nature portfolio

Peer Review File

Development of accessible and scalable maize pollen storage technology

Corresponding Author: Mr Jared Carter

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Development of accessible and scalable maize pollen storage technology” by Carter et al. found that suppressing respiration rate and optimizing storage environment gas exchange can extend the pollen grain storage time, and they developed a useful pollen storage protocol which has potential use for both breeders and scientists. Here are my comments for this manuscript:

Comments：
1. Table S1, the author used “germination rating”, accurate germination ratio could be better (also indicate the number of pollen grain being calculated). Same for other tables in the manuscript.
2. Table S1, the author claimed that the germination rate was inversely correlated with the amount of pollen and talcum carrier mixture, but in the data set, 5 days after storage, we can not see clearly the inverse correlation, and even in 7 days data, the trend is not clear. This may also be solved by using accurate germination ratio instead of rating.
3. “Compound microscopy images of silk that were pollinated with a mix of pollen and talcum showed that the silk surface and trichomes were covered in loose talcum particles with comparatively few pollen grains sticking to the silk during pollination (fig. S4)”, I can not find the information on Fig. S4, wrong figure.
4. “These observations suggested that talcum inhibited silk adherence and therefore, inhibited the pollen tube germination required for fertilization and seed set”, this conclusion from the manuscript is a bit speculated. To confirm this statement, the author could perform a sequential pollination experiment, first with a similar amount of talcum to cover silk, then pollinate the silk with fresh pollen grain and observe the result. In this case, the author can identify whether the reduced adherence of silk is simply caused by talcum on silk or talcum itself has impact on pollen grain and induces less adherence.
5. The author did not specify which ecotype that was selected for this experiment. As the pollen from different ecotypes have distinct characteristics, I would suggest the authors select several (3 or more) commonly used ecotypes, and test with simple in vitro germination experiment and confirm whether the storage method is useful for all ecotypes.
6. The author wrote the manuscript with beautiful words, but some sentences are relatively long and complex, which might not be friendly for non-native speakers, please pay attention to this point in the revision process.

Reviewer #2

(Remarks to the Author)
Maize crossing for seed production requires collecting pollen for pollination in the field. Its pollen vitality is prone to injury by environmental conditions, especially high temperatures, ultraviolet radiation, and dry air, making it difficult to preserve collected pollen for later pollination. In this submission, the authors established a relatively simple maize pollen storage technology by controlling gas exchange and reducing temperature to suppress pollen respiration metabolism, and evaluated the effects of 10 gas and temperature combinations on pollen germination and seed setting rate. Thus, this work has important applications for scientific research and maize hybrid seed production. However, the article only observed the phenotypes of pollen, pollen tubes, and ears, lacking biochemical and molecular level analysis of pollen metabolism processes in order to identify key enzymes or genes involved and improve the longevity of maize pollen through genetic manipulation. Thus, the submission could be accepted after major modifications.

Reviewer #3

(Remarks to the Author)
Comments:
The manuscript entitled "Development of accessible and scalable maize pollen storage technology" by Jared D. Carter and colleagues showed that suppressing respiration rate and optimizing storage environment gas exchange constitute the critical combination of factors for maintaining pollen viability during storage. The authors finally produced a high performing, short-term maize pollen storage protocol that is accessible to diverse global users, a novel approach that addresses the inherent challenges of maintaining pollen viability. This breakthrough is particularly noteworthy for its potential in the improvement of maize germplasm and boost hybrid seed production in maize, which has been a longstanding question due to the short lifespan of maize pollen. This is a well-written manuscript, with well-designed and expertly executed experiments. The methodology is sound and the conclusions are amply supported by the data presented. I have no major concern, only a few intermediate and minor concerns.

Intermediate concerns:
1.The team has tested six CO_2 sequestration agents, could you please elaborate on the selection criteria for these agents and explain why they were chosen instead of other potential alternatives?
2.It has been reported that maize pollen is relatively large and heavy with a diameter ranging between 80 and 125 μm. Whether there is a correlation between pollen size and surface area breathable barrier surface? What factors were considered in this calculation?
3.The use of breathable barriers was a major innovation in this study. Could you provide more details on how the breathable barriers were able to facilitate high pollen performance and maintain optimal O_2 and CO_2 content within the storage vessels?

A few minor concerns are listed below:
1."…, while nickel, tungsten, cobalt, and zinc oxide powder were toxic. " Could you provide more insight about the potential mechanisms of this toxicity? Were there any connections between the chemical properties of these compounds and their influence on pollen viability?
2.The results demonstrate the scalability of the storage protocol using modified cell culture flasks. More considerations required to scale up this technology for large-scale maize breeding and seed production operations should be discussed. What are the potential challenges and how can they be addressed?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for submitting the revised version of your manuscript titled “Development of accessible and scalable maize pollen storage technology”. I have reviewed your revisions and the responses to my previous comments. I am pleased to confirm that you have addressed all the concerns raised, and the manuscript is now clear, well-written and informative.

Reviewer #3

(Remarks to the Author)
I am satisfied with the corrections.

Reviewers' comments	Author Response
Reviewer #1	Author Response
1. Table S1, the author used "germination rating", accurate germination ratio could be better (also indicate the number of pollen grain being calculated). Same for other tables in the manuscript.	We used an ordinal categorical rating scale for pollen tube germination due to high variability in media handling, incubation time, and technique variance between users. I expanded the following section of Materials and Methods (page 33, line 1 through page 34, line 6) to clarify how pollen tube germination plates were imaged, the criteria set for image collection, and the rationale for scoring pollen tube germination using a scale rather than exact counts. **Syngenta maize pollen tube germination media and pollen tube germination scoring** Syngenta maize pollen tube germination is a solid agarose-based media consisting of 100 g L-1 sucrose, 30 g L-1 agarose, 0.37 g L-1 potassium chloride, 0.1 g L-1 boric acid, 0.55 g L-1 calcium chloride, and 0.25 g L-1 magnesium sulfate heptahydrate. Media was adjusted to pH 6.6 prior to autoclaving and was poured into standard 100 mm x 15 mm petri plates after autoclave sterilization. Where possible, media plates were left unwrapped overnight to reduce media surface condensation. Excessive media surface condensation increased pollen grain bursting prior to pollen tube germination. Maize pollen was evenly applied to the media by hand using the same techniques as were used to disperse pollen samples during plant pollination. Pollen was incubated on media plates with the lid closed for minimum of 60 minutes prior to imaging for data collection. Plates were kept out of direct sunlight and away from heat sources during incubation. Plates were not wrapped or sealed during incubation. Plates were incubated under a range of temperature (22 to 35°C) and relative humidity (15 to >80%) conditions based on the laboratory, greenhouse, or field location where work was conducted. During field pollen collection and application, plates were incubated up to 24 hours overnight before being returned to a location with a microscope for imaging. Following incubation, pollen tube germination plates were imaged at 40x magnification. Imaging was conducted using an AmScope 40x-2500x Compound LED Microscope

