ISSS/ISTA webinar on fundamental and applied aspects of seeds

Session 1 - Seed Storage

PD Dr. Manuela Nagel (MN) and Dr. Christina Walters (CW) have answered all the questions that were sent in during the session in detail below.

Cristina Walters: Thank you for such nice and thought-provoking questions!

To Manuela, as you told genotype difference places a major role in seed viability, is there any specific seed morphology related to the long living genotype?

MN: So far, I do not see a clear relationship between seed morphology and genotype. I am sure there is one and I would also assume that there is a strong dependency on seed coat or epi/pericarp but this has not been studied over hundreds of genotypes or perhaps we have not studied the right trait. When we examined the mapping population panel harvested in 1974, there was a tendency for the six row types to have a higher germination than the two row barley genotpyes after 40 years of storage. However, harvest time and weather conditions at harvest time would need to be reviewed and considered. I hope that the data from advanced phenotyping and machine learning approach can help us to elucidate such factors in future.

What were your control samples?

CW: It's not clear whether this question was intended for Manuela or me and also for what experiment? I provide a response- even if Manuela was supposed to answer- because I think the question brings up the problem of making inferences about detectable changes in seeds over time. For some of the experiments I presented, the hypothesis was whether there was a difference between an older (harvested decades ago) and a newer (harvested within a few years) sample. Here we have control over the genetic background (same cultivar or population) and same moisture and temperature storage conditions, but we do not have control over growth year conditions or post-harvest handling. Some of these experiments used multiple harvest years, and that allows us to estimate variation resulting from harvest year for the particular cultivar. In other experiments, the hypothesis was whether there was a difference between a seed stored at a higher and lower temperature or moisture that did or did not, respectively, show symptoms of ageing. Here, we have good control over most of the experimental variables, but we lack an initial value for an analysis (except for germination tests, which were conducted using AOSA rules). The experiments we did made use of one or just a few genetic lines for a number of species, unlike Manuela's experiments that used hundreds of barley lines; hence we are unable to make inferences on genotypic effects on seed aging rates.

MN: I fully agree with Christina, the long-term seed storage experiments bear the problem that you cannot compare with the initial control because it has also aged or has been stored under

different conditions. In our mapping population panel, we can compare to freshly reproduced material and can make use of artificial seed ageing. However, as weather conditions change from year to year and ageing condition are different from long-term seed storage conditions, you will see in the data that this is not an appropriate control. As Christina mentioned the only chance you have is to estimate the effects by using various numbers of accessions and harvest years but even this is sometimes extremely challenging.

The delay in germination, appearance of abnormal seedlings, and eventual death suggests that it is not a sudden transition from good seed to dead seed. Why not use germination speed (or rates) as an indication of the progression of aging?

CW: This could be a question of about how we distinguish organisms that are near the end of their life expectancy versus those for which their life is over. I consider an aging organism still viable, but really, this assessment is based on presence/absence of an emerged radicle within the timeframe of the germination assay (or a +/- TZ stain). For seeds, it's hard to observe aging (the discreet problem), but for non-quiescent organisms, aging symptoms are identifiable (e.g., gray hair and bad eyesight in humans). In seeds, we also tend to conflate mortality of the individual with aging of the sample. For example, a sign of increased mortality is considered to be a sign that aging is occurring rather than a sign that aging has become lethal. True, we could change our metrics of aging to be something before lethality – e.g., time of radicle emergence or growth rate -- and then say that an individual seed that germinates or grows slower than expected is alive but showing signs of aging. But the question of functionality remains: does the individual seed or the collective sample have sufficient health to accomplish what we need it to do? We can define 'sufficient health' however we need – and we can move the bar as technologies for embryo rescue improve – but, I feel that we will always be judging seed health in the context of a yes/no = binary = discrete question of functionality. Personally, I am reluctant to return to a metric that I can't use with a variety of systems, including species that haven't been characterized or samples that have a lot of heterogeneity. I used to use radicle length as a descriptor of health of a stored seed, and that, understandably, got push-back from the seed science community because it wasn't very relatable.

