DPPH in methanol was prepared and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentrations (25, 50,100,µg/mL). Mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \{(A0 - A1)/A0)*100\}$$

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. Triplicates were performed.

3. Results and discussion

3.1 Toxicity assay to determine the effective concentration of TCM

3.1.1 *Monascus purpureus* extract

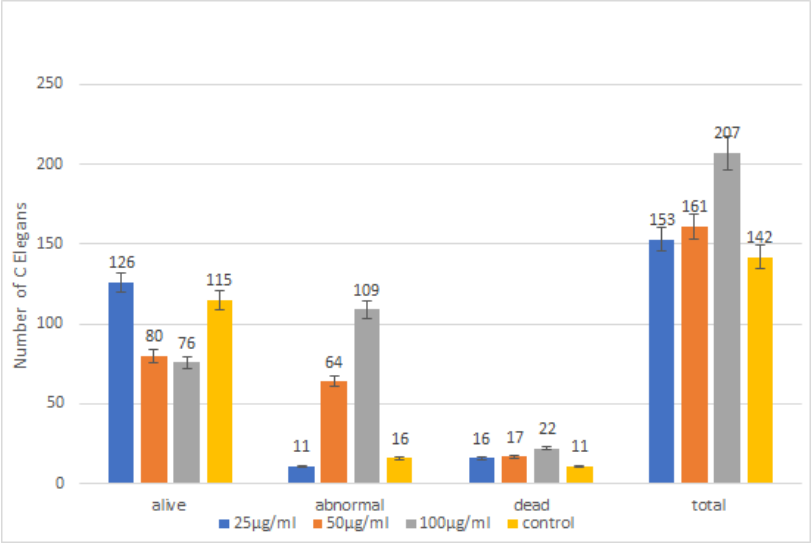

Fig 3.2.1 Effect of different concentrations of *Monascus purpureus* extract/(µg/ml) on the number of worms surviving after 7 days.

Sample size: 663; Blue, orange, grey and yellow represent 25µg/ml, 50µg/ml, 100µg/ml, and water respectively. Number of worms counted 7 days after extract was added. P value between 25µg/ml and control is 0.43

P value between 100µg/ml and control is 0.04

Toxicity assay was done to determine the optimal concentration of *M. purpureus* extract. As seen in Fig. 3.2.1, there was no significant difference in the survival rate of wild type *C. elegans* at an extract concentration of 25µg/ml and sterile water, at 82.35% compared to 80.99% for our control (p value = 0.43). Thus, we decided to use the extract concentration of 25µg/ml for the paralysis assay with *Monascus purpureus* extract.

3.2.2 *Ginkgo biloba* nut extract

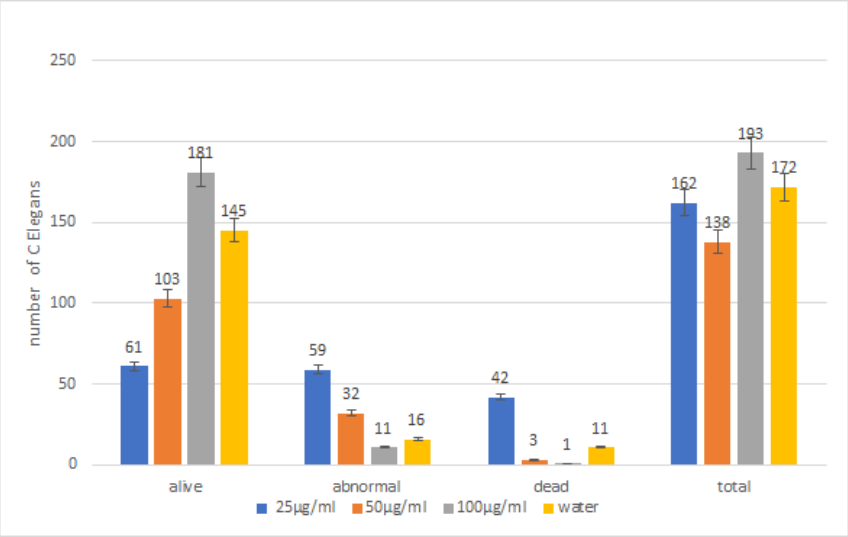

Fig 3.2.1 Effect of different concentrations of *Ginkgo biloba* nut extract/(µg/ml) on the number of worms surviving after 7 days.

Sample size: 665 Blue, orange, grey and yellow represent 25µg/ml, 50µg/ml, 100µg/ml, and water respectively. Number of worms counted 7 days after extract was added. P value between 25µg/ml and control is 0.03. P value between 100µg/ml and control is 0.52

Toxicity assay was done to determine the optimal concentration of *G. biloba* nut extract. As seen in Fig 3.2.1, the survival rate of wild type *C. elegans* at a high extract concentration of 100µg/ml is highest, at 93.78% compared to 80.99% for the control of sterile water (P value= 0.52). Thus, we decided to use the extract concentration of 100µg/ml for the subsequent paralysis assay with *Ginkgo biloba* nut extract as it is the extract with the highest survival rate of *C. elegans* after 7 days.