	equipped with an 18-megapixel camera. Representative images with a minimum of 20 pollen grains per image were collected for each sample. Due to the variability in media handling, incubation, and imaging conditions, the exact count of germinated pollen tubes versus ungerminated or burst pollen grains was difficult to quantify across experiments. In response, the team scored pollen tube germination using an ordinal categorical rating scale. Pollen tube germination was rated by reviewing images and assigning a rating on a 1-5 scale (5 = >≈60% pollen tube germination, 4 = ≈41-60%, 3 = ≈21-40%, 2 = ≈1-20%, 1 = 0% or completely dead and burst pollen). The top rating of "5" covered a larger range of pollen tube germination due to the reduced maximum potential pollen tube germination rates observed under greenhouse conditions. Fresh pollen samples collected under greenhouse conditions rarely exceeded 80% pollen tube germination compared to field collected samples that showed nearly 100% germination. The team hypothesized that this reduced maximum potential pollen tube germination rate under greenhouse conditions was due to accelerated pollen desiccation. Accelerated desiccation was caused by the high rate of air recycling and the use of conditioned, 70% relative humidity air under greenhouse conditions.
2. Table S1, the author claimed that the germination rate was inversely correlated with the amount of pollen and talcum carrier mixture, but in the data set, 5 days after storage, we can not see clearly the inverse correlation, and even in 7 days data, the trend is not clear. This may also be solved by using accurate germination ratio instead of rating.	I altered the main text section introducing "Metabolic gas exchange dictates storage vessel requirements" to accurately reflect the categorical rating system described in Table S1 and removed the "inverse correlation" statement. I also changed the confusing use of grams/liter equivalent pollen volumes per storage vessel to the more straightforward pollen quantity per storage vessel in grams. This change is reflected in Tables S1 and S2. (page 7, lines 2-5) The pollen tube germination rating collected following pollen storage decreased when over one gram of pollen was stored in a 125 ml storage vessel for seven days compared to less than lower quantities of pollen stored for the same duration in identical storage vessels (table S1)
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pollen and talcum showed that the silk surface and trichomes were covered in loose talcum particles with comparatively few pollen grains sticking to the silk during pollination (fig. S4)", I can not find the information on Fig. S4, wrong figure.	
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	carrier mix contained 0.33 ml pollen and 0.17 ml carrier based on the 2:1 ratio of the mixture by volume. To replicate these volumes and separate carrier interaction with silk from carrier interaction with pollen, 0.17 ml of a pollen storage carrier compound was evenly applied to the silks on a maize ear. The same ear was then pollinated by evenly applying 0.33 ml of fresh maize pollen. This fresh pollen was not mixed with any carrier compound and was not stored prior to use. This method of carrier pretreatment followed by fresh pollen application was used to test < 75 µm talcum, 10 µm crystalline silica, and Avicel PH-101®. As a positive control, a set of ears were pollinated with 0.33 ml of fresh pollen and did not receive a carrier pretreatment. To test for airborne pollen contamination, a set of ears were pretreated with < 75 µm talcum and did not receive any fresh pollen application.
5. The author did not specify which ecotype that was selected for this experiment. As the pollen from different ecotypes have distinct characteristics, I would suggest the authors select several (3 or more) commonly used ecotypes, and test with simple in vitro germination experiment and confirm whether the storage method is useful for all ecotypes.	Table S11 (Internal Link) has been added to address this concern. The table is addressed by the following lines in the Main Text, Results section. (page 12, lines 3-7) Experiments over the entire duration of the project demonstrated that pollen storage technology was penetrant across temperate and tropical dent corn lines used in hybrid seed production. Summarized testing results indicated that pollen from over 90% of maize lines across four major heterotic groups survived for five or more days in storage (table S11). Pollen from the single sweet corn line included in testing survived for five days in storage. The temperate and tropical dent corn inbreds tested during the research and early deployment phases of this project were summarized based on their adapted region and major heterotic group. This project prioritized testing modern, commercially relevant inbred parents in order to ensure the pollen storage technology would be effective in the hands of global maize breeding teams. Those teams are now using pollen storage technology as triage when lines will not sync for cross pollination or to move pollen between locations when males are damaged in a crossing block. The authors hope that academic groups will use this published protocol to test pollen

	preservation on more diverse ecotypes and uncover long-lived pollen (>7 days) that we believe is not present in commercial germplasm. Inbred line codes are proprietary and not approved for publication. The line codes have been changed to anonymous identifiers (Syngenta Inbred 1, Syngenta Inbred 2, etc.) in the supplied source data Excel.
6. The author wrote the manuscript with beautiful words, but some sentences are relatively long and complex, which might not be friendly for non-native speakers, please pay attention to this point in the revision process.	Hundreds of minor changes were made during manuscript revision to simplify sentence structure. I eliminated non-scientific English words that are uncommon in spoken language (ex: thereby, thus, therefore, whereby, elucidate). Track changes was left on throughout revision of the v2 draft submitted based on this feedback. An example of revised text is given below. Old: To better understand the challenges of extending maize pollen lifespan while the pollen is held in a metabolically active state, fresh pollen from greenhouse grown inbred and hybrid maize plants was mixed with talcum and stored in airtight jars with a gas sampling rubber septum (Fig. 1A, 1B). Sealed vessels were stored at room temperature (23°C) or were placed into refrigerated storage at 2°C or 6°C at local atmospheric pressure (100.3 kPa). Endpoint storage vessel atmosphere gas analysis (Fig. 1C) was conducted after 1-7 days. Mold growth was visible after four days storage at 23°C (Fig. 1D) and researchers noted odors associated with fermentation. Pollen viability following storage was assessed using pollen tube germination media (Fig. 1E, 1F, 1G). Pollen tube germination on media completely ceased after three days at 23°C, while the samples stored at 2°C retained viability for six days and those at 6°C remained capable of germination for the full seven days. New: (page 6, lines 3-13) Experiments where maize pollen was stored in a metabolically active state were conducted to better understand the challenges of extending maize pollen lifespan. The team mixed fresh pollen from greenhouse grown maize plants with talcum and stored the mixture in airtight jars equipped with a

	gas sampling rubber septum (Fig. 1A, 1B). Sealed vessels were stored at room temperature (23°C) or were placed into refrigerated storage at 2°C or 6°C at local atmospheric pressure (100.3 kPa). Endpoint storage vessel atmospheric gases (Fig. 1C) were measured after 1-7 days. Mold growth was visible after four days storage at 23°C (Fig. 1D) and researchers noted odors associated with fermentation. The team assessed pollen viability following storage using pollen tube germination media (Fig. 1E, 1F, 1G). Pollen tube germination on media completely ceased after three days at 23°C. Samples stored at 2°C retained viability for six days and those at 6°C remained capable of germination for seven days.

