

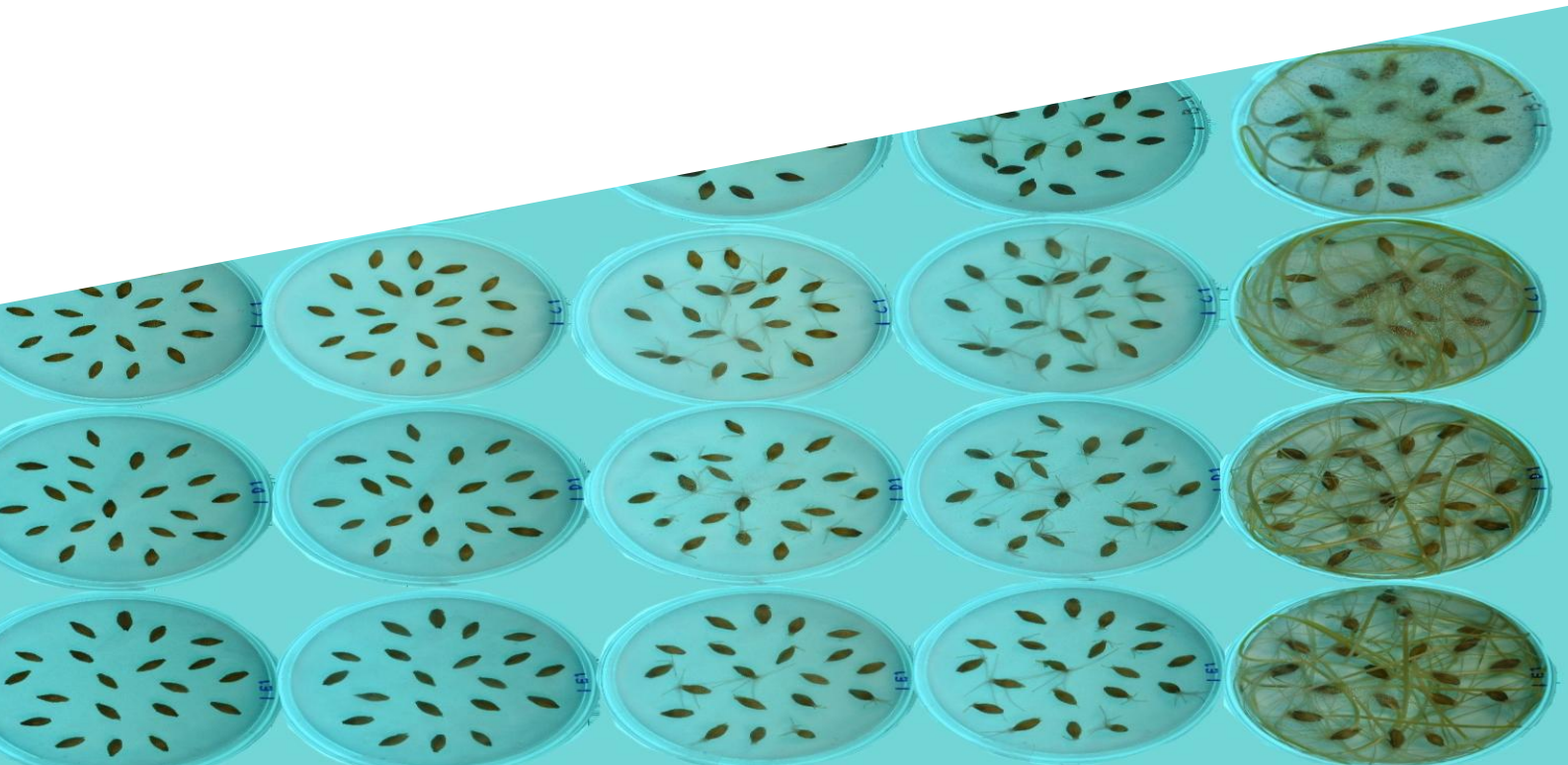


Oxidation status as an indicator of planting value of cereal seeds

Implementing FOX-1 assay on seed quality control

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Oxidation status as an indicator of planting value of cereal seeds: Implementing FOX-1 assay on seed quality control

Oxideringsstatus som en indikator av spjutkraft i stråsåd: Implentering av FOX-1 som metod för kvalitets-kontroll av fröer.

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Abstract

The global population is continuously growing and with reports projecting both heavier droughts and precipitation there is a need for resilient seeds, to ensure food security. Today's germination tests often focus on testing seed viability, which is shown under optimal conditions. Seed vigor is a measurement of seeds performance in field-conditions. Assays to evaluate seed vigor is becoming more relevant as the environment becomes more variable. Cereals is one of the most important calorie sources around the world. This study uses barley (*hordeum vulgare*) as a model crop for cereals. The cultivars of barley used in this study is RGT Planet, Laurette and Lexy, all commercial cultivars. Reactive oxygen species in cells drive seed ageing, making seeds lose vigor faster. The aim of this study is to try to implement the FOX-1 assay as a method to measure seed vigor. It has previously been tested on seeds by Griffo (2023). The FOX-1 assay measures reactive oxygen species using ferrous ion(II)sulphate to bind to peroxides that has leached into the solution from the seeds. It then forms a measurable complex with an indicator called Xylenol Orange. The results showed that the FOX-1 assay was useful to discriminate between seed lots with high and low amounts of reactive oxygen species, correlating to good and poor seed vigor through germination tests. Though, when comparing two seed lots with similar seed vigor it was difficult to predict germination performance through the results of the FOX-1 assay. More research needs to be done to optimize the FOX-1 assay. Future research should investigate possible applications of the assay on freshly harvested seeds and on a variation of seeds.

Keywords: ROS, FOX-1, Barley, Hordeum vulgare, seed vigor

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Abbreviations

Abbreviation	Description
FOX	Ferrous ion Oxidation Xylenol Orange
ISTA	International Seed Testing Association
ROS	Reactive Oxygen Species
SLU	Swedish University of Agricultural Sciences
GSH	Glutathione

1. Introduction

1.1 Background

1.1.1 Global food production and climate change

The human global population has grown rapidly during the last 200 years. It reached one billion globally in the year 1825, roughly 200 000 years after the establishment of us as a species, just to double in size from 3 billion to 6,8 billion between 1960 and 2010 (Cain et al. 2011). This constant increase in the global population puts pressure on harvests to provide adequate food security. Instead, recent projections show there is a risk that crop yields will drop globally due to several factors, a major one being drastic changes in the global climate (Adetunji et al. 2021).

According to the latest IPCC report (2023), global greenhouse gas emissions continue to increase due to multiple reasons, such as unsustainable land- and energy use. Since the previous IPCC report in 2014 (Calvin et al. 2023), instances of extreme weather such as heatwave induced droughts and uneven heavy precipitation have increased (Calvin et al. 2023). The 2023 IPCC report also states, with some uncertainty, that the increase in global agricultural productivity has been slower during the last 50 years because of global climate change (Calvin et al. 2023). Simulations show that with continued emissions of greenhouse gases we will at best limit the increase in global temperature to 1.5°C (2021-2040), with an estimated best/worst case scenario of 1.4-4.4 °C at the turn of the century (2081-2100) (Calvin et al. 2023).