I would also like a metric that makes the math easy. If we used time for radicle emergence, we have the problem of a constant time for an initial period of storage (i.e., we haven't fixed the problem of having no visible signs that aging is occurring), and then that time increases to infinity (dead seeds; math becomes difficult).

It's hard to comment on the canonical sequence of aging from slower to germinate to abnormal seedling to ??? to dead and the time frame that this occurs. We could reason that this occurs in individual seeds because we see this type of sequence in the population during monitor testing, but we can't really observe this in an individual seed. Therefore it's hard to know the interval of time from onset of detectable aging to death. In my opinion, but this is pure speculation, I think the time frame for an individual seed might be narrower than the population of seeds that we test to obtain a deterioration time course. However, I didn't intend to imply that the time frame for the transition defined its discrete qualities (I think it's about the dichotomy of functionality). I could certainly agree that there is a continuous accumulation of damage during storage that leads to a

progressively less healthy seed. That is in fact, our working hypotheses. Other hypotheses could be a rare or chance event finally occurs or sufficient accumulation of a substrate to induce a mortality sequence (both of these might be considered more discrete events in time).

MN: Of course, we can analyse and I also do analyse the various parameters of germination speed. These data provide additional information about the viability state of seeds, the progression of ageing and the genetic regulation (if work on mapping populations). However, these data correlate with each other differently leading to trait specific interpretations which can be rather challenging when looking at different speed data simultaneously. The main problem is, that we cannot compare germination speed data from the long-term stored material to the initial germination speed because those data were not evaluated 40 years ago. As Christina mentioned, we do not have appropriate information about the processes ageing because we do not know what was the initial setup. Overall, also for me the clearest picture comes from the total germination data.

What is the ideal temperature for storing seed in general?

CW: the ideal temperature to store seeds is the one that keeps seeds alive for the duration that you need without costing extra.

MN: I agree, if you want to store genetic diversity incl. thousands of genotypes you need to find a way to keep this material in a high quality for the required amount of time. According to the FAO genebank standards, it has been agreed to dry orthodox seeds at approximately 15 to 25 % RH and – 18 °C but these standards do not reflect the differences of species and genotypes. For some species either higher or lower storage parameters would be ideal for a longer storage period.

Is RNA degradation the cause or effect or loss of viability?

CW: I think we can safely say it is not an effect of lost viability. That is because we don't think either transcription or RNA turnover is occurring in the dry seed – nor are these processes needed. Also, we see changes in RNA before evidence of viability decline.

Our work isn't really designed to identify cause/effect relationships. Initially we set out to detect changes (chemical or physical) in seeds before a drop in viability occurred. We didn't want to use assays, like decreased unsaturation of lipids or increased free fatty acids, because those changes were mostly detected after measurable mortality and we get into questions (ala Bradford) of which seeds in the population were giving the signal. We wanted "crude" assays that focused on classes of molecules rather than a specific analyte. Initially, we wanted to prove that there were plenty of reactions that occur in dry seeds (how does a seed die if nothing is happening?). If we could do this, then measuring the rate of change (reactivity) was our next objective. Why? Because in a dry (= solidified) system, the main barrier to reactions is the limited molecular mobility. We reason that if we see differences of reactivity in different reactions to temperature or

moisture we can infer the nature of the molecular motion needed to effect that reaction. The temperature or moisture dependency that matched best with those dependencies for aging rate would be candidates to study how aging occurred. In effect, we are looking for 'time-keepers' where aging rate and reactivity correlate with storage conditions because that would give us great tools to predict longevity. It would be a bonus if these tools also provided insights about harvest year and genotypic effects. In other words, at the moment, we are looking at reaction kinetics as a proxy for molecular mobility not as a mechanism of aging.