3.2.3 Unusual swarming behaviour of *C. elegans*.

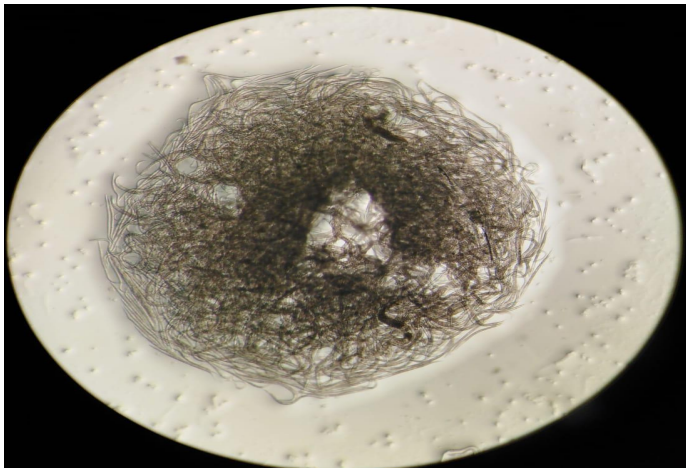

Fig 3.2.3 Image showing the swarming behaviour of the *C. elegans* at high extract concentrations taken at 40x magnification.

A video showing the behavior of the worms could be accessed at
https://drive.google.com/drive/folders/1w4ArPvE8FJzbyTlnlKBKNJrsls0LWbpl?usp=sharing

Interestingly, we observed that half of the plates of *C. elegans* at high extract concentrations of 100µg/ml displayed unusual swarming activity as shown in Fig 3.2.3, where more than100 worms gathered in a circle on the plate, and remained there in such large groups for more than a week. (**Appendix 1.1**)

3.3 Paralysis assay for transgenic *C elegans* treated with *Monascus purpureus* extract

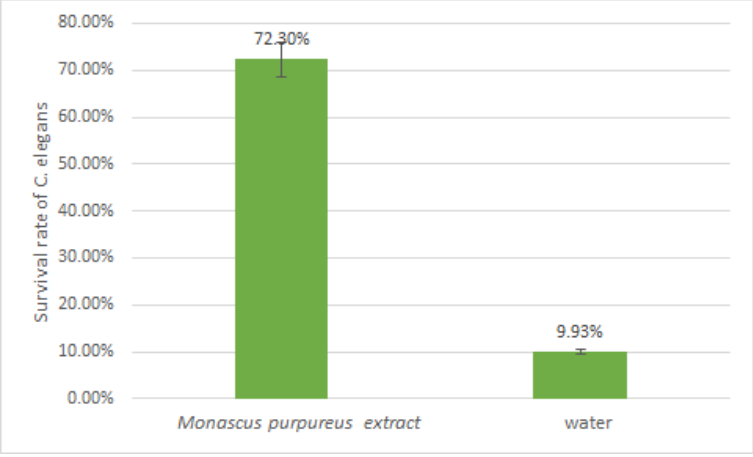

Fig 3.3.1 Effect of *Monascus purpureus* extract on survival rate of *C. elegans* 4176 at 25°C after 7 days

Sample size: 1034 Bar on the left represents survival rate of *C. elegans* 4176 treated with *Monascus purpureus* extract at 25°C Bar on the right represents survival rate of *C. elegans* 4176 with equal volume of water added at 25°C Number of dead worms counted 7 days after upshift of plates to 25°C. P value between *Monascus purpureus* extract and extract is 0.019.

As shown in Fig 3.3.1,the survival rate for *C. elegans* treated with *M. purpureus* extract was significantly higher at 72.30% as compared to our control of water at 9.93% survival rate (p value=0.019) . Thus suggesting that *M. purpureus* extract indeed had a positive impact on the survival rate of the transgenic *C. elegans* that express the amyloid-β protein.