These changes in the global climate leads to an increased exposure of abiotic stress in crops. The negative impact of this is dual as abiotic stress tends to reduce germination and seedling emergence of planted seeds, while mother plants exposed to the same kind of abiotic stress also tend to produce seeds with poor planting value. Previous studies have shown that drought stress in barley mother plants during the time of seed development resulted in shriveled and hard seeds with low seed vigor (Samarah & Alqudah 2011). Abiotic stress causes production of secondary metabolites in plants, taking resources that stun growth and development. Stressors such as drought and extreme temperatures cause more oxidative stress in plants by inducing the production of reactive oxygen species as a means to overcome the effects of the induced secondary metabolites (Adetunji et al. 2021).

Climate change can also be expected to increase yield losses attributed to problems with crop pathogens as geographic distribution patterns and host ranges for these are likely to expand (Singh et al. 2023). This problem is reinforced by the fact that chemical pesticide use is becoming increasingly restricted to preserve biological diversity (European Commission 2022). Also, more sustainable methods to fight pathogens based on physical interventions are at risk since they typically rely on high quality seeds that can withstand them, a resource which is at risk as already pointed out above (Sharma et al. 2015).

1.1.2 Cereals

Cereals such as wheat, oats, rye and barley are all important sources of calories for the global population, making up approximately 45% of the total calorie intake (OECD/FAO 2024). Historically, Swedes consumed a lot of rye and barley in foods such as porridge and bread, grains which could be stored through the scarce winter months. Today wheat is the most consumed and cultivated cereal in Sweden why it is critical for the economy and food security (Lantmännen n.y.).

1.1.3 *Hordeum vulgare*

The seeds used in this study are barley (*Hordeum vulgare*). Barley is considered one of the “ancient grains”, meaning that it is one of the grains that have been an integral part of the human diet for centuries (WWF 2022). Barley makes up ~ 30% of the total amount of harvested grains in Sweden, grown on 14% of the arable area (Lantmännen n.y). A majority of this harvest is used as animal feed, while a large portion of the remaining fraction is used as malting barley to produce beer and whiskey (Harwood 2018). Barley is also a crop that does not require a lot of space to grow, making it a highly efficient crop per area. Crops like barley will therefore be an important part of a future sustainable agricultural landscape (WWF 2022).

Barley is comparably well suited for experimental studies with its diploid and relatively small genome, fully sequenced reference genomes from several cultivars, and established methods for genetic transformation and CRISPR-based gene editing (Zeng et al. 2025). Methods and findings from barley are ideally relevant also for wheat and other cereals, making barley an important model crop (Jayakodi et al. 2020).

1.1.4 Seed vigor

Seed quality is usually tested through standardized germination tests that follow rules specified by organizations such as the International Seed Testing Association (ISTA). Besides being both labor intensive and time consuming, these tests are limited to germination and don't really address seedling emergence and are done under optimal conditions only (Hampton & TeKrony 1995). A consequence of this is that they are poor predictors of how seeds will perform in actual variable field conditions.

Seed vigor is the term for the different capacity seeds can have to establish themselves into seedlings in variable and potentially stressful field conditions (Reed et al. 2022). Since field conditions will become even more extreme and unpredictable due to climate change, being able to assess seed vigor, rather than just seed germination and viability under optimal conditions, will be important for creating climate resilient agriculture.

By the International Seed Testing Association (ISTA), seed vigor is defined as:

"the sum total of those properties of the seed which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence. Seeds which perform well are termed 'high vigour' seeds'" (Hampton och TeKrony, p. 11)

When evaluating seed vigor several parameters are considered: Rate and uniformity of germination; rate and uniformity of emergence as seedlings and how transportation and storage affect their vigor (Hampton & TeKrony 1995). Variation in the seed lots can be explained by different microclimates in the field. For example, topographical differences, such as slants can lead to differences in soil moisture retention. This affects both the crops growing in the field along with the seed maturation process, as well as conditions during planting and the seeds' ability to establish healthy seedlings quickly. Seeds from mother plants that have all experienced ideal conditions should all have the same seed vigor. However, since environmental conditions are never ideal throughout the entire life cycle of the plants, variations in seed vigor are inevitable (Hampton & TeKrony 1995).

Since seed vigor is such a complex agricultural trait it has been difficult to find an adequate method to measure all aspects of it. The most common method is to test different aspects of seed vigor during multiple tests. Only a few tests work for multiple different crops (Reed et al. 2022). One of the most common approaches is to subject the seeds to high humidity and high temperature to speed up the ageing process of the seeds, this enhances their difference in vigor. Then through standard germination tests the seed vigor can be observed (Reed et al. 2022).

Assays to evaluate seeds' potential to germinate during unfavorable conditions are of importance not only to agriculture but also to gene banks (Adetunji et al. 2021). When gene banks choose seeds to conserve important plant genetic traits for the future, it is of utmost importance that the seeds maintain viability throughout storage.

There is a need for effective and fast assays to ensure access to high quality seeds. Environmental stressors have big effects on germination and seedling establishment since these are particularly sensitive life stages for plants. High vigor seeds that can thrive through more variable conditions are needed to ensure rapid seedling establishment in a future with accelerating climate change (Waterworth et al. 2024).

1.1.5 ROS (Reactive Oxygen Species)

Reactive oxygen species (ROS) are a class of highly reactive molecules that are present in living cells. Some of them include hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$) and superoxide (O_2^-) (Gomes & Garcia 2013). ROS production is a common stress response, it is produced in several locations in the cells such as the chloroplast, mitochondria and endoplasmic reticulum (Adetunji et al. 2021). During electron transport processes electrons are transferred to O_2 forming these more reactive oxygen species (Bailly 2004). An excess of these molecules can be toxic to cells since oxidation of biological molecules such as DNA, RNA and lipids can be disruptive to their function (Sano et al. 2016). The oxidation of these molecules in turn tends to facilitate further production of ROS, leading to chain reactions and an amplified effect. To counteract this toxicity, cells employ several antioxidant systems that prevent excess accumulation of ROS as well as utilize repair mechanisms to mend the damage that has already been done (Jeevan Kumar et al. 2015).

Just as ROS presents a challenge for active cells, mature seeds also cope with the same effects in their dry state. However, in dry cells ROS production stems mainly from non-enzymatic reactions such as lipid peroxidation, Amadori and Maillard reactions (Jeevan Kumar et al. 2015). Particularly lipid peroxidation is suggested as an important reaction making the membrane lipids brittle, causing nutrient leakage upon imbibition (Kurek et al. 2019).