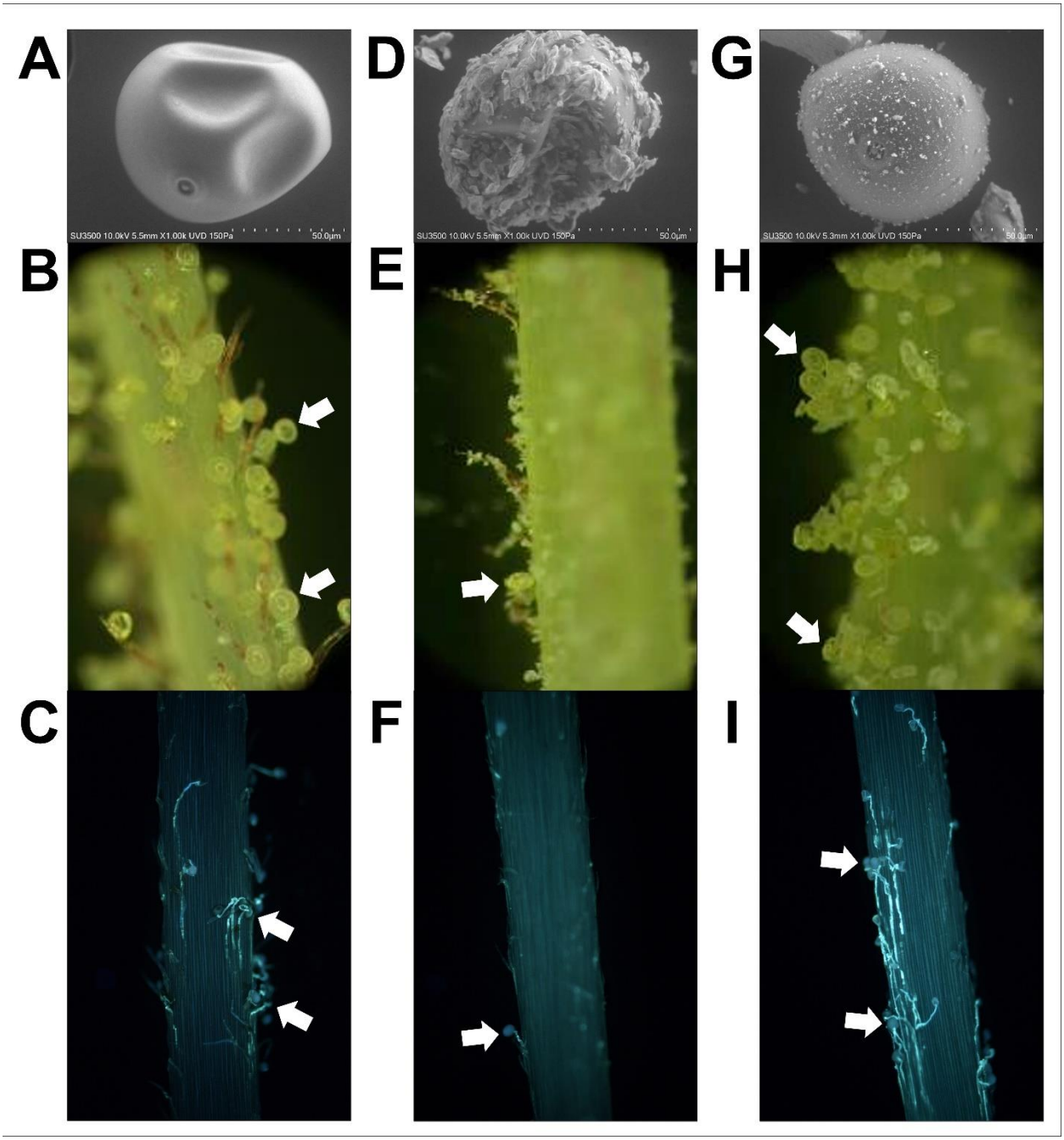

Fig. S6.

Comparative images of maize pollen, maize pollen on silks, and maize pollen on silks stained with aniline blue after pollination. Representative individual pollen grains in images of pollen on silks (**B**, **E**, **H**) and pollen on silks stained with aniline blue (**C**, **F**, **I**) are denoted by white arrows. (**A**) An individual maize pollen grain from a freshly

collected bulk sample (**B**) A maize silk pollinated with freshly collected pollen that shows excellent adherence between pollen grains and silk trichomes. (**C**) A maize silk from the same pollination stained with aniline blue to reveal the pollen tubes growing through the silk. (**D**) An individual maize pollen grain from the same freshly collected bulk sample that was mixed with talcum prior to imaging. The pollen grain surface was covered by talcum particles. (**E**) A maize silk pollinated using pollen mixed with talcum that shows minimal pollen grain adherence and talcum particles completely coating the silk trichomes. (**F**) A maize silk from the same pollination stained with aniline blue to reveal only a single pollen tube. (**G**) An individual maize pollen grain from the same freshly collected bulk sample that was mixed with < 75 µm Florisil® prior to imaging. The pollen grain surface is visible between Florisil® particles. (**H**) A maize silk pollinated using pollen mixed with 75-150 µm Florisil® that showed pollen grains can still adhere to the silk trichomes. (**I**) A maize silk from the same pollination stained with aniline blue to reveal many pollen tubes growing through the silk.

Table S11.

				Maximum Duration of Survival in Pollen Storage		
Ecotype	Adapted Region	Major Heterotic Group	Total Lines Tested	3 Days	5 Days	7 Days
Dent Corn	Temperate	Stiff Stalks	6	0	3	3
Dent Corn	Temperate	Non-Stiff Stalks	7	2	1	4
Dent Corn	Temperate	Iodent	9	0	5	4
Dent Corn	Tropical	N3	3	0	5	ND
Sweet Corn	Temperate	North American Flints	1	0	1	ND

A summary of the maximum observed pollen lifespan in storage for 26 maize inbred lines tested over the course of pollen storage technology development. All inbred lines tested were current or recently relevant maize commercial parent lines. Pollen survival in storage was measured by successful seed set or pollen tube germination. Summarized results include experiments using vacuum, sealed vessel with soda lime, and breathable barrier pollen storage technologies. The two Non-Stiff Stalk lines with pollen that survived for three days in storage were selected for testing based on observed poor pollination performance in hybrid seed production.

Table S13.