3.4 Paralysis assay for transgenic *C. elegans* treated with *Ginkgo biloba* nut extract

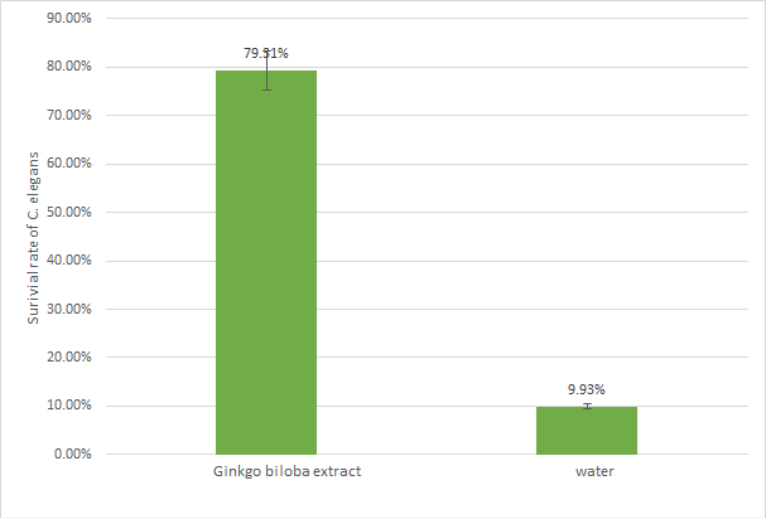

Fig 3.4.1 Effect of *Ginkgo biloba* nut extract on survival rate of *C. elegans* 4176 at 25°C after 7 days

Bar on the left represents survival rate of *C. elegans* 4176 treated with *Ginkgo Biloba* nut extract extract at 25°C Bar on the right represents survival rate of *C. elegans* 4176 with equal volume of water added at 25°C. Number of dead worms counted 7 days after upshift of plates to 25°C. P value between *G. biloba* extract and water is 0.026

As shown in Fig. 3.4.1, There was a significant increase in the survival rate (p value = 0.026) for transgenic C. *elegans* treated with *G. biloba* nut *extract* as compared to our

control.This suggests that *G. biloba* nut extract also had a positive impact on the survival rate of the transgenic *C. elegans* that express the amyloid-β protein. A direct comparison between *M. purpureus* and *G. biloba* extracts could not be made as the concentration of the extracts used were not the same and there could be different concentrations of compounds present in each extract. Further experiments were needed to compare which extract was more effective in improving the survival rate of transgenic *C. elegans*.

3.5 DPPH assay of *Ginkgo Biloba* nut *and Monascus purpureus* extract

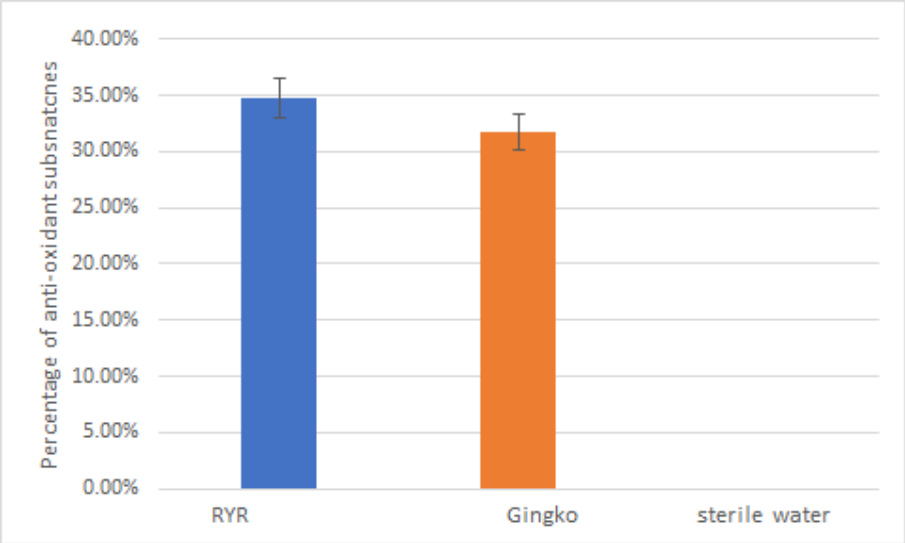

Fig 3.5.1 Effect of type of extract on percentage of antioxidative properties of extract.

Sample size: 3 samples per extract, 5 replicates performed. The extract concentration used was 25μg/ml for both extracts. Sterile water taken be the blank sample. P value between *M. purpureus extract* and water is 0.034, P value between *G. biloba* extract and water is 0.033

The results showed that both TCM extracts contained substances with antioxidative properties. Water was taken to be the blank sample. In comparison, *M. purpureus extract* showed a concentration of 34.8% of antioxidative substance while *Ginkgo biloba* nut extract showed a concentration of 31.8% of antioxidative substances.

Explanation of Results and Discussion

Previous studies have found that *M. purpureus* extracts were able to reverse memory loss as well as prevent Aβ40 infusion and damage in the hippocampus and cortex in rats treated with amyloid β (Aβ) when orally administrated (Zhu et al., 2019), and thus explaining its effect on improving the survival rate of the transgenic worms as shown in our study In addition, another study has found that Ginkgo leaf and nuts have antioxidant and antiapoptotic properties as well as potential inhibiting effects against amyloid-β aggregation (Luo et al., 2002), and thus the extract was effective in improving the survival rate of the transgenic worms Further investigations could be done to isolate the effective chemical compounds from both TCM extract and to test their effects on paralysed worms directly. .