Since most metabolic activity is halted in cells of mature seeds, they must rely on passive antioxidants such as glutathione (GSH) (Kurek et al. 2019).

The dual role of ROS remains true for cells of mature seeds as well. Oxidation is harmful in the seed but is needed in lower amounts to break dormancy and germinate (Bailly et al. 2008).

Besides the seeds' ability to scavenge ROS during its lifetime when it is metabolically active, adequate storage conditions are crucial for maintaining viability. When the seeds dry the cytoplasm is reduced until it reaches a glassy state (Buitink & Leprince 2008). If stored in poor conditions, at high humidity and temperatures, the cytoplasm becomes fluid. During these conditions ROS activity can increase but the repair functions remain inactive (Waterworth et al. 2024). Such conditions drive seed ageing and causes rapid loss in seed viability and vigor (Kurek et al. 2019).

High levels of oxidative products cause deactivation of the antioxidant system and genome damage in cells. Disrupting the redox balance and reparation machinery in the cells, driving seed ageing (Kurek et al. 2019). This central role in seed ageing makes ROS a potential biomarker for seed vigor and viability.

1.1.6 FOX-1 assay

The FOX-1 assay for detection of oxidizing agents was first described by Jiang (1990). The assay is based on the rapid oxidization of Fe^{2+} to Fe^{3+} by H_2O_2 and peroxy radicals, whereafter Fe^{3+} is allowed to react with Xylenol Orange producing a blue/violet product possible to detect spectrophotometrically at 560 nm. The assay is carried out in an acidic environment to prevent autooxidation of Fe^{2+} . The FOX-1 assay has since then been used in different research fields, including detergent research (Jaeger et al. 1994), wine (Bridi et al. 2015) and recently also seeds (Griffo et al. 2023). In the context of seeds, the assay detects oxidating agents leached from the seeds into the solution (Griffo et al. 2023).

As already stated, oxidative status in seeds may be indicative of their vigor which is a crucial determinant of storability, tolerance to treatment against seed-borne pathogens, and planting value. The FOX-1 assay therefore has potential to predict the value of individual cereal seed batches both for seed banks and regular agricultural production.

1.2 Aims and objectives

The aim of this study was to establish the FOX-1 method (Griffo et al., 2023) for ROS measurement in dry cereal seeds in the host laboratory at SLU and to evaluate this method as an indicator of vigor in barley seeds.

If the assay can be confirmed to reliably predict seed vigor it would pave the way for rapid diagnostics of seed treatment tolerance and planting value for the potential benefit of both research and agriculture.

2. Method

2.1 The Seed materials

Seeds of *hordeum vulgare* (c.v. RGT Planet, Laurette & Lexy) were used during both the FOX-1 assay and the germination tests. Seed lot A (RGT Planet, harvested 2025-04-30) had been grown under optimal conditions (22°C/16°C and 16/8 hour day/night cycle) in a research greenhouse. It was used during all initial optimization of the assay to establish a consistency with the method described in Griffo (2023). Since harvest, seed lot A had been stored at -20°C.

Seed lots B-C (c.v. Laurette) and D-E (c.v. Lexy) (Table 1) are commercial seed lots supplied by Lantmännen BioAgri, cultivated and harvested during 2025 in Sweden. As such, their detailed growth- and storage conditions are unknown. Since winter 2025/2026 they have been stored in airtight bags at 6°C.

The ISTA germination tests presented in table 1 were done in an accredited lab (Frökollen). Seed lot B and C were chosen to be tested for the assay since they could be assumed to show big differences, based on their ISTA germination. Seed lot D and E were tested because of their similar ISTA germination tests, to see if seeds with such small difference in ISTA germination could be told apart through assay results.

Table 1. Cultivar, germination and moisture content of the seed lots.

Seed lots	Cultivar	ISTA germination	Moisture content
A	RGT	93%	Unknown
B	Laurette	33%	13,5%
C	Laurette	100%	13,6%
D	Lexy	98%	13,4%
E	Lexy	91%	12,1%

2.2 FOX-1

The solutions for the FOX-1 assay were prepared according to Bridi (2015) and used as described by Griffo (2023). Though slight modifications to the method used by Griffo (2023) were made. 10X stock solutions of Xylenol Orange (1,25mM) and D-Sorbitol (1M), were prepared. For the FOX-1 working solution, in a 50 mL Falcon tube, 5 mL of each stock solutions were added, along with 40 mL milli Q-water. Finally, 0,5 mL ammonium ferrous (II) sulphate (25mM) in sulphuric acid (0.25M) was added to the tube. The tube was gently shaken until

the solution was uniform in colour, forming a yellow-orange solution (figure 1). Exposure to sunlight was kept to a minimum to prevent autoxidation, this was done by covering samples and stock solutions with aluminium foil. For the same reason the FOX-1 working solution was made new every day before use.

To be able to compare and estimate the amount of [ROOH] that could be measured from the seed samples a serial dilution of hydrogen peroxide (H_2O_2) were made. Four reference concentrations were made of: 0 mM, 12,5 mM, 25 mM and 50mM by diluting the 30% H_2O_2 solution in milliQ-water. A reference curve was made as a compliment, each time a seed lot was tested.

10 seeds were used during each repetition of the assay. Each seed was placed in a microcentrifuge tube with 1,5 mL FOX-1 working solution. For the reference solutions, 50 μ L of each reference concentration was pipetted into one tube each and then 1450 μ L of FOX-1 was added. A blank containing solely 1,5 mL of FOX-1 was also prepared.

All samples containing seeds were centrifuged for 3 minutes at 13 000 RCF, to ensure that interference from dust and debris from the seeds as well as antioxidant molecules that might also leech during the assay, were prevented, since this could interfere with the absorbance measurement. 1 mL from each tube, including the reference solutions were pipetted into individual cuvettes. Absorbance was measured at 560 nm according to the method of Griffio (2023) since that is the absorption maximum for the FOX-1 assay product.

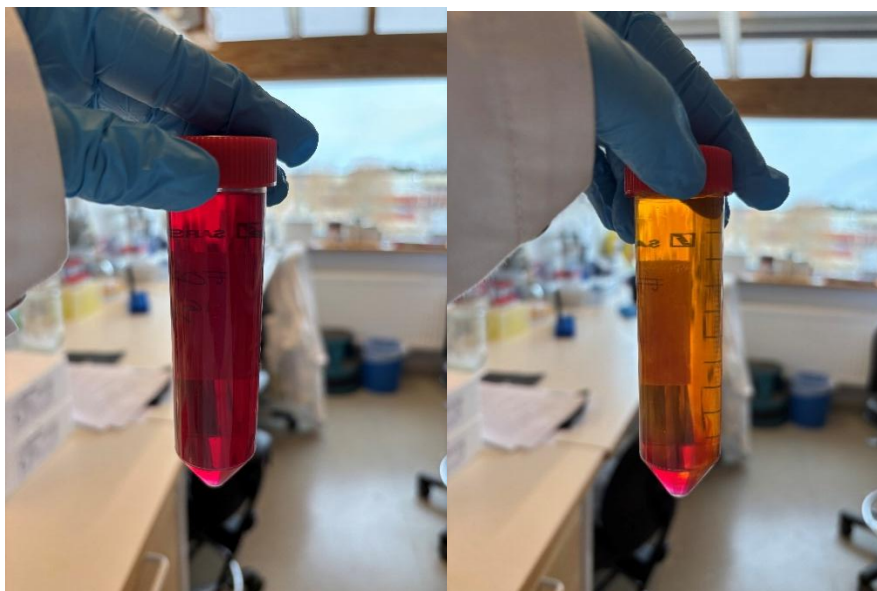


Figure 1: Before (left) and after (right) mixing in ammonium ferrous (II) sulphate in sulphuric acid into the rest of the FOX-1 working solution.