Treatment	Pollen Storage Carrier Used as Silk Pretreatment	Volume of Pollen Storage Carrier as Pretreatment (ml)	Fresh Pollen Volume Applied After Pretreatment (ml)	Seed Set (kernels per ear)
Fresh Pollen Only	NA	NA	0.33	364[a]
Talcum First, Fresh Pollen Second	<75 µm Talcum	0.17	0.33	209[b]
Silica First, Fresh Pollen Second	10 µm Crystalline Silica	0.17	0.33	366[a]
MCC First, Fresh Pollen Second	Avicel PH-101®	0.17	0.33	371[a]
Talcum Only	<75 µm Talcum	0.17	NA	1

Three carrier compounds were applied to silks prior to fresh pollen application to test whether these powders inhibited pollen and silk interaction in pollinations where no pollen storage was involved. Silks were pretreated by evenly coating the silk cluster with 0.17 ml of pollen storage carrier. Pretreatments were followed by an even coating of 0.33 ml fresh maize pollen. Talcum applied to silks as a pretreatment significantly reduced seed set compared to all other treatments. Crystalline silica and Avicel PH-101® microcrystalline cellulose pretreatments did not reduce seed set compared to check pollination using only fresh pollen. Connecting letters reflect Tukey's HSD, $P<0.05$, $N=48$.

Reviewer #3 (Remarks to the Author): Intermediate concerns:	Author Response
1.The team has tested six CO2 sequestration agents, could you please elaborate on the selection criteria for these agents and explain why they were chosen instead of other potential alternatives?	Global accessibility and a favorable safety profile always made soda lime the clear number one choice of CO_2 sequestration agent. The following statement that was present in the Main Text has been modified to further clarify why soda lime was the clear best choice for this protocol. (page 8, lines 11-18) All further testing used soda lime to maintain a ≤0.01% CO2 atmosphere due to soda lime having a lower cost, global availability for purchase, and favorable safety profile compared to alternatives. The following section was added to the Materials and Methods to clarify why move advanced CO_2 sequestration technologies were not tested. (page 36, lines 11-20) Carbon dioxide sequestration technologies were selected based on accessibility, minimal technical complexity, and user safety. Active carbon dioxide scrubbing technologies based on pumped air passing though molecular sieves or amine gas treatment were excluded from testing based on high cost and high technical complexity. Biological carbon dioxide scrubbing using algae or similar photosynthetic organisms was excluded due to high technical complexity and lack of scalability. Continuous or intermittent injection of pure oxygen, nitrogen, or other gas mixtures to dilute the accumulated carbon dioxide were excluded based on high technical complexity and the safety risk posed by compressed gasses. Carbon dioxide sequestration agents were selected for testing based on their ability to work in a sealed storage vessel without electricity, active air movement, and interaction with an operator.
2.It has been reported that maize pollen is relatively large and heavy with a diameter ranging between 80 and 125 μm. Whether there is a correlation between pollen size and surface area breathable barrier surface? What factors were considered in this calculation?	We did not measure pollen grain size in this study. Testing across species with similar, short-lived pollens that are smaller than maize pollen (wheat, rice) would better examine the relationship between pollen metabolic rate, metabolic gas exchange, and pollen grain size. Variation in metabolic gas exchange due to the varying surface area to volume ratio across these pollen types may require varying

	breathable barrier surface areas to maintain a favorable storage vessel atmosphere. Rice and wheat pollens are expected to show a similar rate of high metabolic gas exchange as measured in corn pollen, though we did not assess rice and wheat pollens during the course of this study. I have not observed differences in pollen grain size across maize heterotic groups and inbred vs. hybrid plants, though an absolute difference may certainly exist if measured. My anecdotal observations were that visually detectable differences in maize pollen grain size were reflective of pollen moisture content (dry pollen is smaller than standard fresh pollen at 40x magnification). I inferred this from the collection environment rather than exact pollen moisture content measurements. For example, pollen grains that I collected in greenhouse or field environments as the midday heat approached were slightly smaller compared to pollen collected from the same plants earlier in the day when humidity was higher. These late morning/midday pollen collections were at the end of the natural pollen shedding window. The pollen collected was low in quantity and the pollen tube germination rates were reduced compared to collection conducted under more optimal conditions earlier in the day. These late pollen collections were not used in experiments due to the reduced starting pollen quality.
3.The use of breathable barriers was a major innovation in this study. Could you provide more details on how the breathable barriers were able to facilitate high pollen performance and maintain optimal O2 and CO2 content within the storage vessels?	This was addressed by modifying the original abrupt transition from sealed vessel systems (vacuum, soda lime) to breathable barriers. The modified text below adds context to what inspired breathable barriers and the hypothesized selective gas exchange properties. With this context, the data then describes how we quantified the limited water vapor loss (Table S7) and how seed set from stored pollen gets higher as the storage environment gets closer to normal atmospheric gas concentration (21% O2, <0.1% CO2) in Table S8. (Page 10, lines 2-13) The above experiments clarified that the high metabolic rate of maize pollen was the critical factor limiting scalability. Large pollen storage vessels could preserve > 100 ml of pollen in a field trialing

	context using either vacuum storage or sealing the vessel with added soda lime (fig. S3A, S3B). Scaling this protocol up to pollinate an average seed production field would require thousands of liters of storage vessel space (fig. S4). The team took inspiration from common practices in plant tissue culture where there is a similar challenge of enabling plants to exchange gas with the outside atmosphere while preventing dehydration of the tissue culture media. The team hypothesized that breathable barriers similar to those used in plant tissue culture would limit water vapor loss and allow for passive gas exchange of O2 and CO2 between the storage vessel interior and the surrounding atmosphere. The selective properties of barriers would prevent buildup of CO2 and depletion of O2 while avoiding excessive water vapor loss across the barrier that could lead to pollen death.
Reviewer #3 (Remarks to the Author):	
1."…, while nickel, tungsten, cobalt, and zinc oxide powder were toxic. " Could you provide more insight about the potential mechanisms of this toxicity? Were there any connections between the chemical properties of these compounds and their influence on pollen viability?	I expanded on this topic and added citations for those interested in further reading. The interaction of most metals with pollen was predictable. Inert and/or not bioavailable ferrous metals, metallic aluminum, and titanium metal were non-toxic. Two of the classic toxic metals (Ni &Co) were toxic to pollen. Chromium defied this expectation of toxicity, though the lack of solubility in this context likely prevented any interaction with the pollen. I was predicting 50/50 on tungsten given that tungsten toxicity is rarely relevant, so it was interesting to see it prove toxic in pollen storage. Zinc oxide was the real surprise given that published studies on ZnO nanoparticles have reported that the particles are non-toxic. (Page 15, lines 7-17) High seed set was obtained from pollen stored with ferrous metal powders that were expected to be biologically inert. Similar high seed set was obtained metal powders that have been published as non-toxic to plant surfaces when applied as nanoparticles, including aluminum oxides[28] and titanium[29] powder. Powders of metals known to be toxic to plants including nickel[30] and tungsten[31] were toxic to pollen during storage. Interestingly, cobalt[32] has been published as specifically detrimental to pollen tube growth and was