Limitations

Our study has chosen C. elegans 4176 to be the model organism, so it cannot be confirmed that *G. biloba* and *M. purpureus* will also increase the survival rate of human Alzheimer's disease patients. The time period after which the number of worms were counted was one week, so it is not possible to conclude that *G. biloba* and *M. purpureus* will help provide a long term delay in the onset of Alzheimer's disease. We were unable to do testing for *Hericium erinaceus* extract due to time constraints

4. Conclusion and future work

In summary, our results have suggested that both *M. purpureus* and *G. biloba* nut extracts had a positive impact on the survival rate of worms expressing beta-amyloid, indicating the potential of using these TCM to alleviate the symptoms or improve lives of people with Alzheimer's disease. As both TCMs are readily available and not as toxic to people's health, the treatment could be widely sought after by the 50 million people worldwide who suffer from Alzheimer's disease, as a replacement of the use of synthetic drugs with possible side effects.

Our future work could include the investigation of the combinatorial effects of both herbs on preventing or delaying the onset of Alzheimers' Disease. The ability of other herbs such as *Hericium erinaceus* and *Uncaria rhynchophylla* to delay the onset of paralysis caused by production of TAU protein could be investigated too.

Appendix 1

1.1 Strange swarming behaviour of *C. elegans*

When doing the toxicity assay, we observed that half of the plates of *C. elegans* at high extract concentrations of 100µg/ml displayed unusual swarming activity, where >100 worms gathered in a circle on the plate, as seen in **Fig 3.2.3**

We hypothesized that the high extract concentration caused the worms to display unusual swarming behaviour, as a side effect of our extract. We also proposed that the extract could have caused the OP50 E. coli lawn to grow much thicker, attracting more worms to certain spots on the plate.

After further research, we found that *C. elegans*, including the laboratory strain (N2), forms complex patterns during feeding. When thousands of worms are forced to feed together, aggregation-induced bacterial accumulation and oxygen depletion create unstable conditions and further trigger phase separations. The principle of phase separation is mainly based on a sudden change in the animal's motility (Liu, et al., 2016). We also found that the dynamics of the entire process is controlled by the sensitivity of the oxygen-sensing neurons, which cause the worms to display swarming behaviour .

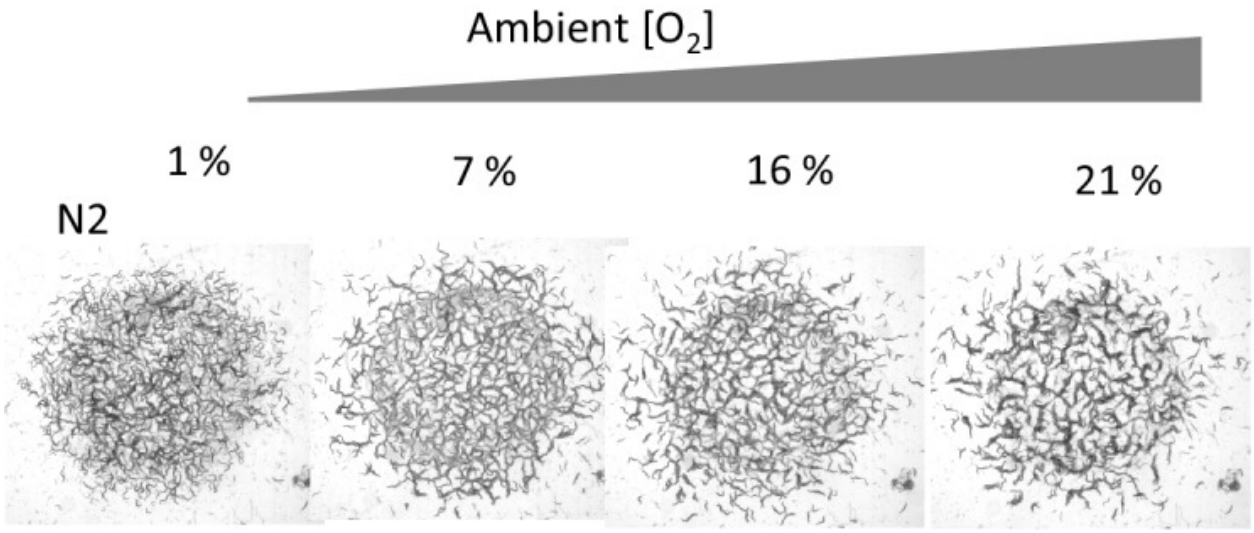

Photo Credit: (Demir et al., 2020)

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