2.3 Germination tests

Instead of using standard germination tests which relies on end-point measurement, germinating seeds were counted continuously during the germination tests to account for the speed of germination and variation within seed lots (Hay et al. 2014).

100 seeds were used for each germination test, divided across 5 petri dishes. Five germination tests were made in total. Each dish contained 3 Whatman[®] qualitative filter papers, Grade 1. To ensure that water was not a limiting factor during the germination process (Jeevan Kumar et al. 2015), 4 mL of H₂O was pipetted into each dish.

20 seeds were placed with their ventral side down onto the filter paper making sure the seed had good contact with the damp paper. The dishes were sealed using parafilm to retain moisture. The dishes were wrapped in aluminium foil to ensure minimum exposure to light, since any residual dormancy might prevent germination under light conditions (Gubler et al. 2008). All dishes were placed in a climate-controlled darkroom with a temperature of 20°C.

Germinated seeds were counted by photographing them at the start of their imbibition (T₀), after 20h(T₁), 22h(T₂), 24h(T₃), 43h(T₄), 46h(T₅), 48h(T₆). Final germination percentage was calculated at 7 days (T₇). Seeds were considered germinated when radicle emergence had reached approximately 1 mm.

2.4 Statistical tests

The germinated seeds were counted at each time point. The percentage of germination at each time point from the five dishes of each seed lot was calculated. The time was made into \log^{10} to concentrate the time points so that the time of germination would make a linear and more comprehensive graph. The percentage of germinated seeds at each time point was made into probits as a part of the cumulative normal distribution curve. A linear regression was made to each seed lot. This was done to see the variance within the seed lot. The equation from the linear regression was then compared to the variance within the FOX-1 tests.

2.4.1 Probit analysis

Since the culminating germination over time follows a sigmoid curve, probit analysis was used to determine the germination behaviour. This is done by calculating the germination probability at each observed time point and

calculating the corresponding z-score. This converts the sigmoid curve into a straight line. Because the culminative germination is of an asymmetrical character, since outlier seeds skews the rights tail of the distribution, the time was converted into \log^{10} .

Linear regression was perform in excel a on the calculated data. Time it took to reach 50% germination ($T^{50}(g)$) could hence be estimated. Since one of the seed lots did not reach 50% germination at all, 20% ($T^{20}(g)$) germination was used to be able to compare the seed lots.

3. Results

3.1 Establishing the FOX-1 assay

Seed lot A was used to optimize the FOX-1 assay in the host laboratory. Several experiments were done to ensure the method could be replicated in the designated laboratory. In the initial experiments 10 seeds were used, as for most of the experiments. The incubation was done at room temperature. There was a great difference in standard deviation between the two initial experiments, as shown in figure 2. All experiments presented below were made using seed lot A.

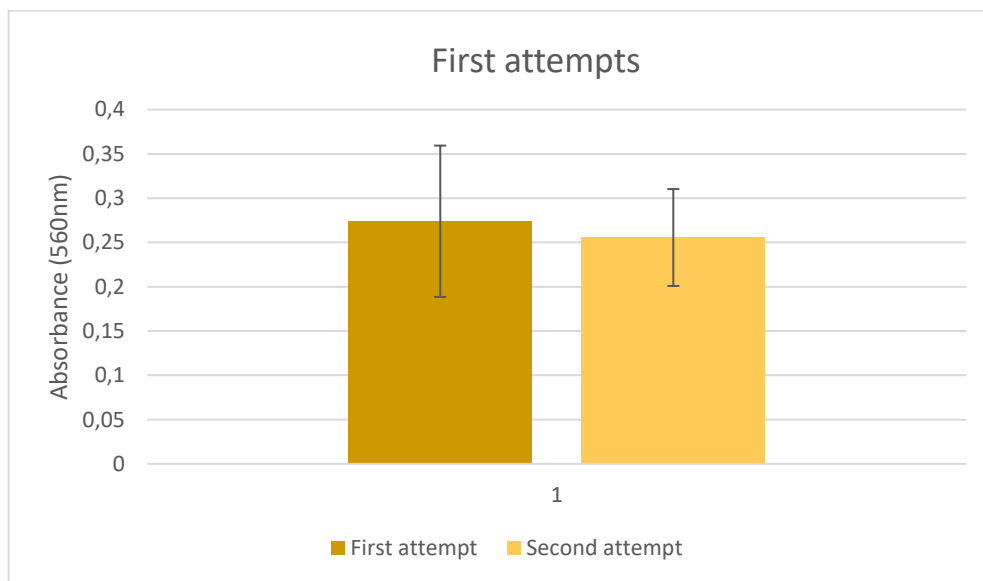


Figure 2: Average absorbance and standard deviation measured from two replicates of the FOX-1 assay applied on seed lot A. Error bars indicate standard deviation. $p = 0,92053$

Because the presence of dust and other particles are common on cereal seeds, we theorized that this might interfere with absorbance measurements. To test this, a batch of seeds were incubated together with enough FOX-1 solution to split the leachate into two fractions. One of them was then centrifuged while the other was not. After this, the absorbance measurement was higher for the centrifuged sample, as seen in figure 3. It is notable that the uncentrifuged sample (figure 3), was clearly lower than the levels measured from the seeds of the initial experiments (figure 2).