	confirmed as toxic to maize pollen in storage. The high toxicity of zinc oxide powder was not expected. Further study is required to understand why this toxicity does not align with other studies on zinc oxide nanoparticles applied to plant tissue[33-34]. The lack of toxicity from chromium[30] powder was also not expected and was hypothesized to reflect the poor solubility of the metal powder that prevented interaction with the pollen. Metal powders were included in this study to further explore our hypothesis that hard materials at specific particle sizes were the key properties defining a pollen storage carrier that was superior to talcum. Further testing was discontinued given the scalability and safety issues with metal powders used at production scale. We immediately dropped testing of silica and other alternatives once we identified that microcrystalline cellulose was the best possible combination of material properties and safety. I am hoping that someone will take inspiration from this use of metal powders mixed with pollen to invent a novel application of the technology.
2.The results demonstrate the scalability of the storage protocol using modified cell culture flasks. More considerations required to scale up this technology for large-scale maize breeding and seed production operations should be discussed. What are the potential challenges and how can they be addressed?	The following sentences were added to the Discussion section to address the key challenge for scale up (collection and application efficiency). Scaling up pollen collection and stored pollen application to production operations is an ongoing effort that the authors hope to publish in full in a future manuscript (2026-2027 timeframe). Portions of this scale-up work are currently the subject of provisional patent applications by Syngenta. This work is not approved for publication in a journal at this time. (Page 18, lines 3-15). Scaling up breathable barrier pollen storage technology to commercial seed production will require mechanized pollen collection and mechanized stored pollen application over thousands of maize seed production hectares. The team has identified collection and application efficiency as the key challenge for mechanization moving forward. Maize plants functioning as the male in seed production release 5-10 million pollen grains over the course of up to one week and production

	scientists can collect only a small fraction of this pollen in a single machine pass. Machine passes and pollen handling must move quickly to avoid pollen dehydration or heat exposure prior to storage, which further challenges collection efficiency. In order to set seed, efficient mechanized pollen application requires dosing the bulk stored pollen into < 1 ml quantities and effectively targeted those doses to the silks of plants acting as the female. Any stored pollen that misses the silks during application is a complete loss. Both the collection and application steps must be efficient to ensure that the cost of seed production does not exceed value of the resulting seed.

Reviewer response to author response.

Reviewers' comments	Author Response
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Thank you for submitting the revised version of your manuscript titled "Development of accessible and scalable maize pollen storage technology". I have reviewed your revisions and the responses to my previous comments. I am pleased to confirm that you have addressed all the concerns raised, and the manuscript is now clear, well-written and informative.	
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	AmScope 40x-2500x Compound LED Microscope equipped with an 18-megapixel camera. Representative images with a minimum of 20 pollen grains per image were collected for each sample. Due to the variability in media handling, incubation, and imaging conditions, the exact count of germinated pollen tubes versus ungerminated or burst pollen grains was difficult to quantify across experiments. In response, the team scored pollen tube germination using an ordinal categorical rating scale. Pollen tube germination was rated by reviewing images and assigning a rating on a 1-5 scale (5 = >≈60% pollen tube germination, 4 = ≈41-60%, 3 = ≈21-40%, 2 = ≈1-20%, 1 = 0% or completely dead and burst pollen). The top rating of "5" covered a larger range of pollen tube germination due to the reduced maximum potential pollen tube germination rates observed under greenhouse conditions. Fresh pollen samples collected under greenhouse conditions rarely exceeded 80% pollen tube germination compared to field collected samples that showed nearly 100% germination. The team hypothesized that this reduced maximum potential pollen tube germination rate under greenhouse conditions was due to accelerated pollen desiccation. Accelerated desiccation was caused by the high rate of air recycling and the use of conditioned, 70% relative humidity air under greenhouse conditions.
2. Table S1, the author claimed that the germination rate was inversely correlated with the amount of pollen and talcum carrier mixture, but in the data set, 5 days after storage, we can not see clearly the inverse correlation, and even in 7 days data, the trend is not clear. This may also be solved by using accurate germination ratio instead of rating.	I altered the main text section introducing "Metabolic gas exchange dictates storage vessel requirements" to accurately reflect the categorical rating system described in Table S1 and removed the "inverse correlation" statement. I also changed the confusing use of grams/liter equivalent pollen volumes per storage vessel to the more straightforward pollen quantity per storage vessel in grams. This change is reflected in Tables S1 and S2. (page 7, lines 2-5) The pollen tube germination rating collected following pollen storage decreased when over one gram of pollen was stored in a 125 ml storage vessel for seven days compared to less than lower quantities of pollen stored for the same duration in identical storage vessels (table S1)

3. “Compound microscopy images of silk that were pollinated with a mix of pollen and talcum showed that the silk surface and trichomes were covered in loose talcum particles with comparatively few pollen grains sticking to the silk during pollination (fig. S4)”, I can not find the information on Fig. S4, wrong figure.	Thank you for catching this typo. The correct reference to fig. S6 (Internal Link) was added.
4. “These observations suggested that talcum inhibited silk adherence and therefore, inhibited the pollen tube germination required for fertilization and seed set”, this conclusion from the manuscript is a bit speculated. To confirm this statement, the author could perform a sequential pollination experiment, first with a similar amount of talcum to cover silk, then pollinate the silk with fresh pollen grain and observe the result. In this case, the author can identify whether the reduced adherence of silk is simply caused by talcum on silk or talcum itself has impact on pollen grain and induces less adherence.	Please see the new Table S13 (Internal Link) and main text description quoted below. I took the opportunity to perform the experiment you described with talc and two higher performing pollen storage carriers (silica and microcrystalline cellulose. This proved to be an excellent demonstration of how talc inhibits pollination performance compared to silica and MCC. It was fortunate that extra plants were flowering in our research greenhouse to run this experiment. Pollen storage technology has been fully deployed to field teams within Syngenta and we are no longer resourced to grow plants in support of this manuscript. This experiment is described as occurring chronologically last in the main text. Main text: (Page 16, lines 9-16) The team conducted a final test of the optimal pollen storage carrier hypothesis by testing whether the presence of carrier compounds on maize silks prior to pollen application inhibited pollination in the same manner as was observed when pollen grains were coated in the carriers prior to pollination (table S13). These results demonstrated that application of talcum to silks prior to applying fresh pollen without carrier significantly reduced seed set compared to fresh pollen alone. Application of 10 µm silica or Avicel® PH-101 prior to fresh pollen application did not have a negative impact on seed set. Materials and Methods (page 42, lines 10-21) Inhibition of pollination by carrier compounds present on silks was accomplished by pretreating silks with an even coating of carrier compound prior to applying fresh pollen. A standard hand pollination