As mentioned above, our working hypothesis for aging is accumulated damage that weakens the seed, and then maybe a chance or minor event that transitions the seed from alive to dead. I feel that every molecule in the cell is subject to deterioration – some may be more resilient (slower to lose integrity) and some may be more important to the cells' functioning (less degradation needed to see larger effects). So aging might involve elements of random, nonspecific events (i.e., the time-keeping) and perhaps some specific events. We can use the random events to anchor the math and perhaps give a direction to seek those specific events. Narrowing the possibilities of a specific reaction is important because otherwise finding a small reaction amongst a multitude -- if all molecules in the cell are subject to deterioration – is truly a needle-in-the-haystack venture. The RNAseq experiments turn out to be an amazing tool to examine specificity of reactions. And, of course, there is a bit of implied significance that certain mRNAs must survive storage to effect translation needed for germination (ala Job).

Thanks for the excellent presentation Dr. Christina Walters and Dr. Manuela Nagel. To Walters, Is there any differential degradation of RNA from different seed parts like is it different/ less in seed coat compared to embryo?

CW: thank you. We measured RIN in different seed parts of Williams '82 soybean: seed coat, cotyledon, embryonic axis, plumule. Not surprising we found the axis to yield more RNA per mass than cotyledons; also axes had slightly higher RIN than cotyledons. However, RIN values for the two tissues among all assays were highly correlated, which would lead to the idea that degradation rates were similar, though we didn't test this directly. It's a good idea and we can, so thanks for the pointer. With regards to seed coats and plumules. We abandoned analyses of those tissues after initial characterization because RNA yields were low (seed coats) and RIN values varied among replicates (both tissue types). Also RIN in seed coats tended to be lower.

Have you tested if RNA extraction method influence the RIN Value?

CW: After trying a few approaches with fresh soybean seeds, we settled on Qiagen's RNeasy plant mini kit based on RNA yield and processing ease (RIN wasn't affected too much). Recently we've tried a Trizol and Chloroform extraction to increase RNA yield in Poaceae seeds. RIN values on fresh soybean cotyledons are indistinguishable using the two methods.

Hi Christina, thanks for the interesting talk. I just wonder, did your group considered the RNA of endophytic microorganisms in the seeds that you might have extracted, which contributed to the total RNA used for further analysis?

CW: thanks and interesting question. We do believe that endophytes are present in stored seeds and I am working with Ilenys Perez-Diaz to explore that. So, I would not exclude the possibility that microbial RNA is included in our total RNA extraction. My understanding is that rRNA from plants elutes at a slightly different time than prokaryotes' on the Bioagilent chip. Bioagilent's proprietary RIN formula heavily depends on rRNA peaks and we are using the formula developed for plant samples rather than the ones available for eukaryotic or prokaryotic samples. That our conclusions are similar when we explore mRNAs identified from the soybean library is encouraging too.

Could RNA degradation quantification be also a good candidate for seed testing ie an indicator of seed ageing during seed production according to environmental or biotic stress met during seed maturation ?

CW: We are hoping that some level of RNA testing provides information about initial seed quality. I call it our dead vs. dormant study and results are pretty mixed at the moment. In our hands, we see high quality RNA when we extract from immature or germinated seeds that were killed by desiccation, freezing, or cutting. We also don't see a nice correlation between kinetics of RIN and viability decline under humid conditions, which may not bode well for assessing processing mishaps. I'm intrigued by the soybean transcripts that appear to degrade faster than most of the others, and think we might explore that a bit in quest of something that relates to late maturation events.

How we can check RNA Integrity from dry seeds, as seeds deattched from mother plant there will be no RNA activity

CW: Unlike metabolizing cells that rapidly turn over RNA (through RNAases), most RNAs remain intact in dry seeds and we can extract and characterize them, much as one might do for DNA, enzymes, etc. Activity, per se, such as transcription and translation is not required for detection and characterization of these molecules. It is intriguing to think that seed cells are packed full of all the molecules they need for germination; just add water to stimulate activity. It is also intriguing to think that with time these molecules degrade and lose the capacity to do their jobs.