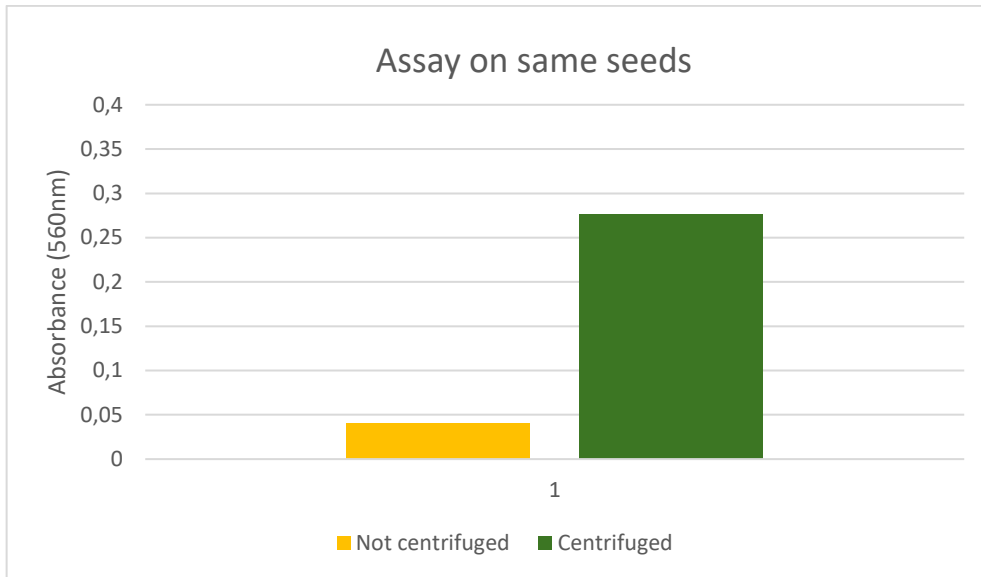


Figure 3: Difference in absorbance when not centrifuging and centrifuging the same sample.

To further investigate the correlation between centrifugation and higher absorbance, the next experiment used a bigger sample size of 20 seeds. The samples were all incubated at the same time and at room temperature. 10 tubes were chosen at random after incubation to be centrifuged. As in the batch test, the absorbance measurements were higher for the centrifuged samples (figure 4). The standard variation was lower for the centrifuged samples.

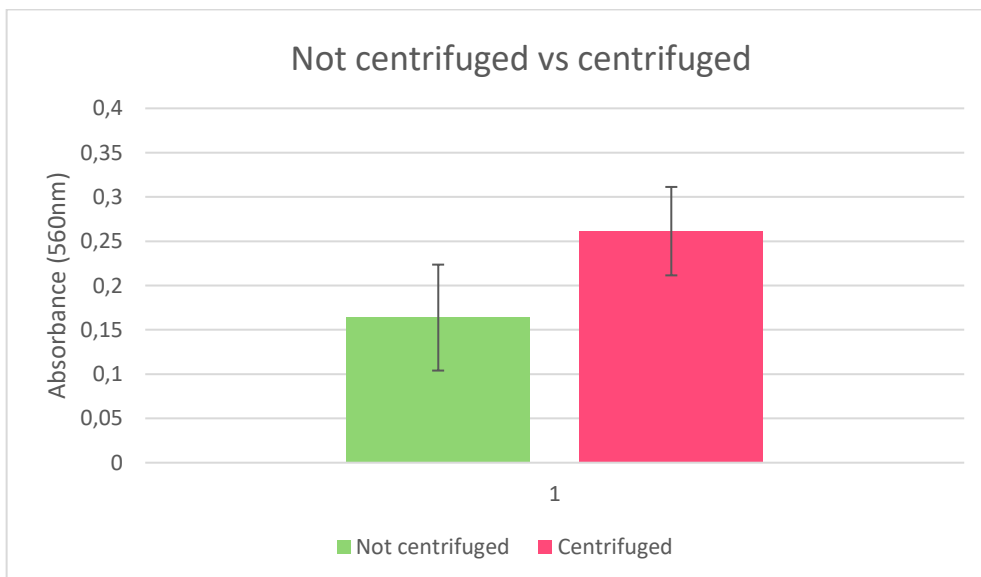


Figure 4: Average absorbance measured from two replicates of the FOX-1 assay applied on seed lot A, one without centrifugation and one with. Error bars indicate standard deviation. $p = 0.000965$.

Three experiments on three different days were conducted using our tried modifications. This was done to examine the stability of the assay results with our modifications. As before, a sample size of 10 seeds was used during each test. Variation was still observed between the experiments, in both average absorbance and standard deviation (figure 5).

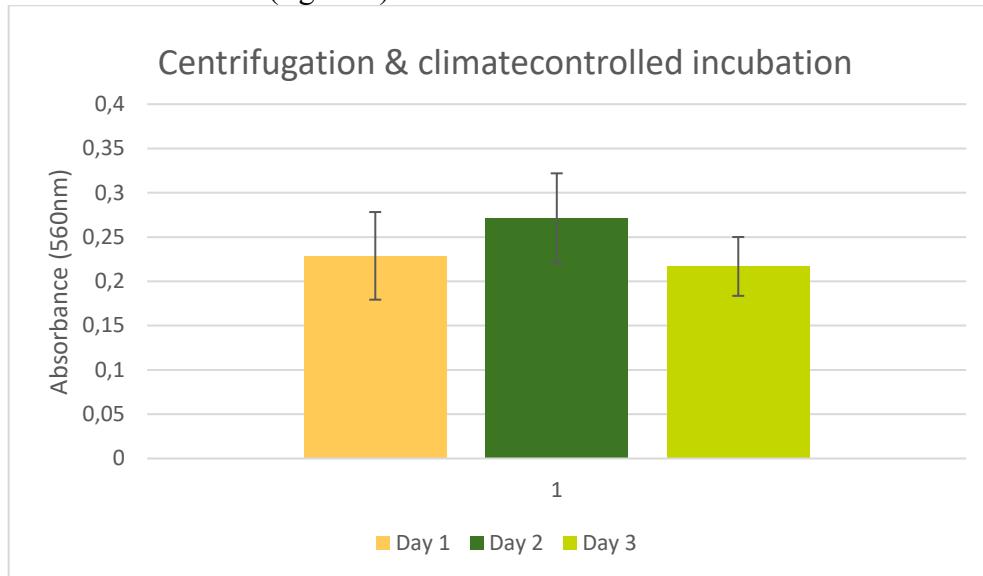


Figure 5: Average absorbance of three replicates of FOX-1, with centrifugation and incubation at 28°C. Error bars indicate standard deviation.

3.2 Calculated results from FOX-1 & germination tests

For the results from the FOX-1 assay, the average of two replicates from each seed lot B-E was calculated. For the germination tests the percentages and the regression analysis were made from the germination results of 400 seeds. 100 seeds from each seed lot.

Table 2. Results from FOX-1 assay and germination tests. 100 seeds were used for each germination test.

Seedlots	FOX-1		Germination				
	Average abs (560nm)	Stand.dev	k	m	(h) 20% germ.	(h) 50% germ.	Tot. germ %
B	0,57	0,24	2,55	-4,85	37,01	not reached	0,29
C	0,15	0,05	4,86	-7,06	18,96	28,29	0,9
D	0,17	0,06	8,66	-12,24	20,71	25,93	0,99
E	0,18	0,09	6,76	-9,81	21,21	28,29	0,95

3.2.1 Seed lot B & C

Our optimized FOX-1 assay was tested on seed lots B and C, which have known significant differences in germination. According to ISTA tests performed at an accredited lab (Frökollen) seed lot B had a germination percentage of 33% whilst seed lot C had 100% (table 1). These two seed lots are both of the cultivar “Laurette”, which enables us to compare them without having to account for genetic variability.