	using stored pollen applied 0.5 ml of stored pollen and carrier mix. The 0.5 ml of stored pollen and carrier mix contained 0.33 ml pollen and 0.17 ml carrier based on the 2:1 ratio of the mixture by volume. To replicate these volumes and separate carrier interaction with silk from carrier interaction with pollen, 0.17 ml of a pollen storage carrier compound was evenly applied to the silks on a maize ear. The same ear was then pollinated by evenly applying 0.33 ml of fresh maize pollen. This fresh pollen was not mixed with any carrier compound and was not stored prior to use. This method of carrier pretreatment followed by fresh pollen application was used to test < 75 µm talcum, 10 µm crystalline silica, and Avicel PH-101®. As a positive control, a set of ears were pollinated with 0.33 ml of fresh pollen and did not receive a carrier pretreatment. To test for airborne pollen contamination, a set of ears were pretreated with < 75 µm talcum and did not receive any fresh pollen application.
5. The author did not specify which ecotype that was selected for this experiment. As the pollen from different ecotypes have distinct characteristics, I would suggest the authors select several (3 or more) commonly used ecotypes, and test with simple in vitro germination experiment and confirm whether the storage method is useful for all ecotypes.	Table S11 (Internal Link) has been added to address this concern. The table is addressed by the following lines in the Main Text, Results section. (page 12, lines 3-7) Experiments over the entire duration of the project demonstrated that pollen storage technology was penetrant across temperate and tropical dent corn lines used in hybrid seed production. Summarized testing results indicated that pollen from over 90% of maize lines across four major heterotic groups survived for five or more days in storage (table S11). Pollen from the single sweet corn line included in testing survived for five days in storage. The temperate and tropical dent corn inbreds tested during the research and early deployment phases of this project were summarized based on their adapted region and major heterotic group. This project prioritized testing modern, commercially relevant inbred parents in order to ensure the pollen storage technology would be effective in the hands of global maize breeding teams. Those teams are now using pollen storage technology as triage when lines will not sync for cross pollination or to move pollen between locations when males are damaged in a

	crossing block. The authors hope that academic groups will use this published protocol to test pollen preservation on more diverse ecotypes and uncover long-lived pollen (>7 days) that we believe is not present in commercial germplasm. Inbred line codes are proprietary and not approved for publication. The line codes have been changed to anonymous identifiers (Syngenta Inbred 1, Syngenta Inbred 2, etc.) in the supplied source data Excel.
6. The author wrote the manuscript with beautiful words, but some sentences are relatively long and complex, which might not be friendly for non-native speakers, please pay attention to this point in the revision process.	Hundreds of minor changes were made during manuscript revision to simplify sentence structure. I eliminated non-scientific English words that are uncommon in spoken language (ex: thereby, thus, therefore, whereby, elucidate). Track changes was left on throughout revision of the v2 draft submitted based on this feedback. An example of revised text is given below. Old: To better understand the challenges of extending maize pollen lifespan while the pollen is held in a metabolically active state, fresh pollen from greenhouse grown inbred and hybrid maize plants was mixed with talcum and stored in airtight jars with a gas sampling rubber septum (Fig. 1A, 1B). Sealed vessels were stored at room temperature (23°C) or were placed into refrigerated storage at 2°C or 6°C at local atmospheric pressure (100.3 kPa). Endpoint storage vessel atmosphere gas analysis (Fig. 1C) was conducted after 1-7 days. Mold growth was visible after four days storage at 23°C (Fig. 1D) and researchers noted odors associated with fermentation. Pollen viability following storage was assessed using pollen tube germination media (Fig. 1E, 1F, 1G). Pollen tube germination on media completely ceased after three days at 23°C, while the samples stored at 2°C retained viability for six days and those at 6°C remained capable of germination for the full seven days. New: (page 6, lines 3-13) Experiments where maize pollen was stored in a metabolically active state were conducted to better understand the challenges of extending maize pollen lifespan. The team mixed fresh pollen from

	greenhouse grown maize plants with talcum and stored the mixture in airtight jars equipped with a gas sampling rubber septum (Fig. 1A, 1B). Sealed vessels were stored at room temperature (23°C) or were placed into refrigerated storage at 2°C or 6°C at local atmospheric pressure (100.3 kPa). Endpoint storage vessel atmospheric gases (Fig. 1C) were measured after 1-7 days. Mold growth was visible after four days storage at 23°C (Fig. 1D) and researchers noted odors associated with fermentation. The team assessed pollen viability following storage using pollen tube germination media (Fig. 1E, 1F, 1G). Pollen tube germination on media completely ceased after three days at 23°C. Samples stored at 2°C retained viability for six days and those at 6°C remained capable of germination for seven days.