Seed lot B has a significantly higher measured absorbance compared to seed lot C, 0,57 nm on average. From table 2 we can also see that this seed lot has the highest variation within the lot, with an average standard deviation of 0,24. The slope value of seed lot B was low, 2,55x. This means that there was high variation of measured absorbance between the seeds in the lot. The variation is visualized by the more flat slope in figure 7. Seed lot B took the longest to reach a 20% germination, at the calculated time of 37,01 hours after imbibition. Seed lot B did not reach 50% germination at all and reached a total germination of 29%.

Seed lot C had an average absorbance of 0,15 nm, which is significantly lower than seed lot B. The average standard deviation of seed lot C was 0,05. The slope-value calculated from the germination tests was quite low at 4,86x. Which shows that there was quite a bit of variation in the germination success within the seed lot. This seed lot reach 20% germination in 18,96 h. The total germination success was 90%. The difference in variation between seed lot B and C can be seen in the variation of slope steepness from the linear equation in figure 7 and 8. The big difference in both standard deviation and average absorbance measurements can be visualized in figure 6.

After incubation samples from seed lot B had turned a dark violet (see figure 9). Whilst samples from seed lot C still had a yellow-ish color, shown in figure 10.

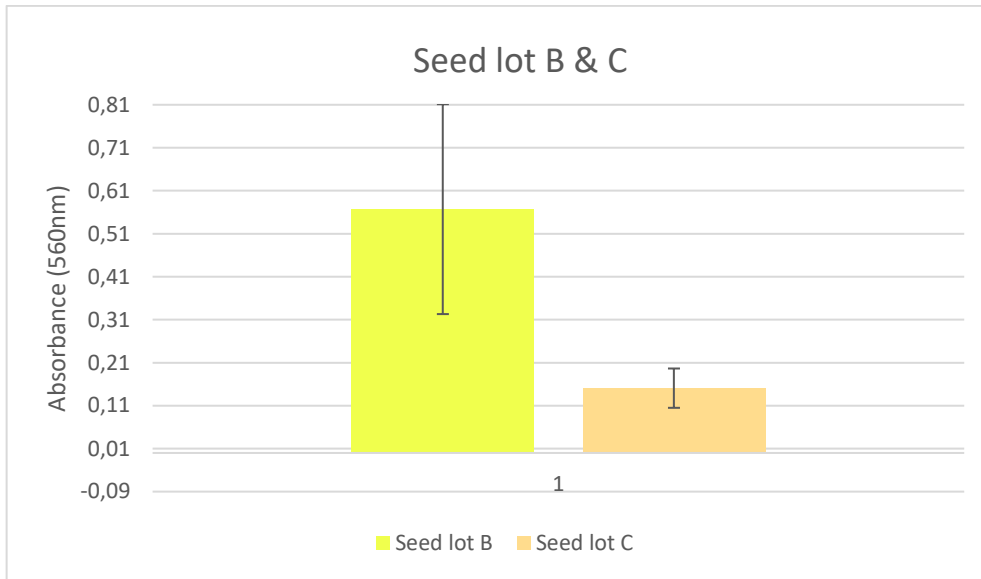


Figure 6: Absorbance measurements using FOX-1 assay on seed lot B & C. $p=0,000146$

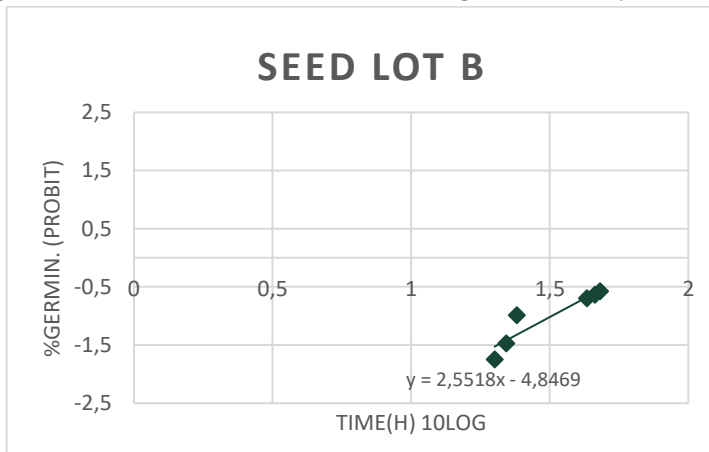


Figure 7: Germination of seed lot B as probits at each time point.

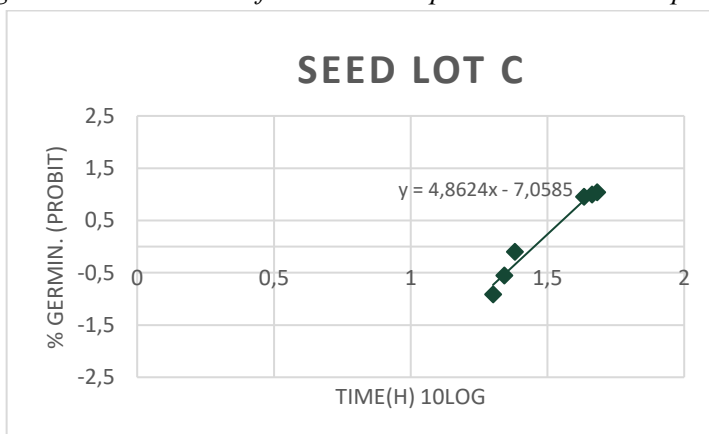


Figure 8: Germination of seed lot C as probits at each time point.



Figure 9: Sample 2B after 45 min incubation.



Figure 10: Sample 2C after 45 min incubation

3.2.2 Seed lot D & E

Seed lot D and E were both of the cultivar “Lexy”. The optimized version for the host laboratory was used on seed lot D and E with the objective of comparing the FOX-1 results between two seed lots with similar germination percentages. Additionally, since the standard germination test is poor when comparing similar seed lots, observing the speed of germination provided an additional measure to distinguish these seed lots. This was done to see if the FOX-1 assay could discriminate between such subtle differences in vigor.

According to ISTA tests performed at an accredited lab (Frökollen) seed lot D had a germination percentage of 98% whilst seed lot E had 91% (table 1).

Seed lot D had an average of 0,17 nm in absorbance with an average standard deviation 0,06. The slope-value (figure 12) was the highest of all of the measured seed lots with 8,66x, which shows low variation. Seed lot D reached 50% germination after 25,93 hours, which is faster than seed lot E. Seed lot D had a total germination success of 99%, which was the highest measured.

Seed lot E had an average measured absorbance of 0,18 nm during the FOX-1 assay. It had quite a high average standard deviation of 0,09 (figure 11). The calculated variation from the germination test is 6,76x. This seed lot reached 50% germination after 28,29 hours. Total germination reached 95%. The difference in variation can be visualized as the difference in slope in figure 12 and 13. Seed lot D had less variation and hence a steeper slope. The average absorbance was very similar between the seed lots, 0,17412 for D and 0,177895 for E (figure 11). But, the average standard deviation differed between the lots (figure 11). After

incubation the samples turned a similar light violet, samples from seed lot E turned slightly darker (figure 14 & 15).

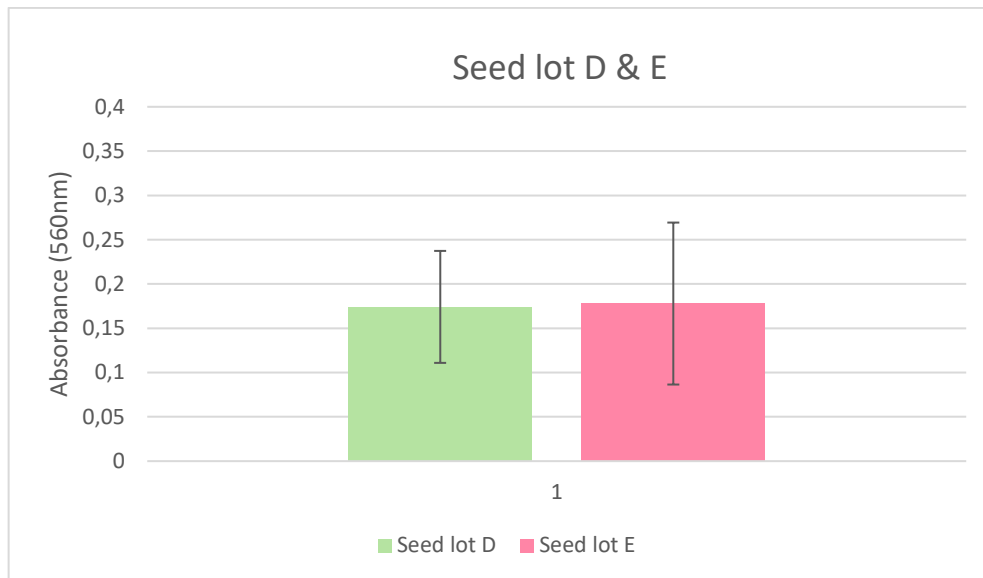


Figure 11: Absorbance measurements from the FOX-I assay used on seed lot D & E. $p=0,137731$

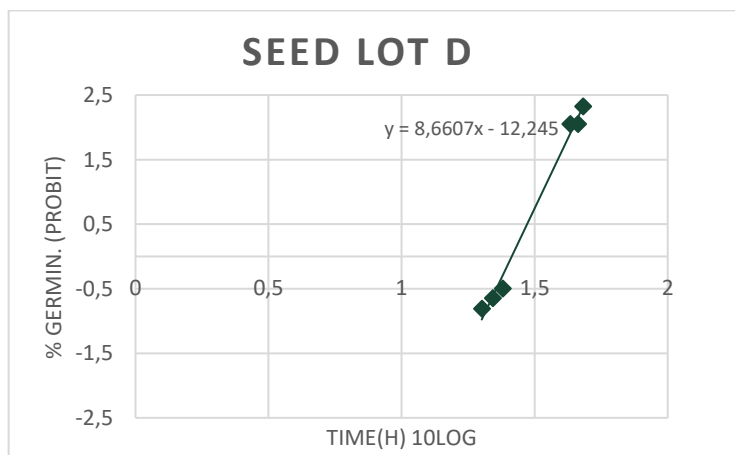


Figure 12: Germination of seed lot D as probits at each time point.

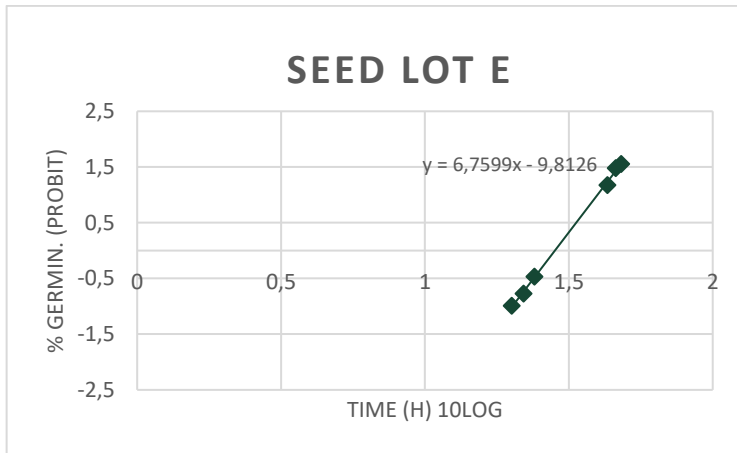


Figure 13: Germination of seed lot E as probits at each time point.

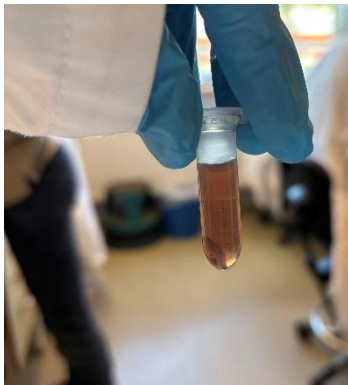


Figure 14: Sample 2D after 45 min incubation.



Figure 15: Sample 2E after 45 min incubation.

4. Discussion

4.1 Germination tests

Germination tests were conducted to be able to compare the results from the FOX-1 assay to the actual germination rate of the different seed lots. Since the test was done in a controlled environment, it is still unknown how the seeds would germinate in field-conditions. This makes the results of the FOX-1 assay difficult to relate to the seed vigor the seeds could display in field-conditions. Since the germination tests recorded multiple time points, it gave more information on the variation within the seed lot. This could be representative of possible uneven emergence in the field from the same seed lot.

The seeds used in this experiment were orthodox seeds, meaning they have a low moisture content and can therefore be stored for a long time. The moisture content in the seeds is similar (table 1), meaning it is unlikely to represent a variable that could cause results being different between the lots.

In total the germination tests were done on five different seed lots. The seed lots came from three different cultivars of barley. The mother plants providing the seeds for seed lot B-E had all been growing in field conditions, with unknown abiotic stresses. Seed lot A was grown in a research greenhouse under optimal conditions. They were hence expected to have different germination rates and seed vigor. It is also unknown how the seeds in seed lot B-E had been stored by the farmers before drying or their conditions during harvest, which could also affect germination. In general, comparisons of results between different cultivars are difficult to draw firm conclusions from.

The germination test was time consuming. It took 7 days in total with the first two days being more intense, since the seed lots were photographed every second to third hour. On a bigger scale, a simpler and less time-consuming method to measure germination would save a lot of resources, FOX-1 could be such a method even though its reliability still needs to be determined.