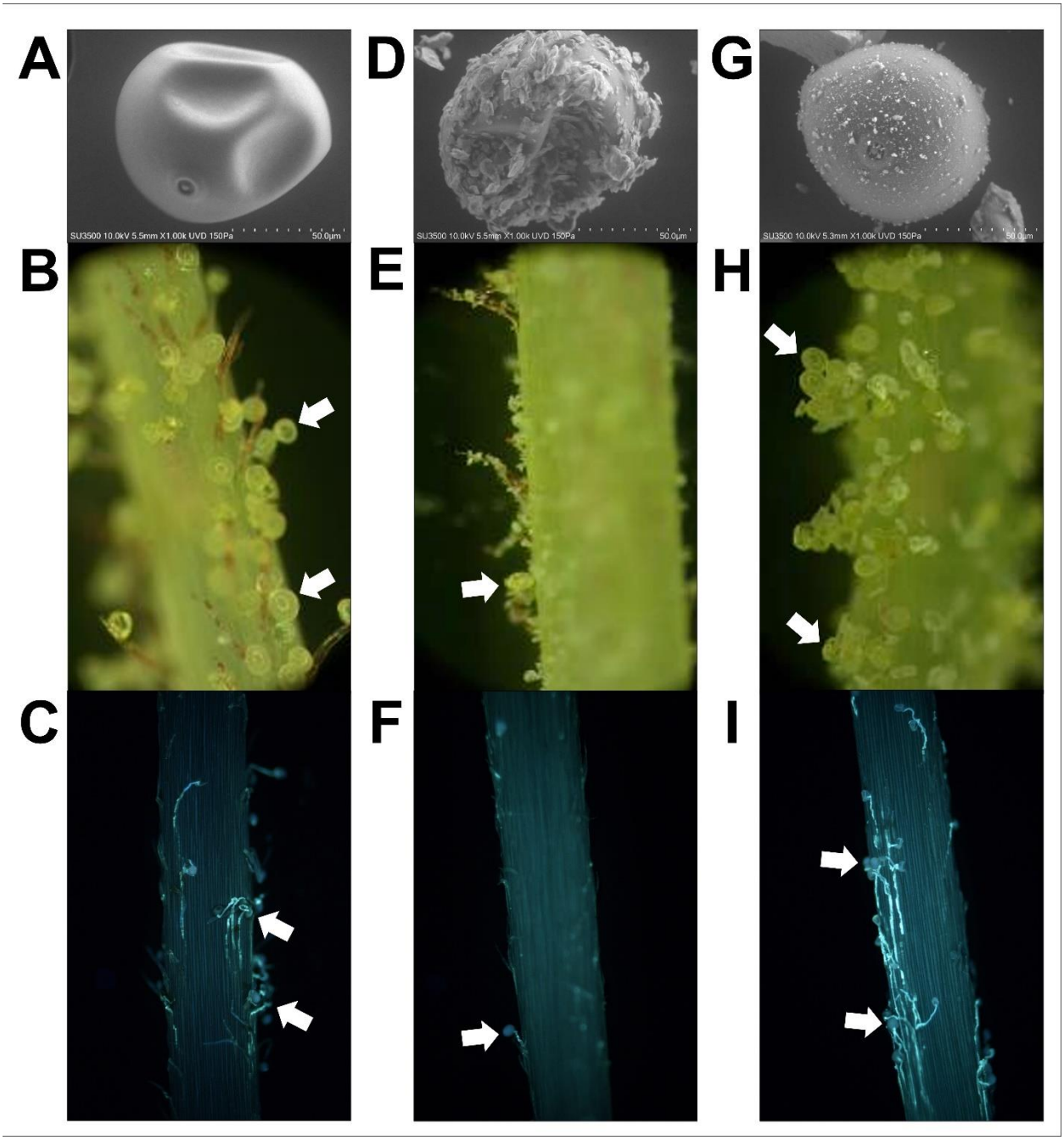

Fig. S6.

Comparative images of maize pollen, maize pollen on silks, and maize pollen on silks stained with aniline blue after pollination. Representative individual pollen grains in images of pollen on silks (**B**, **E**, **H**) and pollen on silks stained with aniline blue (**C**, **F**, **I**) are denoted by white arrows. (**A**) An individual maize pollen grain from a freshly

collected bulk sample (**B**) A maize silk pollinated with freshly collected pollen that shows excellent adherence between pollen grains and silk trichomes. (**C**) A maize silk from the same pollination stained with aniline blue to reveal the pollen tubes growing through the silk. (**D**) An individual maize pollen grain from the same freshly collected bulk sample that was mixed with talcum prior to imaging. The pollen grain surface was covered by talcum particles. (**E**) A maize silk pollinated using pollen mixed with talcum that shows minimal pollen grain adherence and talcum particles completely coating the silk trichomes. (**F**) A maize silk from the same pollination stained with aniline blue to reveal only a single pollen tube. (**G**) An individual maize pollen grain from the same freshly collected bulk sample that was mixed with < 75 µm Florisil® prior to imaging. The pollen grain surface is visible between Florisil® particles. (**H**) A maize silk pollinated using pollen mixed with 75-150 µm Florisil® that showed pollen grains can still adhere to the silk trichomes. (**I**) A maize silk from the same pollination stained with aniline blue to reveal many pollen tubes growing through the silk.

Table S11.

				Maximum Duration of Survival in Pollen Storage		
Ecotype	Adapted Region	Major Heterotic Group	Total Lines Tested	3 Days	5 Days	7 Days
Dent Corn	Temperate	Stiff Stalks	6	0	3	3
Dent Corn	Temperate	Non-Stiff Stalks	7	2	1	4
Dent Corn	Temperate	Iodent	9	0	5	4
Dent Corn	Tropical	N3	3	0	5	ND
Sweet Corn	Temperate	North American Flints	1	0	1	ND

A summary of the maximum observed pollen lifespan in storage for 26 maize inbred lines tested over the course of pollen storage technology development. All inbred lines tested were current or recently relevant maize commercial parent lines. Pollen survival in storage was measured by successful seed set or pollen tube germination. Summarized results include experiments using vacuum, sealed vessel with soda lime, and breathable barrier pollen storage technologies. The two Non-Stiff Stalk lines with pollen that survived for three days in storage were selected for testing based on observed poor pollination performance in hybrid seed production.

Table S13.

Treatment	Pollen Storage Carrier Used as Silk Pretreatment	Volume of Pollen Storage Carrier as Pretreatment (ml)	Fresh Pollen Volume Applied After Pretreatment (ml)	Seed Set (kernels per ear)
Fresh Pollen Only	NA	NA	0.33	364[a]
Talcum First, Fresh Pollen Second	<75 µm Talcum	0.17	0.33	209[b]
Silica First, Fresh Pollen Second	10 µm Crystalline Silica	0.17	0.33	366[a]
MCC First, Fresh Pollen Second	Avicel PH-101®	0.17	0.33	371[a]
Talcum Only	<75 µm Talcum	0.17	NA	1

Three carrier compounds were applied to silks prior to fresh pollen application to test whether these powders inhibited pollen and silk interaction in pollinations where no pollen storage was involved. Silks were pretreated by evenly coating the silk cluster with 0.17 ml of pollen storage carrier. Pretreatments were followed by an even coating of 0.33 ml fresh maize pollen. Talcum applied to silks as a pretreatment significantly reduced seed set compared to all other treatments. Crystalline silica and Avicel PH-101® microcrystalline cellulose pretreatments did not reduce seed set compared to check pollination using only fresh pollen. Connecting letters reflect Tukey's HSD, $P<0.05$, $N=48$.

Reviewer #3 (Remarks to the Author): Intermediate concerns:	Author Response
1.The team has tested six CO2 sequestration agents, could you please elaborate on the selection criteria for these agents and explain why they were chosen instead of other potential alternatives?	Global accessibility and a favorable safety profile always made soda lime the clear number one choice of CO_2 sequestration agent. The following statement that was present in the Main Text has been modified to further clarify why soda lime was the clear best choice for this protocol. (page 8, lines 11-18) All further testing used soda lime to maintain a ≤0.01% CO2 atmosphere due to soda lime having a lower cost, global availability for purchase, and favorable safety profile compared to alternatives. The following section was added to the Materials and Methods to clarify why more advanced CO_2 sequestration technologies were not tested. (page 36, lines 11-20) Carbon dioxide sequestration technologies were selected based on accessibility, minimal technical complexity, and user safety. Active carbon dioxide scrubbing technologies based on pumped air passing though molecular sieves or amine gas treatment were excluded from testing based on high cost and high technical complexity. Biological carbon dioxide scrubbing using algae or similar photosynthetic organisms was excluded due to high technical complexity and lack of scalability. Continuous or intermittent injection of pure oxygen, nitrogen, or other gas mixtures to dilute the accumulated carbon dioxide were excluded based on high technical complexity and the safety risk posed by compressed gasses. Carbon dioxide sequestration agents were selected for testing based on their ability to work in a sealed storage vessel without electricity, active air movement, and interaction with an operator.
2.It has been reported that maize pollen is relatively large and heavy with a diameter ranging between 80 and 125 μm. Whether there is a correlation between pollen size and surface area breathable barrier surface? What factors were considered in this calculation?	We did not measure pollen grain size in this study. Testing across species with similar, short-lived pollens that are smaller than maize pollen (wheat, rice) would better examine the relationship between pollen metabolic rate, metabolic gas exchange, and pollen grain size. Variation in metabolic gas exchange due to the varying surface area to volume ratio across these pollen types may require varying