Seed lot B had the highest amount of variation within the lot, which can be seen by the more level slope in figure 7. Since the seeds germinated slower (see table 2) and over multiple time points, the trendline becomes flatter. Seed lot D had a lower amount of variation (figure 12), meaning the majority of the seeds had germinated at the same or close time points, making the trendline steeper. As mentioned, such low in-lot variation is likely associated with even emergence under field conditions, a highly desired trait of cereal seed lots.

4.2 FOX-1 assay

Initially, the results from the absorbance measurement on seed lot A, which was used to optimize the FOX-1 assay, showed a lot of variation. We theorized that uneven occurrence of dust and debris could affect the absorbance. To test this theory two samples were made from the same incubated seed-sample, one was centrifuged and one was not. Using the same seeds guarantee equal amounts of possible contaminating particles. We expected the centrifuged sample to have a lower absorbance since any dust particles should gather as a pellet at the bottom of the tube. Instead, the absorbance was higher in the centrifuged sample. This surprising result could be because biological substances with an antioxidizing effect is leaching out of the seed along with the ROS during incubation. This possible antioxidant might have a higher density than the FOX-1 working solution, separating it from the rest of the sample during centrifugation. The experiment was then done on a bigger sample size, 10 centrifuged and 10 not centrifuged. This was done to establish the correlation between centrifuging and higher absorbance. As shown in figure 4 the centrifuged samples continued to have a higher average absorbance. We did three replications of the experiment under the same conditions to see if the centrifugation caused less variation. As seen in figure 5, the samples still varied.

Although the initial tests were promising in terms of replicating the results (figure 2), the difference in absorbance of the uncentrifuged samples from figure 4 led us to theorize about another factor causing variation between experiments. Since incubation was conducted in the host laboratory, any fluctuations in room temperature might make the reactions go faster or slower during incubation. The samples were therefore instead incubated in a climate controlled darkroom at 28°C. We believed the higher temperature in the climate controlled room could make the reaction go faster. This would make the absorbance measurements overall higher. The experiments carried out with both climate controlled incubation and centrifugation still varied between the experiments, seen in figure 5. The present variation could indicate that the sample sizes need to be bigger in order to stabilize the results or possibly that other biological substances are affecting the assay.

4.3 Seed lot B

Seed lot B was the only one to not reach 50% germination. It measured a high average absorbance at 0,57 nm. Meaning seed lot B contained almost four times the amount of ROS compared to seed lot C (table 2) Seed lot B was also the only seed lot to mold during the germination test. A couple of seeds in the lot started to mold as early as T3 (24h). This could be because the high accumulation of ROS has caused too much oxidative damage to DNA and RNA molecules (Adetunji et al. 2021). If too much damage is done before imbibition the seed is unable to repair the damage and will be more susceptible to pathogens. Another possible explanation is inappropriate storage and handling of the seeds. Storage conditions are unknown before they reached Lantmännen, BioAgri. The FOX-1 assay was able to discriminate this higher ROS in seed lot B, making it useful to detect seed lots unsuitable for storage.

4.4 Correlation between germination test and FOX-1

Germination tests were conducted to test if ROS measurement in seed could be predictive of germination performance as a proxy for seed vigor.

Seed lot B had the highest average by far, at 0,57 in absorbance compared to the other seed lots where the second highest was 0,21 in absorbance at 560 nm. Seed lot B also had the lowest germination success at 29% in total.

The seed lot with the lowest absorbance measured, seed lot C, had the second lowest total germination, 90%. This could be because ROS is an important factor in alleviating seed dormancy, although dormancy is not expected to be a significant factor this long after harvest. The concentration at which ROS induces phytotoxicity to the seeds is unclear, so too low or too high concentrations of ROS could result in lower germination. This means that the ROS might have been too low to alleviate seed dormancy in some of the seeds in lot C, therefore the total germination was a bit lower.

Within the “Laurette” cultivar, seed lot B had the highest standard deviation and also the flattest curve compared to seed lot C (figure 7 &8) . When looking at the “Lexy” cultivar, seed lot E had the highest standard deviation and also a more flatter slope (figure 12 & 13).

4.5 The future for FOX-1

The FOX-1 assay successfully discriminated between seed lots with substantial differences in ROS. Comparing seed lot B and C, which had a big difference in both absorbance during the FOX-1 assay and in their germination. The FOX-1 assay could thus successfully discriminate between seeds with substantially different concentrations of ROS, in a manner correlating with their seed vigor as estimated in germination tests. In these more extreme cases, the FOX-1 assay therefore seems to be useful.

When comparing seed lots with only small differences in seed vigor, the FOX-1 assay is currently not precise enough to tell them apart. Seed lots with only a slight difference in seed vigor, lots D and E, showing an almost 10% difference in total germination, gave similar average absorbance measured with FOX-1 (see table 2).

As the method is today, I think the FOX-1 assay seemingly gives a quite clear discrimination between seed lots with substantial differences in vigor. When used on seed lots with similar vigor it is difficult to differentiate them, making the assay less useful in that regard. Our optimization of the assay still gave variation in results. This could be because we used too small sample sizes or possibly because other biological processes in the seed that could interfere with the FOX-1 working solution, skewing the measured absorbance. I believe the FOX-1 assay could be a feasible method to exclude seed lots with poor storability and vigor.

4.6 Error sources & future research

When performing the statistical tests, since seed lot A had multiple rounds of measurement to choose from, one was chosen at random. The calculated average and standard deviation might have been slightly different for seed lot A if another repetition of assay was chosen.

The earliest time points that germination was recorded was after 20h. Some radicles had already emerged at that timepoint. The seeds could have been checked earlier to catch any earlier emergents. The seeds could also have been photographed after 48h, catching any later germination to ensure the correct total germination.

In future research I think it would be useful for the assay if more optimization was carried out to establish a more certain average within the seed lots. Because of time limitations this study only repeated the FOX-1 assay twice on seed lot B-E. More experiments were done to seed lot A since it was used to optimize the assay. For a more representative average of the seed lots I think the assay should have to be carried out more times on each seed lot and with bigger sample sizes.

Self-oxidization of the FOX-1 working solution is a limitation to the assay. When the solution oxidizes it turns a darker violet colour, which is apparent in

samples with high amounts of ROS. This can be seen when comparing sample 2B and 2C (figure 9 & 10). I believe making FOX-1 less susceptible to self-oxidization is an important step for it to be a useful tool for research and agriculture.

Another interesting application would be to test if the assay can distinguish ROS levels in freshly harvested seeds, where dormancy is more of a challenge. It would also be useful to investigate at which concentrations of ROS is too low, resulting in the seed not being able to mobilize seed reserves and alleviating seed dormancy (Gomes & Garcia 2013), making some seeds unable to germinate. There also needs to be a better understanding at which level ROS induces phytotoxicity since that is a limiting factor of germination success.

More empirical research is needed to establish the correlation between the results of the FOX-1 assay and germination during field-conditions. This is needed to be able to determine their seed vigor in a variable field-environment in comparison to the FOX-1 results.

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