	breathable barrier surface areas to maintain a favorable storage vessel atmosphere. Rice and wheat pollens are expected to show a similar rate of high metabolic gas exchange as measured in corn pollen, though we did not assess rice and wheat pollens during the course of this study. I have not observed differences in pollen grain size across maize heterotic groups and inbred vs. hybrid plants, though an absolute difference may certainly exist if measured. My anecdotal observations were that visually detectable differences in maize pollen grain size were reflective of pollen moisture content (dry pollen is smaller than standard fresh pollen at 40x magnification). I inferred this from the collection environment rather than exact pollen moisture content measurements. For example, pollen grains that I collected in greenhouse or field environments as the midday heat approached were slightly smaller compared to pollen collected from the same plants earlier in the day when humidity was higher. These late morning/midday pollen collections were at the end of the natural pollen shedding window. The pollen collected was low in quantity and the pollen tube germination rates were reduced compared to collection conducted under more optimal conditions earlier in the day. These late pollen collections were not used in experiments due to the reduced starting pollen quality.
3.The use of breathable barriers was a major innovation in this study. Could you provide more details on how the breathable barriers were able to facilitate high pollen performance and maintain optimal O2 and CO2 content within the storage vessels?	This was addressed by modifying the original abrupt transition from sealed vessel systems (vacuum, soda lime) to breathable barriers. The modified text below adds context as to what inspired breathable barriers and the hypothesized selective gas exchange properties. With this context, the data then describes how we quantified the limited water vapor loss (Table S7) and how seed set from stored pollen gets higher as the storage environment gets closer to normal atmospheric gas concentration (21% O2, <0.1% CO2) in Table S8. (Page 10, lines 2-13) The above experiments clarified that the high metabolic rate of maize pollen was the critical factor limiting scalability. Large pollen storage vessels could preserve > 100 ml of pollen in a field trialing

	context using either vacuum storage or sealing the vessel with added soda lime (fig. S3A, S3B). Scaling this protocol up to pollinate an average seed production field would require thousands of liters of storage vessel space (fig. S4). The team took inspiration from common practices in plant tissue culture where there is a similar challenge of enabling plants to exchange gas with the outside atmosphere while preventing dehydration of the tissue culture media. The team hypothesized that breathable barriers similar to those used in plant tissue culture would limit water vapor loss and allow for passive gas exchange of O2 and CO2 between the storage vessel interior and the surrounding atmosphere. The selective properties of barriers would prevent buildup of CO2 and depletion of O2 while avoiding excessive water vapor loss across the barrier that could lead to pollen death.
Reviewer #3 (Remarks to the Author):	
1."…, while nickel, tungsten, cobalt, and zinc oxide powder were toxic. " Could you provide more insight about the potential mechanisms of this toxicity? Were there any connections between the chemical properties of these compounds and their influence on pollen viability?	I expanded on this topic and added citations for those interested in further reading. The interaction of most metals with pollen was predictable. Inert and/or not bioavailable ferrous metals, metallic aluminum, and titanium metal were non-toxic. Two of the classic toxic metals (Ni &Co) were toxic to pollen. Chromium defied this expectation of toxicity, though the lack of solubility in this context likely prevented any interaction with the pollen. I was predicting 50/50 on tungsten given that tungsten toxicity is rarely relevant, so it was interesting to see it prove toxic in pollen storage. Zinc oxide was the real surprise given that published studies on ZnO nanoparticles have reported that the particles are non-toxic. (Page 15, lines 7-17) High seed set was obtained from pollen stored with ferrous metal powders that were expected to be biologically inert. Similar high seed set was obtained metal powders that have been published as non-toxic to plant surfaces when applied as nanoparticles, including aluminum oxides[28] and titanium[29] powder. Powders of metals known to be toxic to plants including nickel[30] and tungsten[31] were toxic to pollen during storage. Interestingly, cobalt[32] has been published as specifically detrimental to pollen tube growth and was

	confirmed as toxic to maize pollen in storage. The high toxicity of zinc oxide powder was not expected. Further study is required to understand why this toxicity does not align with other studies on zinc oxide nanoparticles applied to plant tissue[33-34]. The lack of toxicity from chromium[30] powder was also not expected and was hypothesized to reflect the poor solubility of the metal powder that prevented interaction with the pollen. Metal powders were included in this study to further explore our hypothesis that hard materials at specific particle sizes were the key properties defining a pollen storage carrier that was superior to talcum. Further testing was discontinued given the scalability and safety issues with metal powders used at production scale. We immediately dropped testing of silica and other alternatives once we identified that microcrystalline cellulose was the best possible combination of material properties and safety. I am hoping that someone will take inspiration from this use of metal powders mixed with pollen to invent a novel application of the technology.
2.The results demonstrate the scalability of the storage protocol using modified cell culture flasks. More considerations required to scale up this technology for large-scale maize breeding and seed production operations should be discussed. What are the potential challenges and how can they be addressed?	The following sentences were added to the Discussion section to address the key challenge for scale up (collection and application efficiency). Scaling up pollen collection and stored pollen application to production operations is an ongoing effort that the authors hope to publish in full in a future manuscript (2026-2027 timeframe). Portions of this scale-up work are currently the subject of provisional patent applications by Syngenta. This work is not approved for publication in a journal at this time. (Page 18, lines 3-15). Scaling up breathable barrier pollen storage technology to commercial seed production will require mechanized pollen collection and mechanized stored pollen application over thousands of maize seed production hectares. The team has identified collection and application efficiency as the key challenge for mechanization moving forward. Maize plants functioning as the male in seed production release 5-10 million pollen grains over the course of up to one week and production

	scientists can collect only a small fraction of this pollen in a single machine pass. Machine passes and pollen handling must move quickly to avoid pollen dehydration or heat exposure prior to storage, which further challenges collection efficiency. In order to set seed, efficient mechanized pollen application requires dosing the bulk stored pollen into < 1 ml quantities and effectively targeted those doses to the silks of plants acting as the female. Any stored pollen that misses the silks during application is a complete loss. Both the collection and application steps must be efficient to ensure that the cost of seed production does not exceed value of the resulting seed.